Tumor and Stem Cell Biology

GLI1 Interferes with the DNA Mismatch Repair System in Pancreatic Cancer through BHLHE41-Mediated Suppression of MLH1

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Abstract

The mismatch repair (MMR) system is indispensable for the fidelity of DNA replication, the impairment of which predisposes to the development and progression of many types of cancers. To date, GLI1 transcription factor, a key molecule of the Hedgehog signaling pathway, has been shown to regulate the expression of several genes crucial for a variety of cancer cell properties in many types of cancers, including pancreatic ductal adenocarcinoma (PDAC), but whether GLI1 could control the MMR system was not known. Here, we showed that GLI1 and GLI2 indirectly suppressed the expression of MLH1 in PDAC cells. Through GLI1 target gene screening, we found that GLI1 and GLI2 activated the expression of a basic helix-loop-helix type suppressor MLH1 through a GLI-binding site in the promoter. Consistent with a previous report that BHLHE41 suppresses the MLH1 promoter activity, we found that the activation of GLI1 led to the BHLHE41-dependent suppression of MLH1, and a double knockdown of GLI1 and GLI2 conversely increased the MLH1 protein in PDAC cells. Using TALEN-based modification of the MLH1 gene, we further showed that GLI1 expression was indeed associated with an increased tolerance to a methylating agent, methylnitrosourea cooperatively with a lower copy number status of MLH1. Finally, GLI1 expression was immunohistochemically related positively with BHLHE41 and inversely with MLH1 in PDAC cells and precancerous lesions of the pancreas. On the basis of these results, we propose that GLI1 depresses the MMR activity and might contribute to the development and progression of PDAC. Cancer Res; 73(24); 7313–23. ©2013 AACR

Introduction

As with many other cancers, pancreatic ductal adenocarcinoma (PDAC) is thought to develop primarily due to an accumulation of genomic mutations, which would be monitored and repaired by the mismatch repair (MMR) system in the normal cells (1, 2). A DNA mismatch is first recognized by either MutS-α (MSH2 and MSH6) or MutS-β (MSH2 and MSH3). These proteins then recruit an MLH1-containing complex with either PMS2 (MutL-α), PMS1 (MutL-β), or MLH3 (MutL-γ), which activates a downstream repair cascade. This MMR system is crucial for the fidelity of DNA replication, and the impairment of this system attenuates the stability of the genome, resulting in increases in the base substitution mismatches and insertion or deletion (indel) events in particular genomic sequences, called microsatellites. Microsatellites are tandem repeats of short (1–6 base pairs) DNA motifs that are distributed throughout the genome, including the TP53, TGFBR2, and BRCA2 genes. Therefore, impairment of the MMR system leads to microsatellite instability (MSI) with predisposition to cancer development (1, 2). Indeed, the autosomal dominant hereditary nonpolyposis colorectal cancer (HNPPC) or Lynch syndrome, which is characterized by germline mutations in one allele of an MMR-related gene, usually the MLH1 gene, highly predisposes to a variety of cancer types (such as PDAC), as a consequence of homozygous or compound heterozygous inactivation of the MMR-related genes (2–4). These cancers that lack MMR activity are characterized by a high frequency of MSI. In contrast, the majority of cases of sporadic PDAC retain at least one allele of MLH1 in the cancer genome. It was generally thought that a single copy of the wild-type allele of a caretaker gene such as DNA repair or mitotic checkpoint genes, including MLH1, might be sufficient to perform its proper function and that both alleles need to be inactivated to impair the activity, as observed in HNPPC cases. However, high-throughput sequencing studies have revealed that mutations in caretaker genes were not frequent in many types of sporadic cancers and indicated that mutations in caretaker genes might not account for the presence of genomic instability of many sporadic cancers (5). Furthermore, a recent whole-exome sequencing study revealed that the deletion of...
one copy of the MLH1 gene correlated with an increased rate of somatic indel mutations, indicating that the MLH1 gene is haploinsufficient for full MMR activity in PDAC (6), whereas the molecular mechanism for this haploinsufficiency was not understood.

