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***MIIP* haploinsufficiency induces chromosomal instability and promotes tumour progression in colorectal cancer**

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Abstract

The gene encoding Migration and Invasion Inhibitory Protein (*MIIP*), located on 1p36.22, is a potential tumour suppressor gene in glioma. In this study, we aimed to explore the role and mechanism of action of *MIIP* in colorectal cancer (CRC). *MIIP* protein expression gradually decreased along the colorectal adenoma-carcinoma sequence and was negatively correlated with lymph node and distant metastasis in 526 colorectal tissue samples ($P < 0.05$ for all). Analysis of The Cancer Genome Atlas (TCGA) data showed that decreased *MIIP* expression was significantly associated with *MIIP* hemizygous deletion ($P = 0.0005$) that was detected in 27.7% (52/188) of CRC cases, and associated with lymph node and distant metastasis ($P < 0.05$ for both). We deleted one copy of the *MIIP* gene in HCT-116 CRC cells using zinc finger nuclease technology and demonstrated that *MIIP* haploinsufficiency resulted in increased colony formation and cell migration and invasion, which was consistent with the results from siRNA-mediated *MIIP* knockdown in two CRC cell lines ($P < 0.05$ for all). Moreover, *MIIP* haploinsufficiency promoted CRC progression *in vivo* ($P < 0.05$). Genomic instability and spectral karyotyping assays manifested that *MIIP* haploinsufficiency induced chromosomal instability (CIN). Besides modulating the downstream proteins of APC/C^{Cdc20}, securin and cyclin B1, *MIIP* haploinsufficiency inhibited topoisomerase II (Topo II) activity and induced chromosomal missegregation. Therefore, we report that *MIIP* is a novel potential tumour suppressor gene in CRC. Moreover, we characterized the *MIIP* gene as a novel CIN suppressor gene, through altering the stability of mitotic checkpoint proteins and disturbing Topo II activity.

Keywords

MIIP; colorectal cancer; progression; chromosomal instability; topoisomerase

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both men and women and is the third leading cause of cancer death in the United States [1]. Despite early screening and the introduction of new chemotherapeutic strategies, CRC survival rates have not substantially improved during the past 20 years. The 5-year survival rate is less than 10% for patients with stage IV CRC [2]. It has been commonly accepted that most CRCs undergo a neoplastic progression of the adenoma-carcinoma sequence, accompanied by an accumulation of successive genetic alterations [3, 4]. The chromosomal instability (CIN), mutator phenotype/mismatch repair, and hypermethylation phenotype (CIMP+) pathways have been demonstrated to be important molecular pathogenesis processes underlying tumorigenesis and progression of CRC [5, 6]. However, the molecular events that contribute to the development and progression of CRC are likely far more extensive than previously appreciated [7].

The gene encoding Migration and Invasion Inhibitory Protein (*MIIP*) is located on chromosome 1p36.22 and was found to be a potential tumour suppressor gene in glioma [8]. In our previous studies, *MIIP* was initially identified, in a yeast-two-hybrid screen, as a binding partner for insulin-like growth factor binding protein 2 and was shown to inhibit glioma cell invasion [9]. Our subsequent work demonstrated that *MIIP* can also interact with

Cdc20, which is involved in the regulation of mitotic progression [8]. However, the function of MIIP in CRC remains unclear.

In this study, we provide evidence that *MIIP* haploinsufficiency is associated with CRC progression in TCGA and an independent cohort. The effect of *MIIP* haploinsufficiency/downregulation in CRC is then manifested *in vitro* and *in vivo*. Subsequent molecular studies demonstrate that *MIIP* haploinsufficiency leads to a weakened mitotic checkpoint and inhibits the decatenation activity of Topo II, promoting chromosomal missegregation and CIN. Therefore, we report that *MIIP* is a novel tumour suppressor gene and CIN suppressor gene in CRC and is a potential target for CRC treatment.

Materials and Methods

CRC patients and data collection

We collected 526 formalin-fixed, paraffin-embedded colorectal tissue samples (including 287 carcinomas, 145 adenomas, and 94 samples of histologically normal mucosa which were more than 5 cm distant from the corresponding cancer or adenoma tissues, from the Department of Pathology at Tianjin Medical University Cancer Institute and Hospital, after we received institutional review board approval from Tianjin Medical University Cancer Institute and Hospital (Tianjin, China) and obtained consent from every patient. All of the samples were from inpatients undergoing surgical operation from January 2000 to December 2004, and none of the patients had received any chemotherapy or radiotherapy before operation. The cohort included 53 sets of matched surgical samples (carcinomas, adenomas, and non-neoplastic mucosae from the same patient). The clinical information is shown in Supplementary Table S1. Tissue microarrays (TMA) were constructed using a manual tissue microarray instrument (Beecher Instruments) equipped with a 2.0 mm punch needle, as described in our previous study [10].

Copy number data from an Affymetrix SNP 6.0 array, whole exome mutation data, and clinical information, were obtained from the open- and controlled-access tiers of the TCGA data portal, with National Cancer Institute approval. The clinical details and patient selection criteria can be found in the recent TCGA report [11]. Alignment of the sample identifiers yielded 188 CRC cases for which information was available at the time of data retrieval.

Immunohistochemical analysis

We performed an immunohistochemical analysis for MIIP on TMA sections using a rabbit anti-human MIIP antibody (1:300, HPA044948; Sigma-Aldrich, St. Louis, MO, USA), as previously described [12]. MIIP-positive samples were defined as those with brown staining in the cytoplasm. The staining intensity of MIIP was graded on a scale from 0 to 3 (0 for no staining, 1 for light yellow, 2 for yellow, and 3 for brown). The percentage of cells with immunoreactivity was scored on a scale from 0 to 3 (0 for no positive cells, 1 for estimated <25% positive, 2 for 25%-50% positive, and 3 for >50% positive). The staining intensity and percentage of immunoreactivity scores were then multiplied to obtain the total score (0, 1, 2, 3, 4, 6, or 9). MIIP expression was classified as negative (–), weakly positive (++), moderately positive (+++), or strongly positive (++++) when the total score was 0, 1-3, 4-6, or

9, respectively. High MIIP expression was defined as (+++), and low expression was defined as (-), (+), or (++) (Supplementary Figure S1A).

Fluorescence *in situ* hybridization analysis

A dual-colour fluorescence *in situ* hybridization (FISH) analysis was performed on 4 µm TMA sections using a Spectrum-Green-labelled chromosome 1 centromeric probe (CEP 1) and a Spectrum-Orange-labelled locus-specific *MIIP* probe (Empire Genomics, Buffalo, New York, USA), as described previously [13]. Fluorescence signals were captured by a computer-controlled digital camera and processed by ProgRes CapturePro software (NatureGene, USA). Sequential digital images were captured by a stack motor for each fluorescence filter, and the resulting images were reconstructed with blue, green, and red pseudo-colours. The FISH sections were evaluated simultaneously by two observers. Typically, 30-90 cells were enumerated in each case. In the areas where cell borders were clear, the numbers of red and green signals per nucleus were evaluated. If there were two *MIIP* gene (red) signals in more than 80% of nuclei, the case was considered *MIIP*-diploid. *MIIP*hemizygous deletion was defined as the presence of only one red signal in at least 20% of nuclei, and *MIIP*homozygous deletion was defined as both red signals having been lost at least 20% of nuclei with at least one green signal.

Zinc finger nuclease-mediated *MIIP* gene deletion

A *MIIP*-knockout zinc finger nuclease kit (*MIIP*-ZFN) was purchased from Sigma (St. Louis, MO, USA; #CKOZFN13330-1KT). We used the Amaxa Nucleofector system to transfect HCT116 cells with *MIIP*-ZFN constructs (Lonza, Köln, Germany). In brief, 2×10^6 HCT116 cells per transfection were washed twice with PBS and resuspended in 100 µl of Nucleofection Solution V. *MIIP*-pZFN1 (2.5 µg) and *MIIP*-pZFN2 (2.5 µg) were mixed with the cells; the mixture was transferred to a 2 mm electroporation cuvette and nucleofected on a Nucleofector with the D032 program. The cells were then transferred to pre-warmed medium in a six-well plate and incubated for 3 days. Ninety-six single colonies were prepared from the transfected cells and seeded in a 96-well plate. Two replica plates were prepared: one was used to harvest genomic DNA and perform genotyping (ZFN primer F: TCTGGGAGGAAAGGGGTTAG; ZFN primer R: ACCAGTGGTTCAGGCTCTGT), and the other was used as a working plate. After genotyping and sequencing, the *MIIP* knockout clones were used to test MIIP protein expression by Western blot analysis.

Cell migration and invasion assay

Cell migration and invasion assay was performed as described previously [14].

Orthotopic CRC mouse model

Male athymic nude mice (6 weeks old) were from the specific pathogen-free animal colony of the Department of Experimental Radiation Oncology and housed in the specific pathogen-free animal facility of the Department of Veterinary Medicine at The University of Texas MD Anderson Cancer Center (Houston, Texas). All mouse experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee. HCT116 *MIIP*-diploid (HCT 116_ *MIIP* +/+) and *MIIP*-deleted (HCT 116_ *MIIP* +/-_1 and

HCT 116_ *MIIP* +/–_2) cells were harvested, washed in serum-free medium, and resuspended in HBSS. Each mouse (10 per group) was anesthetized with a ketamine/ xylazine solution; the caecum was exteriorized using a small left abdominal flank incision, and 2×10^6 cells, suspended in 50 μ l of HBSS, were injected into the caecal wall using a 30-gauge needle. To prevent leakage, a cotton swab was held gently over the injection site. The injected caecum was returned to the abdominal cavity, and the wound was closed with wound clips (CellPoint Scientific, Inc., Gaithersburg, MD). Mice were weighed weekly and monitored for primary tumour development and metastasis; they were killed when most mice demonstrated signs of morbidity. The caecum, liver, and large lymph and omentum nodes were collected for histological analysis.

Chromosomal analysis

Exponentially growing cells were exposed to colcemid (0.04 μ g/ml) for 1 h at 37° C. The cells were trypsinised, washed with PBS, and resuspended in hypotonic (75 mM KCl) for 20 min at ambient temperature. Cells were fixed in a methanol and acetic acid (3:1 v/v) mixture for 15 min and washed three times in the fixative. Air-dried preparations were created, and the slides were stained with 4% Giemsa. The cells were analysed for several parameters, including chromosome aberrations (as evidenced by chromosome and chromatid-type breaks), fragments, tetraploidy, and fusion. At least 40 metaphases were analysed from each sample. Images were captured using a Nikon 80i microscope equipped with karyotyping software from Applied Spectral Imaging, Inc. (Vista, CA).

Spectral karyotyping

Spectral karyotyping was performed according to the manufacturer's protocol using Human Paint probes (Applied Spectral Imaging, Carlsbad, CA). Images were captured using a Nikon 80i microscope equipped with spectral karyotyping software (Applied Spectral Imaging).

Co-immunoprecipitation and Western blot analysis

Co-immunoprecipitation and Western blot analysis were performed as described previously [8]. Detailed information is provided in Supplementary methods.