The zinc finger transcription factors, GLI1, GLI2, and GLI3, are known as downstream effectors of the Hedgehog signaling and thought to be crucial for many aspects of mammalian development (7, 8). In addition, the upregulation of GLI1 and GLI2 has been well documented to be indispensable for invasion and metastasis (9–11), chemoresistance (12), epithelial–mesenchymal transition (EMT; ref. 13), and the stemness of cancers (14–19). Given that the transcriptional target gene(s) of GLI1 and GLI2 might be responsible for these cancer cell properties, many attempts have been made to find the GLI target genes, but the full range of targets has not been identified (9, 10, 12, 13, 15, 16, 18, 20–23).

We recently established a stable PDAC cell line harboring a GLI1–estrogen receptor (ER) chimeric transgene. Treating these cells briefly with β-estradiol (E2) rapidly upregulates GLI1 target genes (10). In the present study, we used this cell line with a gene expression microarray analysis to screen putative target genes of GLI1 and uncovered BHLHE41 as a direct target gene of GLI1. BHLHE41 is a member of the basic helix-loop-helix (bHLH) protein family (23) and functions as a transcriptional suppressor by binding to the E-box sequence (CANNTG) in the promoter of various genes (24–26). Indeed, BHLHE41 was recently shown to suppress the transcriptional activity of MLH1 through the E-box sequence in its promoter (27). Consistent with this finding, we also showed here that GLI1 suppressed the expression of MLH1 through upregulation of BHLHE41 in PDAC cells. Furthermore, by using the methylnitrosourea (MNU) tolerance assay (28, 29), we demonstrated that GLI1 functionally depressed the activity of the MMR system cooperatively with the low copy number status of MLH1 gene. These results suggest that GLI1 interferes with the MMR activity of PDAC cells, which might account for the molecular mechanism of the haploinsufficiency of MLH1 gene and contribute the development and progression of PDAC.

Materials and Methods

Cells

The human PDAC cell lines, PANC-1, KP-4, MIAPaCa2, PA-TU-8902, and PA-TU-8988T, were obtained from the Health Science Research Resources Bank, Japan Health Sciences Foundation (Osaka, Japan), the RIKEN BioResource Center (Tsukuba, Japan), and the German Collection of Microorganisms and Cell Cultures (DSMZ). The stable cell line PANC-1GLI1ER and its control PANC-1ER have been previously described (10). The expression vector of human BHLHE41 was constructed from pcDNA3.1 plasmid (Invitrogen). The lentivirus vectors of human GLI1 and its control luciferase were constructed from CSII-EF-MCS plasmid, which was kindly provided by Dr. H. Miyoshi (RIKEN BioResource Center, Tsukuba, Japan).

Luciferase reporter assay

The promoter regions of human BHLHE41 and human MLH1 genes were PCR amplified and cloned into the firefly luciferase reporter vector pGL4-basic (Promega). A mutation in the GLI-binding site (GBS) was introduced using the GeneTailor Site-directed Mutagenesis System (Invitrogen). The luciferase reporter assays were conducted using the Dual-Glo luciferase system, along with cotransfection with a control Bonilla luciferase expression vector (Promega), as previously reported (10, 30).