Topo II activity assays

DNA Topo II activity was measured by decatenating kinetoplast DNA (kDNA) with a Topo II assay kit (TopoGEN, Port Orange, FL). In brief, equal volumes of buffers A (0.5 M Tris-HCl, pH=8, 1.5 M NaCl, 100 mM MgCl₂, and 5 mM dithiothreitol) and B (20 mM ATP in water) were mixed to make a fresh 5 $\times$ complete assay buffer. Each 20 μ l of reaction mixture contained 4 μ l of 5 $\times$ complete assay buffer, 200 ng of kDNA, and 100 ng (or 300 ng) of nuclear extract. To determine whether Topo II activity was regulated by MIIP specifically, we used a mixture of 0.25 IU of Topo II α (TopoGEN) and different doses of purified MIIP protein (OriGene, Rockville, MD) to replace the nuclear extracts. This reaction was kept at 37° C for 30 min and stopped with 4 μ l of 5 $\times$ Stop buffer. The mixture was loaded in 1% agarose gel and run for 1 h at 50 V by electrophoresis. The catenated and decatenated kDNA in the gel was stained with ethidium bromide and photographed under ultraviolet

illumination. Topo II activity was quantified on the basis of the amount of decatenated kDNA using Image J software from the National Institutes of Health website (<http://rsb.info.nih.gov/ij/>).

Statistical analysis

We used Student's *t*-test, analysis of variance, chi-square test, Wilcoxon rank-sum test, and Fisher's exact test with SPSS and R 2.10.0 software. Statistical significance was defined as $P < 0.05$. The Benjamini-Hochberg multiple testing correction [15] was used to estimate the false discovery rate.

Results

MIIP downregulation accompanies clinical CRC tumorigenesis and progression

To explore the role of MIIP protein in CRC tumorigenesis and progression, we performed immunohistochemical analysis of MIIP on the TMAs derived from patient samples that included 526 cases of colorectal mucosa, adenoma, and carcinoma tissues from Tianjin Cancer Hospital. The immunohistochemical staining could be evaluated in 518 cases (271 CRCs, 153 adenomas and 94 normal mucosae) (Supplementary Figure S1B). MIIP expression was highest in non-neoplastic mucosa; the levels were lower in adenomas and lowest in carcinomas in the same patient (Figure 1A). This significantly decreased MIIP expression during the mucosa-adenoma-carcinoma sequence was confirmed not only in the 52 cases with matched samples of these three tissue types from the same patient ($P < 0.05$, Figure 1B), but also in the whole 518 cases of colorectal tissue that could be evaluated on TMA ($P < 0.05$, Figure 1C). Detailed clinical correlation analyses revealed that among the 271 CRC patients, a low level of MIIP expression was associated with lymph node ($P = 0.003$) and distant metastasis ($P = 0.020$) (Figure 1D; Supplementary Table S1).

MIIP hemizygous deletion is associated with MIIP downregulation and CRC progression

To determine the genomic status of *MIIP*, we performed a GISTIC analysis of copy number data derived from an Affymetrix SNP 6.0 array analysis of TCGA CRC cases. Among the 188 CRC patients with complete genomic and clinical data, *MIIP* mutation was detected in three diploid cases, including two missense and one frame-shift mutation (Figure 2A), while *MIIP* deletion was found in 52 cases (27.7%). Moreover, all of the cases with *MIIP* deletion had hemizygous deletions. The analysis of the relationship between *MIIP* deletion and expression subtypes (designated by TCGA) showed that *MIIP* hemizygous deletion was more frequent in CIN cases than invasive and MSI/CIMP cases ($P < 0.001$, Supplementary Figure S2A). In addition, *MIIP* deletion demonstrated striking mutual exclusivity with high levels of MSI (MSI-H) ($P = 0.001$, Supplementary Figure S2B). An analysis of the RNA-seq data in the same 188 cases revealed that *MIIP* transcript levels were significantly downregulated in the CRC cases with *MIIP* hemizygous deletion ($P = 0.0005$, Figure 2A). *MIIP* hemizygous deletion was significantly associated with lymph node metastasis ($P = 0.04$) and distant metastasis ($P = 0.02$) (Figure 2B, Supplementary Table S2). In addition, we did not observe an association between *MIIP* hemizygous deletion and the deletion or mutation of other well-established genes in CRC, including *TP53*, *APC*, *PIK3CA*, *KRAS*, *NRAS*, *TGFB2*, *SMAD4*, *MSH6*, and *MSH3* (Supplementary Figure S2C), suggesting that

loss of *MIIP* function may occur mainly through *MIIP* hemizygous deletion which is an important molecular event independent of the well-established genetic events in CRC.

To validate results from the TCGA cohort, we performed FISH analysis of the *MIIP* gene on the TMAs (Figure 2C). FISH results could be assessed in 231 CRC, 137 adenoma and 85 normal mucosa cases. Among the 231 CRC available for FISH analysis on TMA, *MIIP* hemizygous deletion, *MIIP* diploid, and *MIIP* gain were detected in 50 (21.6%), 178 (77.1%), and 3 (1.3%) cases. *MIIP* hemizygous deletion was associated with lymph node ($P=0.007$) and distant metastasis ($P=0.009$) (Figure 2D). Among the 453 colorectal cases available for FISH analysis on TMA, *MIIP* hemizygous deletion, *MIIP* diploid, and *MIIP* gain were detected in 61 (13.5%), 388 (85.7%), and four (0.9%) cases. The ratio of *MIIP* hemizygous deletion was highest in adenocarcinoma and lowest in non-neoplastic mucosa ($P<0.05$ for all, Figure 2E). Moreover, among the 231 CRC patients and 453 colorectal cases available for both FISH and immunohistochemical analysis on TMA, the *MIIP* hemizygous deleted cases showed significantly lower *MIIP* expression ($P<0.001$ for both, Supplementary Table 3). The above results suggest that loss of *MIIP* function may occur mainly through *MIIP* hemizygous deletion and be important in CRC tumorigenesis and progression.