Quantitative RT-PCR

Quantitative real-time PCR (qRT-PCR) was performed using a StepOnePlus real-time PCR system (Applied Biosystems) in conjunction with probes for TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer’s protocol.

siRNA and antibodies

The 21-nucleotide duplex siRNAs for human GLI1 (siGLI1), GLI2 (siGLI2), and control (siControl) were previously reported (10), siRNAs for human BHLHE41 were synthesized as follows: siBHLHE41-1, 5'-GGACUGGACUAUUCUCUUTT-3' and 5'-AAAGGAAUAGUCCAGUCCCTT-3'; siBHLHE41-2, 5'-CACCAAGGAUACCAAAATT-3' and 5'-UUUGAUGGUACCCU-GUGTTT-3'. Antibodies were purchased as follows: anti-BHLHE41 antibody NBP1-19613 (Novus Biologicals), anti-MLH1 antibody ES05 (Dako), anti-MSH2 antibody GTX101175 (GeneTex), anti-MSH6 antibody GTX111661 (GeneTex) and anti-PMS2 antibody ab110638 (Abcam), and anti-GAPDH antibody (Santa Cruz Biotechnology).

Chromatin immunoprecipitation assay

PANC-1 cells were transiently transfected with either FLAG-tagged GLI1 expression vector or a mock vector. After a brief cross-linking with formaldehyde and sonication, the cell lysate...
GLI1 Suppresses MLH1 through BHLHE41

Results

BHLHE41 is a target of GLI1 in PDAC cells

We previously established two stable cell lines, PANC-1GLI1ER and its control PANC-1ER, from the human PDAC cell line PANC-1. PANC-1GLI1ER expresses a chimeric transgene composed of the enhanced GFP (EGFP)-tagged amino-terminal half (1–401aa) of GLI1, including its zinc finger DNA-binding domain, fused to the AF2 domain (281–599aa) of the mouse ER1, whereas PANC-1ER cells harbor only EGFP-tagged AF2 domain (Fig. 1A; ref. 10). PANC-1GLI1ER cells, but not PANC-1ER cells, are expected to quickly upregulate GLI1 target genes following E2 treatment. To find putative target genes of GLI1, we used these cells for a gene expression (cDNA) microarray screen. We extracted total RNA samples from PANC-1GLI1ER and PANC-1ER cells either with or without E2 treatment and then made their cDNA probes for the screening. E2 treatment was limited to 3 hours to reduce the likelihood that secondary targets of GLI1 were affected. We first listed the genes showing more than a 2-fold change by E2 treatment in either PANC-1GLI1ER or PANC-1ER cells. Many genes overlapped between the lists from PANC-1GLI1ER and PANC-1ER cells. We eliminated these overlapping genes from the consideration because their changes might be due to E2 treatment itself (Fig. 1A). As a result, we found 31 upregulated and 18 downregulated genes by E2 treatment in PANC-1GLI1ER and PANC-1ER cells. We considered these to be candidate GLI1 target genes (Supplementary Table S1). From these candidates, we selectively confirmed the upregulation of BHLHE41, Neutrinin (NRTN), and Cytokeratin 16 (KRT16) as well as a known GLI1 target Patched1 (PTCH1) by qRT-PCR (Fig. 1B). In the present study, we focused on BHLHE41 because, as a bHLH-type transcriptional suppressor, BHLHE41 was reported to suppress the expression of MLH1 through the E-box sequence in the promoter (27) and because we expected that GLI1 would be involved in the regulation of MLH1 through BHLHE41. To confirm that the expression of BHLHE41 would really be regulated by GLI1, we transiently transfected PDAC cell lines with either EGFP-tagged full length of GLI1 (EGFP-GLI1) or EGFP expression vector (Fig. 1C). In all the tested human PDAC cell lines, EGFP-GLI1 upregulated the expression of BHLHE41 as well as PTCH1. It was known that GLI1 and GLI2 share many target genes (10, 20, 21), and we also found either GLI1 or GLI2 activated the BHLHE41 reporter construct (see below), indicating a redundant role of GLI1 and GLI2 in the regulation of BHLHE41 expression. Consistently, we observed a downregulation of BHLHE41 by a double knockdown of GLI1 and GLI2 (Fig. 1D). Altogether, these pieces of evidence supported that BHLHE41 was a GLI1 target gene in PDAC cells.

GLI1 directly activates BHLHE41 expression in PDAC cells

Because BHLHE41 was identified as an upregulated gene in the short-term (3 hours) E2-treated PANC-1GLI1ER, we expected that BHLHE41 would be a direct target of GLI1 in PDAC cells. Indeed, it was confirmed that similar to PTCH1, upregulation of BHLHE41 was detected as early as 3 hours after E2 treatment in PANC-1GLI1ER cells, but not in PANC-1ER, whereas JAG2, an
indirect target of GLI1 (10), was induced by 24 hours after treatment (Fig. 2A). Furthermore, pretreatment with a protein synthesis inhibitor cycloheximide (CHX) did not block the E2-induced upregulation of BHLHE41 in PANC-1GL1ER cells (Fig. 2A). Next, we examined the promoter region of the human BHLHE41 gene. Within the 5' flanking region 2 kb upstream of the translational start site (the ATG is located at nucleotide position +1126 to −1117 (5'-CAGGGTGGCT-3'; Supplementary Fig. S1A). This is well conserved across species and similar to a complementary sequence of previously reported GBS consensus sequence (5'-GACCACCCA-3'; Supplementary Fig. S1B; refs. 21, 22). Therefore, we generated luciferase reporter constructs containing fragments of nucleotides −1728 to +291 of the human BHLHE41 promoter region and examined their responsiveness to GLI1 and GLI2 (Fig. 2B). We found that either GLI1 or GLI2 induced the activation of the reporter constructs containing the −1728 to +291 fragment, or the −1155 to +291 fragment, but not the −1115 to +291 fragment or shorter fragments. Neither GLI1- nor GLI2-dependent activation was observed with the reporter construct harboring a mutated GBS (Fig. 2B). To further confirm the GBS-dependent activation, we generated artificial constructs containing two tandem copies of the −1165 to −1076 fragment flanked by the thymidine kinase minimum promoter (TK) and tested their responsiveness to GLI1 and GLI2 (Fig. 2C). As expected, only the reporter construct containing a wild-type GBS was activated by GLI1 and GLI2. Finally, we performed a chromatin immunoprecipitation (ChIP) assay using PANC-1 cells that were transiently transfected with a FLAG-tagged GLI1 expression vector. We found that the GBS-containing DNA fragment of the BHLHE41 gene was coprecipitated with FLAG-GLI1 to a similar extent to the GBS fragment of PTCH1 gene (Fig. 2D), supporting the notion that GLI1 directly activates the BHLHE41 promoter through the GBS in PDAC cells.

GLI1 suppresses MLH1 through BHLHE41 upregulation in PDAC cells

Previously, BHLHE41 was shown to suppress the expression of MLH1 by direct binding to the E-box sequence of the MLH1 promoter (27). Consistent with this, we also confirmed that BHLHE41 suppressed the reporter activity of the human MLH1 promoter (Fig. 3A) and reduced the amount of MLH1 protein in PDAC cells (Fig. 3B). Given that GLI1 upregulated the expression of BHLHE41 in PDAC cells (Fig. 1C) and that GLI1 suppressed the activity of an MLH1 reporter (Fig. 3A), we assumed that GLI1 would indirectly regulate the MLH1 expression through BHLHE41 upregulation in PDAC cells. Indeed, we first found that a double knockdown of GLI1 and GLI2 increased the amount of MLH1 protein, as opposed to BHLHE41 protein, in PDAC cells (Fig. 3C).
a transient transfection of EGFP-GLI1 decreased MLH1 protein and that this reduction was abrogated by BHLHE41 knockdown (Fig. 3D). These lines of evidence indicated that GLI1 is involved in the reduction of MLH1 through BHLHE41 upregulation in PDAC cells. Interestingly, neither GLI1 transfection nor BHLHE41 knockdown changed the amounts of other MMR proteins, such as MSH2, MSH6, and PMS2, indicating that GLI1 and BHLHE41 regulate only MLH1 expression (Fig. 3D).