***MIIP* haploinsufficiency promotes colony formation, migration, and invasion**

To identify the role of *MIIP* hemizygous deletion in CRC, we deleted *MIIP* in HCT116 CRC cells using ZFN technology. After genotyping 96 single clones from transfected HCT116 cells with ZFN-*MIIP* constructs, followed by sequencing validation, we found three with *MIIP* hemizygous deletion (*MIIP*+/−) (Figure 3A, lower left panel; Figure 3B). In all three *MIIP*+/− clones, *MIIP* expression was approximately 50% lower than in parental cells (Figure 3A, lower right panel). To obtain clones with homozygous deletion of *MIIP*, we further transfected the *MIIP*+/− clones with ZFN-*MIIP* constructs. We genotyped 180 clones but did not identify an *MIIP* clone with homozygous deletion (*MIIP*−/−), suggesting that *MIIP* is required for cell viability in HCT116 cells. Using the first two confirmed *MIIP* heterozygous deleted clones (*MIIP*+/−_1 and *MIIP*+/−_2), we performed a soft agar assay. Compared with HCT116 parental cells, significantly more colonies were found in both *MIIP*+/− clones ($P=0.001$ for both, Figure 3C). Both *MIIP*+/− HCT116 clones had augmented migration and invasion ($P<0.05$ for all, Figure 3D, E).

We observed a similar increase in colony formation when we used siRNA to downregulate *MIIP* expression in both HCT116 and DLD1 CRC cells (Supplementary Figure S3A,B). *MIIP* knockdown also increased the migration and invasion rates of HCT116 and DLD1 cells (Supplementary Figure S3C-3F).

***MIIP* haploinsufficiency promotes tumour growth and progression in an orthotopic mouse model**

To gain deeper insight into *MIIP*'s role in CRC progression, we used an orthotopic mouse model. We injected HCT116 *MIIP*-diploid (*MIIP*+/+) or *MIIP* haploinsufficiency cells (*MIIP*+/−_1 or *MIIP*+/−_2) into the caecum of nude mice (10 mice per group, three groups). Seven that had been injected with HCT116_ *MIIP*+/+ cells, eight with HCT116_ *MIIP*+/−_1 cells, and nine with HCT116_ *MIIP*+/−_2 cells survived all surgical

procedures. Tumours grew in the caecal wall at similar frequencies in the three groups: six mice (86%) in the HCT116_ *MIIP*^{+/+} group (Figure 4A), seven mice (88%) in the HCT116_ *MIIP*^{+/-}₁ group, and eight mice (89%) in the HCT116_ *MIIP*^{+/-}₂ group (Figure 4B). The *MIIP*^{+/-} cells, however, produced more tumours than did the *MIIP*^{+/+} cells (Figure 4C) and resulted in more frequent lymph node and omental metastases (Figure 4B, D). Moreover, liver metastases were found only in mice injected with HCT116_ *MIIP*^{+/-} cells, including two mice in the HCT116_ *MIIP*^{+/-}₁ group and three in the HCT116_ *MIIP*^{+/-}₂ group (Figure 4B, E). The xenograft model provided strong evidence of the causal role of *MIIP* haploinsufficiency in CRC tumour growth and metastasis.

***MIIP* haploinsufficiency results in a weakened mitotic checkpoint and induces CIN in CRC**

In our previous study, we demonstrated that *MIIP* is a key mitotic regulatory protein that modulates cyclin B1 stability by inhibiting the activity of anaphase promoting complex (APC/C), a ubiquitin ligase E3 in mitosis [8]. Besides cyclin B1, securin is one of the downstream proteins of APC/C^{Cdc20} and critical for sister chromosome segregation and stability [16]. In this study, we determined whether *MIIP* targets the downstream proteins of APC/C^{Cdc20} that are involved in mitosis in CRC cells. Overexpression of *MIIP* via an adenoviral expression system in CRC cells resulted in increased cyclin B1 and securin stability (Supplementary Figure S4A,B). A mitotic transition assay revealed that HCT116_ *MIIP*^{+/-} cells transitioned more quickly from the G2/M phase to the G1 phase than did parental cells (Supplementary Figure S4C,D).

Because dysregulation of mitotic transition and sister chromosome segregation leads to CIN [17], we next determined whether *MIIP* haploinsufficiency induces CIN in CRC cells. HCT116 cells have MSI-H and a relatively normal chromosomal composition that represents a diploid karyotype [18]. Therefore, HCT116 cells provide an opportunity to directly test the causal relationship between *MIIP* haploinsufficiency and CIN. A chromosomal analysis of HCT116_ parental, HCT116_ *MIIP*^{+/-}₁, and HCT116_ *MIIP*^{+/-}₂ cells identified more chromosomal aberrations and chromosomal breaks in both *MIIP*^{+/-} clones than in HCT116_ *MIIP*^{+/+} cells (Figure 5A, 5B). A spectral karyotyping assay further verified that *MIIP* haploinsufficiency led to more chromosome aberrations (Figure 5C, 5D).