**GLI1 and GLI2 correlate with the increased tolerance to MNU**

Next, we examined whether the expression of GLI1 and GLI2 would influence the function of the MMR system in PDAC cells. The MMR system is known not only to reverse the replication errors but also to induce apoptotic cell death in response to a certain type of DNA damage. For instance, DNA modification, such as an \( O^\beta \)-methylguanine induced by a methylating agent MNU, is sensed by MutS-a, followed by the MLH1-containing MutL-a complex that leads to an apoptotic cell death. Conversely, cells lacking proper MMR activity acquire resistance to MNU. Although the mechanism of the damaged DNA-induced cell death is not precisely understood, it has been shown that an increased tolerance to MNU correlates with a reduced MMR activity (28, 29). Therefore, we used this MNU tolerance assay to estimate the role of GLI1 in the MMR system. Among the PDAC cell lines tested in the study, PANc-1 and KP-4 cells highly expressed GLI1, GLI2, and BHLHE41, whereas PA-TU-8902 and PA-TU-8988T cells showed very weak expressions of these genes by qRT-PCR (Fig. 4A). And consistent with the above results, GLI1/2-expressing PANC-1 and KP-4 showed lower expression of MLH1 than PA-TU-8902 and PA-TU-8988T. Therefore, we first compared the tolerance of these PDAC cells with MNU and found that GLI1-expressing PANC-1 and KP-4 showed a greater tolerance than PA-TU-8902 and PA-TU-8988T (Fig. 4B).

**GLI1 and copy number status of MLH1 show a combined effect on the tolerance to MNU**

In Fig. 4A, we found a lower expression of MSH2 in PANC-1 and KP-4 than PA-TU-8902 and PA-TU-8988T. However, as mentioned above, neither GLI1 transfection nor BHLHE41 knockdown changed the protein level of MSH2 (Fig. 3D). Furthermore, MLH1 was severely reduced than MSH2 in PANC-1 and KP-4 cells, indicating the possibility that the GLI1-dependent decrease of MLH1 might be more potent than the lower MSH2 in the increased tolerance to MNU. Therefore, we focused in this study on a role of GLI1 and...
MLH1 in the control of the MNU tolerance. First, we examined the copy number of the MLH1 gene using TaqMan copy number variation assay (Applied Biosystems) and found that PANC-1, KP-4, and PA-TU-8902 cells harbor a lower copy number status of the MLH1 gene than PA-TU-8988T cells (Supplementary Fig. S2A). Therefore, to examine whether GLI1-related tolerance might be affected by the copy number status of MLH1 gene, we established the stable cell lines (TALEN clone1 and clone2), which harbored the targeted allele of MLH1 gene, from the parental PA-TU-8988T cell line by TALEN-based homologous recombination (Fig. 5A). Briefly, parental PA-TU-8988T cells were transfected with two TALEN vectors (31) in conjunction with a targeting vector containing the puromycin resistance gene and DNA fragments of MLH1 (Fig. 5A). These TALEN vectors were previously reported to induce a DNA double-strand break around the first methionine codon (ATG) of the MLH1 gene (arrowheads in Fig. 5A). After puromycin selection, clones with a successful recombination in MLH1 were identified by genomic PCR (arrows for primers in Fig. 5A; data in Supplementary Fig. S2B) and Southern blot analysis (Supplementary Fig. S2C). We then transduced either GLI1-expressing or control luciferase-expressing lentiviruses to parental PA-TU-8988T cells or GLI1-transduced parental PA-TU-8988T cells (Fig. 5D and Supplementary Fig. S2D for data of TALEN clone1). These pieces of evidence indicate that GLI1 impaired the activity of the MMR system in combination with the low copy number status of MLH1 gene, at least when estimated in the MNU tolerance assay.

GLI1 and BHLHE41 expression inversely correlates with MLH1 in pancreatic tissues

Finally, we examined the expression of GLI1, BHLHE41, and MLH1 in precancerous pancreatic intraepithelial lesions (PanIN) and PDAC by immunohistochemistry (Fig. 6A for representative photos; Fig. 6B and C and Supplementary Tables S2–S6 for statistical results). As shown in our previous report (10), GLI1 was not detectable in the normal pancreatic duct but became detectable at low levels in low-grade PanIN (PanIN-1A and PanIN-1B), then increased in high-grade PanIN (PanIN-2 and PanIN-3) and PDAC (Fig. 6A and Supplementary Table S2). BHLHE41 expression was faintly detectable in the normal duct and low-grade PanIN, in which we assumed that BHLHE41 was weakly induced by transcriptional regulator(s) other than GLI1. BHLHE41 expression was then increased in high-grade PanIN and PDAC, and this correlated with the increased expression of GLI1 (Fig. 6A and Supplementary Table S3). In contrast, MLH1 expression was found strongly in the normal duct and low-grade PanIN, in which we assumed that BHLHE41 was weakly induced by transcriptional regulator(s) other than GLI1. BHLHE41 expression was then increased in high-grade PanIN and PDAC, and this correlated with the increased expression of GLI1 (Fig. 6A and Supplementary Table S3). In contrast, MLH1 expression was found strongly in the normal duct and low-grade PanIN, in which we assumed that BHLHE41 was weakly induced by transcriptional regulator(s) other than GLI1. BHLHE41 expression was then increased in high-grade PanIN and PDAC, and this correlated with the increased expression of GLI1 (Fig. 6A and Supplementary Table S3). Using a semiquantitative scoring of the staining intensity on a three-tiered scale (negative, 0; weak, 1; strong, 2), the expression levels of BHLHE41 and MLH1 were statistically related positively and inversely with GLI1, respectively (Fig. 6B and C and Supplementary Table S3).
Tables S5 and S6). These lines of evidence supported our \textit{in vitro} results using cultured PDAC cells and suggested that GLI1 and BHLHE41 repress the MLH1 expression.

**Discussion**

GLI1 and GLI2 are upregulated in a variety of human cancers and thought to participate in the development and progression of PDAC (7). Indeed, GLI1 was shown to be indispensable for KRAS-dependent pancreatic epithelial transformation in a genetically modified mouse model (32) and essential for the survival and maintenance of the transformed phenotype of human PDAC cell lines (33). Moreover, GLI1 is known to upregulate a variety of genes crucial for many properties of cancer cells, such as \textit{CYR61} (9), \textit{MUC5AC} (10), and \textit{MMP9} (11) for invasion and metastasis; \textit{ABCG2} for chemoresistance (12); \textit{SNAI1} for EMT (13); \textit{BCL2} for antiapoptosis (34); and \textit{BMI1} (15, 18) and \textit{NANOG} (16) for stemness. These GLI1 target genes, together with many other targets found by comprehensive screens (20–22), highlight a pivotal role of GLI1 in cancer biology, but it was still unknown whether GLI1 might be linked to the regulation of caretaker genes, which should be important in the development and progression of cancer. In the present study, we generated a list of candidates of direct target genes of GLI1 by using PANC-1GLI1ER cells and control PANC-1ER cells (Supplementary Table S1) and focused on BHLHE41 to test the hypothesis that GLI1 participates in the regulation of the MMR system.