***MIIP* haploinsufficiency interrupts the activity of Topo II**

The dramatic alterations from the above chromosomal analysis and spectral karyotyping assay suggest that *MIIP*'s role in maintaining genome stability also involves genes that are involved in regulating chromosomal remodelling. Studies have demonstrated that Topo II α is a critical regulator of sister chromatid unwinding and separation during mitosis [19-21]. Abnormal regulation of Topo II α results in incorrect chromatid separation and breakage [22, 23]. At the cellular level, an anaphase bridge is a common phenotype when Topo II α is abnormally regulated [24]. We next evaluated the anaphase cells in the HCT116_ *MIIP*^{+/+} and HCT116_ *MIIP*^{+/-} cell lines and observed more chromosomal bridges in the HCT116_ *MIIP*^{+/-} cells (Figure 6A, B). We thus sought to determine the possible regulatory relationship between *MIIP* haploinsufficiency and topoisomerase activity. The co-immunoprecipitation assay showed that both Topo II α and Topo II β were detected in the precipitated complex with *MIIP* (Figure 6C). In addition, both Topo II α and Topo II β were

downregulated in *MIIP*^{+/-} cells (Figure 6D). A Topo II activity assay was performed by decatenating kinetoplast DNA (kDNA) as a readout (the cut kDNA migrates into an agarose gel; the uncut catenated DNA remains at the top of the well). As shown in Figure 6E, when purified MIIP was added in increasing doses to the system, with a fixed amount of purified Topo II, catenated DNA was cut in a MIIP dose-dependent manner. Moreover, the density of decatenated kDNA was significantly lower in HCT116_ *MIIP*^{+/-} cells than in *MIIP*^{+/+} cells at two different doses of nuclear proteins (Figure 6F). Therefore, our results provide evidence that *MIIP* haploinsufficiency suppresses Topo II protein level and disturbs the activity of topoisomerases, which is a known cause of CIN.

Etoposide, an inhibitor of TopoII, has been used to treat CRC after failure of front-line therapy [25]. We examined the percentage of sub-G₁ of HCT116_ *MIIP*^{+/+} and HCT116_ *MIIP*^{+/-} cells after treatment with different doses of etoposide at two time points using flow cytometry. The results showed that *MIIP*^{+/-} cells were more sensitive to etoposide than *MIIP*^{+/+} cells (Supplementary Figure S5).

Discussion

According to its genetic alterations, CRC can be broadly classified as having CIN or MSI [26]. CIN, which is characterized by structural or numerical chromosomal alterations, is detected in 85% of CRCs [27]. CIN can induce cancer development and progression and is associated with poor prognosis and drug resistance in CRC [28-31]. Recently, Burrell et al. identified three CIN-suppressor genes that are related to DNA replication stress on chromosome 18q in CRC [18]. *MIIP* is located in 1p36, which is also a region that is commonly deleted in CRC [32, 33]. In this study, we provided functional evidence showing that *MIIP* haploinsufficiency induced CIN in chromosomally stable HCT116 cells. In these experiments, because the *MIIP* gene was the only gene deleted using the ZFN technology, these results strongly suggest that *MIIP* haploinsufficiency is a key driving event for CIN.

CIN can be induced by defective mitotic checkpoints, abnormal chromosome attachment to the mitotic spindle, and pre-mitotic defects that affect chromosome structure [34-36]. In our previous study, we observed that MIIP modulated the stability of cyclin B1, a key mitotic checkpoint protein, by interacting with Cdc20 and inhibiting APC/C^{Cdc20} activity [8]. Our current data demonstrated that besides cyclin B1, MIIP controlled the degradation of securin which is another downstream target of APC/C^{Cdc20} and is critical for sister chromosome segregation and stability. *MIIP* haploinsufficiency would weaken the role of these mitotic check proteins, resulting in abnormal chromosomal separation. We also found that MIIP interacted with TopoII and enhanced its ability to cut double-stranded DNA to release the torsional stress accumulated during mitosis. Thus, *MIIP* haploinsufficiency leads to decreased TopoII activity during mitosis, ultimately causing highly stressed and intertwined chromatids and aberrant chromosomal breaks.

We detected a positive association between *MIIP* haploinsufficiency and metastasis in TCGA CRC cases and an independent validation cohort of CRC patients. To gain insight into whether CIN causes metastasis or metastasis causes genomic instability; we used an orthotopic mouse model and *MIIP* heterozygously-deleted HCT116 cells. HCT116 itself,

which contains two copies of *MIIP*, was not metastatic. However, when one copy of *MIIP* was deleted, leading to decreased *MIIP* expression, the cells became metastatic. Thus, our data support the notion that specific genetic alterations can promote cancer metastasis [26]. However, because we have also demonstrated that *MIIP* inhibits cellular migration and invasion, *MIIP* haploinsufficiency may promote cancer metastasis independent of CIN. Genomic instability and metastasis may be two parallel processes that are intertwined.

In summary, in this study, the combined genomic analyses, *in vitro* molecular studies, and an *in vivo* mouse model provided strong evidence that *MIIP* is a potential tumour suppressor gene in CRC tumorigenesis and progression. Moreover, loss of *MIIP* function may occur mainly through *MIIP* haploinsufficiency. *MIIP* haploinsufficiency led to deleterious unbalance in stabilities of key mitotic checkpoint proteins and reduced activity of Topo II, thus causing aberrant mitosis and chromosome missegregation and finally induced CIN. Given the common loss of 1p36 in multiple cancer types and the importance of CIN as a hallmark of cancer [37-43], *MIIP* haploinsufficiency is likely a common driver event in cancer development and progression. Understanding the mechanisms regulating *MIIP* may improve the clinical management of all CIN cancers. Our data in this study suggests that *MIIP* loss may expose a vulnerability of CRC cells to Etoposide. Further investigations of the modulation of *MIIP* in the Topo network will help us identify CRC patients who are responsive to Topo inhibitors thus improving the therapeutic efficacy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Siegel R, Ma J, Zou Z, et al. Cancer statistics, 2014. *CA Cancer J Clin.* 2014; 64:9–29. [PubMed: 24399786]
2. Chew MH, Teo JY, Kabir T, et al. Stage IV colorectal cancers: an analysis of factors predicting outcome and survival in 728 cases. *J Gastrointest Surg.* 2012; 16:603–612. [PubMed: 22009464]
3. Ilyas M, Straub J, Tomlinson IP, et al. Genetic pathways in colorectal and other cancers. *Eur J Cancer.* 1999; 35:1986–2002. [PubMed: 10711241]
4. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. *N Engl J Med.* 1988; 319:525–532. [PubMed: 2841597]
5. Simons CC, Hughes LA, Smits KM, et al. A novel classification of colorectal tumors based on microsatellite instability, the CpG island methylator phenotype and chromosomal instability: implications for prognosis. *Ann Oncol.* 2013; 24:2048–2056. [PubMed: 23532114]