E2 treatment of PANC-1GLI1ER cells, but not PANC-1ER cells, indeed rapidly upregulated the expression of \textit{PTCH1}, a well-known GLI1 target. In addition, \textit{ARC} (activity-regulated cytoskeleton-associated protein), \textit{PI3} (peptidase inhibitor 3, skin derived), and \textit{KRT16}, which were previously reported as GLI1 targets (35, 36), were also upregulated in our experiments (\textit{KRT16} in Fig. 1B and Supplementary Table S1), suggesting that our experimental system would properly function to identify the target genes of GLI1. However, to the best of our knowledge, many other candidate genes in the list have not been previously identified as GLI1 targets. GLI1 and GLI2 are able to form a heteromeric complex with many transcription factors. For example, a physical interaction between GLI1 and ZIC2 or JUN modulates the transcriptional activity and/or diversity of GLI1 target genes (37–39), and GLI1 interferes with the formation of the MyoD–E12 complex to abrogate muscle-specific gene expression and myogenic differentiation (40). In PANC-1GLI1ER cells, E2-induced transcriptional activation depends on the homodimerization of the GLI1-ER transgene product by bridging the AF2 domain of ER. Therefore, our system is not designed to detect the target genes regulated by a heteromeric complex of GLI1 or an “off-target” effect of GLI1, similar to MyoD–E12 signaling. Incidentally, we previously identified a gel-forming mucin, \textit{MUC5AC}, as a direct target gene of GLI1 (10). In the present study, \textit{MUC5AC} was indeed upregulated 1.32-fold at 3 hours and 2.06-fold at 24 hours after E2 treatment in PANC-1GLI1ER cells (data not shown). However, it was not more than 2-fold upregulated at 3 hours after E2 treatment, so \textit{MUC5AC} was not included in Supplementary Table S1.

\textit{BHLHE41} was first identified from independent experiments as a differentially expressed gene, named human \textit{DEC} (differentially expressed in chondrocytes), \textit{mouse stra} (stimulated with retinoic acid), and \textit{rat sharp} (split and hairy related protein). It belongs to the class E group of the bHLH
family, which includes the closely related BHLHE40/DEC1/STRA13 and the transcriptional repressor Hairy/Enhancer-of-split [E(Spl)]/HES proteins. Although the Hairy/E(Spl)/HES proteins bind the N-box sequence (CACGCG), BHLHE41 and BHLHE40 were shown to bind and suppress the transcriptional activity of the E-box (CANNTG)-containing promoter. For example, BHLHE41 suppresses the E-box–containing promoter activity of the cholesterol 7α-hydroxylase CYP7A gene in HepG2 cells (26) and the E-box–regulated expression of myogenic genes in myoblast C2C12 cells (24).

In the suprachiasmatic nucleus of the mouse brain, bhlhe41 and bhlhe40 were revealed to be regulators of the molecular clock by repressing Clock/Bmal1-induced transactivation of Per1 by either associating with Bmal1 protein or competing for the E-box of the Per1 promoter (25). Intriguingly, BHLHE40 was shown to bind and suppress the E-box–containing promoter activity of the BHLHE41 gene, and it is also likely that BHLHE41 suppresses its own expression through an auto-feedback loop because the bHLH domain of BHLHE41 shares a high degree of homology (97% of the protein) with BHLHE40 (23, 41). Indeed, it was revealed that BHLHE40 and BHLHE41 showed a rhythmic or oscillating expression in the suprachiasmatic nucleus of the mouse brain (25) and in various peripheral tissues (heart, kidney, liver, and skeletal muscle) of rat (26) due to the feedback loop regulation. Here, we showed that GLI1 activates BHLHE41 expression in PDAC cells, but we currently do not know whether the activation of BHLHE41 and the downregulation of MLH1 might also show this oscillating pattern. However, a gene-targeting experiment using single-stranded oligodeoxyribonucleotides proved that a transient (up to 2 days) but not constitutive suppression of MLH1

Figure 5. GLI1 shows a combined effect with the copy number status of MLH1 gene on the increased tolerance to MNU. A, targeting of the MLH1 gene by using TALEN-based homologous recombination. PA-TU-8988T cells were transfected with TALEN vectors, which induced a DNA double-strand break specific to the human MLH1 gene (arrowheads), in conjunction with a targeting vector. After puromycin selection, clones with a successful recombination of MLH1 gene were identified by genomic PCR (arrows for PCR primers; Supplementary Fig. S2B) and Southern blot analysis (Supplementary Fig. S2C). Blue and red marks, probes for Southern blot analysis. B, qRT-PCR of the cloned cells harboring the targeted allele of MLH1 gene (TALEN clone1 and clone2) and their parental PA-TU-8988T, which were transduced by lentivirus either for HA-tagged GLI1 (gray columns) or control luciferase (white columns) expression. The data represent a relative change of MLH1 expression of these cells along with PANC-1 and KP-4 cells (black columns) compared with the luciferase-transduced parental PA-TU-8988T cells (data are shown on a log2 scale). Columns, mean values; bars, SD. C, immunoblot analysis of lentivirus-transduced TALEN clones and their parental PA-TU-8988T cells. D, MNU tolerance assay of lentivirus-transduced TALEN clone2 and parental PA-TU-8988T. TALEN clone1 showed similar results to TALEN clone2 (Supplementary Fig. S2D). Left, data represent mean ± SD from three independent experiments. Right, columns, mean values; bars, SD.
made embryonic stem cells permissive for the nucleotide substitution (42). This suggests that suppressed MLH1, even if it is oscillating, might not be sufficient to steadily maintain the activity of the MMR system, which leads to the increased risk of mutations.

The mutator hypothesis predicted that mutations in caretaker genes are frequent and early events during cancer development because the subsequent genomic instability in the genome should be due to the inactivation of the caretaker (43). It was also predicted that mutation or inactivation of caretaker genes is recessive, and a null mutation or inactivation should be required for the loss of caretaker’s function, as observed in HNPCC cases (2). Indeed, a whole-exome sequencing of PDAC cell lines harboring MLH1 homozygous deletions revealed a dramatically elevated rate of somatic substitutions and indels (6). However, high-throughput sequencing studies of sporadic cancers have revealed that the inactivation of caretaker genes is infrequent and does not account for the presence of genomic instability in many sporadic cancers. Therefore, it was proposed that “oncogene-induced DNA replication stress” should be considered to explain the presence of genomic instability in many sporadic cancers (5). Coincidentally, the above whole-exome sequencing study reported a 10.5-fold increase of somatic indels in PDAC cell lines harboring the loss-of-heterozygosity (LOH) of MLH1 gene, indicating the haploinsufficiency of the MLH1 gene in PDAC cells, but the molecular mechanism of the haploinsufficiency remained unclear (6). In the present study, we uncovered a role for GLI1 in the suppression of MLH1 through BHLHE41 upregulation. We cannot say that GLI1 is a dominant suppressor of the MLH1 expression because we found that transduced GLI1 and the copy number status of MLH1 gene cooperatively depressed MLH1 expression and also increased the tolerance of cells to MNU. Therefore, the activation of GLI1 may correspond to “oncogene-induced stress” only for the MMR system of MLH1-LOH PDAC cases, which were reported as 3% to 15% of all sporadic PDACs (44). Intriguingly, we also found that the GLI1-BHLHE41 axis was not so potent to other MMR proteins such as MSH2, MSH6, and PMS2. Consequently, a fascinating possibility to us is that other oncogenic signals might suppress other MMR genes and the combination of activated GLI1 (for MLH1 suppression) and these unidentified signals (that suppress other MMR gene) cooperatively depresses the MMR activity in a similar manner to that as observed in PDAC cases with a compound heterozygous inactivation of MMR genes. We believe this hypothesis should be investigated in the future by the extensive analysis using a large number of PDAC cases and cell lines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: S. Inaguma, K. Kasai
Development of methodology: S. Inaguma, H. Murakami, K. Kasai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Inaguma, M. Riku, M. Hashimoto, S. Saga, H. Ikeda
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Inaguma, K. Kasai
Writing, review, and/or revision of the manuscript: S. Inaguma, K. Kasai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Riku, M. Hashimoto
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