6. Yamagishi H, Kuroda H, Imai Y, et al. Molecular pathogenesis of sporadic colorectal cancers. *Chin J Cancer*. 2016; 35:4. [PubMed: 26738600]
7. Domingo E, Ramamoorthy R, Oukrif D, et al. Use of multivariate analysis to suggest a new molecular classification of colorectal cancer. *J Pathol*. 2013; 229:441–448. [PubMed: 23165447]
8. Ji P, Smith SM, Wang Y, et al. Inhibition of gliomagenesis and attenuation of mitotic transition by MIIP. *Oncogene*. 2010; 29:3501–3508. [PubMed: 20418911]
9. Song SW, Fuller GN, Khan A, et al. Iip45, an insulin-like growth factor binding protein 2 (IGFBP-2) binding protein, antagonizes IGFBP-2 stimulation of glioma cell invasion. *Proc Natl Acad Sci U S A*. 2003; 100:13970–13975. [PubMed: 14617774]
10. Sun Y, Yokoi K, Li H, et al. NGAL expression is elevated in both colorectal adenoma-carcinoma sequence and cancer progression and enhances tumorigenesis in xenograft mouse models. *Clin Cancer Res*. 2011; 17:4331–4340. [PubMed: 21622717]
11. Comprehensive molecular characterization of human colon and rectal cancer. *Nature*. 2012; 487:330–337. [PubMed: 22810696]
12. Liu G, Sun Y, Ji P, et al. MiR-506 suppresses proliferation and induces senescence by directly targeting the CDK4/6-FOXM1 axis in ovarian cancer. *J Pathol*. 2014; 233:308–318. [PubMed: 24604117]
13. Sun B, Sun Y, Wang J, et al. The diagnostic value of SYT-SSX detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) for synovial sarcoma: a review and prospective study of 255 cases. *Cancer Sci*. 2008; 99:1355–1361. [PubMed: 18460022]
14. Sun Y, Hu L, Zheng H, et al. MiR-506 inhibits multiple targets in the epithelial-to-mesenchymal transition network and is associated with good prognosis in epithelial ovarian cancer. *J Pathol*. 2015; 235:25–36. [PubMed: 25230372]
15. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Royal Statistical Society Series B*. 1995; 57:289.
16. Waizenegger IC, Hauf S, Meinke A, et al. Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell*. 2000; 103:399–410. [PubMed: 11081627]
17. Nojima H. Cell cycle checkpoints, chromosome stability and the progression of cancer. *Hum Cell*. 1997; 10:221–230. [PubMed: 9573481]
18. Burrell RA, McClelland SE, Endesfelder D, et al. Replication stress links structural and numerical cancer chromosomal instability. *Nature*. 2013; 494:492–496. [PubMed: 23446422]
19. Robinson MJ, Martin BA, Gootz TD, et al. Effects of quinolone derivatives on eukaryotic topoisomerase II. A novel mechanism for enhancement of enzyme-mediated DNA cleavage. *J Biol Chem*. 1991; 266:14585–14592. [PubMed: 1650363]
20. Chen GL, Yang L, Rowe TC, et al. Nonintercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA topoisomerase II. *J Biol Chem*. 1984; 259:13560–13566. [PubMed: 6092381]
21. Hashash N, Johnson AL, Cha RS. Topoisomerase II- and condensin-dependent breakage of MEC1ATR-sensitive fragile sites occurs independently of spindle tension, anaphase, or cytokinesis. *PLoS Genet*. 2012; 8:e1002978. [PubMed: 23133392]
22. Warsi TH, Navarro MS, Bachant J. DNA topoisomerase II is a determinant of the tensile properties of yeast centromeric chromatin and the tension checkpoint. *Mol Biol Cell*. 2008; 19:4421–4433. [PubMed: 18701701]
23. Wang LH, Schwarzbraun T, Speicher MR, et al. Persistence of DNA threads in human anaphase cells suggests late completion of sister chromatid decatenation. *Chromosoma*. 2008; 117:123–135. [PubMed: 17989990]
24. Dawlaty MM, Malureanu L, Jeganathan KB, et al. Resolution of sister centromeres requires RanBP2-mediated SUMOylation of topoisomerase IIalpha. *Cell*. 2008; 133:103–115. [PubMed: 18394993]
25. Jeremic B, Acimovic L, Mijatovic L. Carboplatin and etoposide in advanced colorectal carcinoma. A phase II study. *Cancer*. 1993; 71:2706–2708. [PubMed: 8467452]

26. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature*. 1998; 396:643–649. [PubMed: 9872311]
27. Hung KE, Chung DC. New insights into the molecular pathogenesis of colorectal cancer. *Drug Discov Today Dis Mech*. 2006; 3:439. [PubMed: 20151020]
28. Stoler DL, Chen N, Basik M, et al. The onset and extent of genomic instability in sporadic colorectal tumor progression. *Proc Natl Acad Sci U S A*. 1999; 96:15121–15126. [PubMed: 10611348]
29. Shih IM, Zhou W, Goodman SN, et al. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. *Cancer Res*. 2001; 61:818–822. [PubMed: 11221861]
30. Nowak MA, Komarova NL, Sengupta A, et al. The role of chromosomal instability in tumor initiation. *Proc Natl Acad Sci U S A*. 2002; 99:16226–16231. [PubMed: 12446840]
31. Watanabe T, Kobunai T, Yamamoto Y, et al. Chromosomal instability (CIN) phenotype, CIN high or CIN low, predicts survival for colorectal cancer. *J Clin Oncol*. 2012; 30:2256–2264. [PubMed: 22547595]
32. Tanaka K, Yanoshita R, Konishi M, et al. Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. *Oncogene*. 1993; 8:2253–2258. [PubMed: 8101648]
33. Bomme L, Bardi G, Pandis N, et al. Chromosome abnormalities in colorectal adenomas: two cytogenetic subgroups characterized by deletion of 1p and numerical aberrations. *Hum Pathol*. 1996; 27:1192–1197. [PubMed: 8912830]
34. Thompson SL, Compton DA. Chromosomes and cancer cells. *Chromosome Res*. 2011; 19:433–444. [PubMed: 21190130]
35. Janssen A, van der Burg M, Szuhai K, et al. Chromosome segregation errors as a cause of DNA damage and structural chromosome aberrations. *Science*. 2011; 333:1895–1898. [PubMed: 21960636]
36. Crasta K, Ganem NJ, Dagher R, et al. DNA breaks and chromosome pulverization from errors in mitosis. *Nature*. 2012; 482:53–58. [PubMed: 22258507]
37. Caren H, Fransson S, Ejekkar K, et al. Genetic and epigenetic changes in the common 1p36 deletion in neuroblastoma tumours. *Br J Cancer*. 2007; 97:1416–1424. [PubMed: 17940511]
38. Ichimura K, Vogazianou AP, Liu L, et al. 1p36 is a preferential target of chromosome 1 deletions in astrocytic tumours and homozygously deleted in a subset of glioblastomas. *Oncogene*. 2008; 27:2097–2108. [PubMed: 17934521]
39. Loukopoulou P, Shibata T, Katoh H, et al. Genome-wide array-based comparative genomic hybridization analysis of pancreatic adenocarcinoma: identification of genetic indicators that predict patient outcome. *Cancer Sci*. 2007; 98:392–400. [PubMed: 17233815]
40. Alvarez AA, Lambers AR, Lancaster JM, et al. Allele loss on chromosome 1p36 in epithelial ovarian cancers. *Gynecol Oncol*. 2001; 82:94–98. [PubMed: 11426968]
41. Tsukamoto K, Ito N, Yoshimoto M, et al. Allelic loss on chromosome 1p is associated with progression and lymph node metastasis of primary breast carcinoma. *Cancer*. 1998; 82:317–322. [PubMed: 9445188]
42. Girgis AH, Iakovlev VV, Beheshti B, et al. Multilevel whole-genome analysis reveals candidate biomarkers in clear cell renal cell carcinoma. *Cancer Res*. 2012; 72:5273–5284. [PubMed: 22926558]
43. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]

Figure 1. MIIP protein downregulation accompanies clinical CRC development and progression MIIP expression was evaluated using immunohistochemical staining on TMAs. **A.**

Respective images of MIIP expression in normal mucosa, adenoma, and adenocarcinoma from the same patient. Scale bars in the top panel, 500 μ m. Scale bars in the lower panel, 100 μ m. **B.** A statistical analysis revealed that MIIP expression was significantly decreased during the mucosa-adenoma-carcinoma sequence in the 52 sets of matched samples. **C.** A statistical analysis revealed that MIIP expression was highest in non-neoplastic mucosa, lower in adenoma, and lowest in carcinoma in 518 cases. **D.** A statistical analysis revealed that low MIIP expression was correlated with lymph node metastasis and distant metastasis in 271 CRC patients.

Figure 2. *MIIP* hemizygous deletion is associated with *MIIP* downregulation and CRC progression in TCGA CRC cases and an independent cohort

A and B. The genomic analysis of *MIIP* and the association between *MIIP* deletion and metastasis in TCGA CRC cases. Among the 188 CRC patients with complete genomic and clinical data, *MIIP* mutation was detected in three diploid cases, and *MIIP* hemizygous deletion was found in 52 cases. *MIIP* mRNA expression levels were significantly lower in *MIIP*-hemizygous cases than in *MIIP*-diploid CRC within the TCGA data (A). Positive associations were present between *MIIP* hemizygous deletion and lymph node and distant metastasis in TCGA (B). **C-E.** FISH analysis for 526 colorectal tissue samples in Tianjin cohort. Dual-colour FISH analysis of TMA sections using a Spectrum-Green-labelled CEP 1 and a Spectrum-Orange-labelled locus-specific *MIIP* probe (Empire Genomics, Buffalo, New York, USA). The FISH sections were evaluated in 30-90 cells of each case. Respective FISH images from normal mucosa and CRC (C). Two pairs of green and red signals (*MIIP* diploid) were detected in one nucleus of normal mucosa (N), whereas two copies of green signals and one copy of the red signal (*MIIP* hemizygous deletion) were detected in each nucleus of CRC cells. The statistical analysis showed that *MIIP* hemizygous deletion was correlated with lymph node metastasis and distant metastasis (D). Among the 453 colorectal cases available for FISH analysis on TMA, the ratio of *MIIP* hemizygous deletion gradually increased during the mucosa-adenoma-carcinoma sequence (E).

Figure 3. *MIIP* haploinsufficiency promotes colony formation, cell migration, and invasion

A. ZFN-mediated *MIIP* deletion in HCT116 cells. Top panel: A pair of ZFNs (ZFN-1 and ZFN-2) was designed to target the *MIIP* gene at exon 7; each zinc finger array recognized 18 nt of the *MIIP* target site to ensure specificity. Primers 1 and 2, used for genotyping, are described in the Materials and Methods. Lower panel: Three clones containing *MIIP* deletion (*MIIP* +/-) were identified by genotyping (left), with identification of the 317-bp fragment. *MIIP* expression was lower in the three *MIIP* +/- clones, as determined by Western blotting analysis (right). **B.** *MIIP* haploinsufficiency was confirmed by sequencing. The data revealed a 38 bp deletion around the ZFN-targeted site. **C.** *MIIP* haploinsufficiency increased colony formation in soft agar. Respective images of HCT116 *MIIP*-diploid and *MIIP*-haploinsufficient cells after growing in soft agar for 2 weeks (top panel). The number of colonies represents the mean $\pm$ SEM for triplicate tests. **D and E.** *MIIP* haploinsufficiency increased cell migration and invasion. Respective images of HCT116 *MIIP*-diploid and *MIIP*-haploinsufficient cells migrating through an 8- μ m pore size membrane (migration assay) after 20 h of incubation (D, top panel) and invading through an 8- μ m pore size membrane coated with Matrigel (invasion assay) after 24 h of incubation (E, top panel). The migrating or invading cell numbers on each filter were counted, and data were plotted as the fold change by defining the number in parental cells as 1. The fold change of migrating or invading cells is the mean $\pm$ SEM for triplicate tests.

Figure 4. *MIIP* haploinsufficiency promotes tumour growth and progression in a CRC orthotopic mouse model

HCT116_ *MIIP*^{+/+}, HCT116_ *MIIP*^{+/-}_1, and HCT116_ *MIIP*^{+/-}_2 cells were injected into the caecal walls of nude mice. **A.** Photographs of the nude mice injected with HCT116_ *MIIP*^{+/+} cells, their orthotopic tumours, and the respective histological images. Orthotopic tumours (red circle) were found, with invasion of tumour cells (arrows) in the muscularis propria of the caecal wall (arrowheads). **B.** In the mice injected with HCT116_ *MIIP*⁻ haploinsufficient cells, lymph node and omental metastases (green circles) and liver metastases (green arrows) were found in addition to orthotopic tumours (red circle). In the orthotopic tumours, HCT116_ *MIIP*^{+/-} cells (arrows) invaded into the mucosa (arrowheads) from the caecal wall. **C.** *MIIP* haploinsufficiency induced more tumours in the cecum in the CRC orthotopic mouse model. Tumour number is the mean $\pm$ SEM. **D.** Histological images of lymph node metastasis in mice injected HCT116_ *MIIP*⁻ haploinsufficient cells. Arrows and arrowheads represent metastatic tumour cells and lymphocytes respectively in lymph nodes. **E.** Liver metastasis is shown in a mouse injected with HCT116_ *MIIP*⁻ haploinsufficient cells. Arrows represent cancer cells, and arrowheads represent liver tissue. Scale bars: 100 μ m.

Figure 5. *MIIP* haploinsufficiency induces chromosome instability in CRC cells

A and B. The genomic instability analysis for HCT116 *MIIP*-diploid and *MIIP*-haploinsufficient cells. The representative metaphase of HCT116_ *MIIP* +/+, HCT116_ *MIIP* +/–_1 and HCT116_ *MIIP* +/–_2 cells (A). The red arrows indicate chromosome breaks; the blue arrows indicate chromosome fusion. Compared with *MIIP*-diploid cells, *MIIP*-haploinsufficient cells had increased chromosomal abnormalities, especially more chromosome breaks and fusions (B). At least 40 metaphases were analysed from each sample. **C and D.** The spectral karyotyping (SKY) analysis for HCT116 *MIIP*-diploid cells and *MIIP*-haploinsufficient cells. Example of spectral karyotypes of HCT116_ *MIIP* +/+ and HCT116_ *MIIP* +/– cells (C). Arrows show deletions (green) and structural aberrations (red). Compared with *MIIP*-diploid cells, *MIIP*-haploinsufficient cells had more chromosome aberrations (D). Approximately 10-15 metaphase spreads per sample were analysed in each experiment. Chromosome aberrations per metaphase is the mean $\pm$ SEM for triplicate tests.

Figure 6. *MIIP* haploinsufficiency reduces the decatenation activity of topo II and induces incorrect chromatid separation

A. The respective images of anaphase analysis of HCT116_ *MIIP*^{+/+} and HCT116_ *MIIP*^{+/-} cells. **B.** A statistical analysis of three independent experiments showed that the percentage of abnormal anaphase cells in counted *MIIP*-haploinsufficient cells was significantly higher than for *MIIP*-diploid cells. **C.** Co-immunoprecipitation experiments. HCT116 nuclear extract was subjected to immunoprecipitation with an antibody to *MIIP* and analysed for co-immunoprecipitation of Topo II α , and Topo II β . Both Topo II α and Topo II β were detected in the precipitated complex. **D.** Western blot assay for Topo II α and Topo II β of *MIIP*-diploid and *MIIP*-haploinsufficient cells. Compared with *MIIP*-diploid cells, both Topo II α and Topo II β levels were decreased in *MIIP*-haploinsufficient cells. **E.** TopoII activity assay. Differing amounts of the purified TopoII enzyme and human recombinant *MIIP* protein were used, as indicated. The uncut catenated kDNA remained at the top of the well; the cut kDNA (decatenated kDNA) migrated into an agarose gel (top panel). The decatenated kDNA bands were shown in enhanced UV exposure in the middle panel. Quantification using Image J software revealed that the density of decatenated Kdna (lower panel). **F.** Topo II activity assay for HCT-116 *MIIP*-diploid and *MIIP*-haploinsufficient cells. The decatenated kDNA bands are shown using enhanced UV exposure in the middle panel. Quantification using Image J software revealed that the density of decatenated kDNA was significantly lower in HCT116 *MIIP*-haploinsufficient cells than in *MIIP*-diploid cells at both doses of nuclear proteins (lower panel).