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Original Article

ATMIN enhances invasion by altering PARP1 in MSS colorectal cancer

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Abstract: Genomic instability is a key cancer indicator. It results from defects in the DNA damage response (DDR) and increased replication stress. Herein, we examined how ataxia-telangiectasia mutated interactor (ATMIN), a DDR pathway involved in mismatch repair-proficient (microsatellite stability [MSS]), acts in colorectal carcinoma (CRC). Firstly, *ATMIN* mRNA expression was detected in CRC specimens with MSS characteristics, and the effects of ectopic ATMIN expression and ATMIN knockdown on invasion abilities were gauged in MSS cell lines. To understand the molecular mechanism, co-immunoprecipitation analyses in vitro were employed. Interestingly, ATMIN expression was positively correlated with advanced stages ($P < .001$), lymph node metastases ($P = .002$), and deeper invasion ($P = .037$) in MSS tumors; and significantly changed the cell motility in vitro. In the high-throughput analysis, ATMIN was found to act on the Wnt signaling pathway via PARP1. PAPR1 inhibition, in turn, significantly decreased invasion abilities resulting from ATMIN overexpression in cancer cell. Taken together, ATMIN, which alters the Wnt signaling pathway regulating CRC progression, plays as a crucial prognostic factor in MSS tumors.

Keywords: ATMIN, PARP1, MSS, Wnt

Introduction

One of the most prevalent cancers globally, colorectal carcinoma (CRC) ranks second in causes of cancer deaths in the United States [1, 2]. Its characteristic traits of local invasion and distal metastasis mean that the five-year survival rate stands at only 55% [3-5]. CRCs fall into one of two groups: microsatellite stability (MSS) and microsatellite instability (MSI). Approximately 80-85% of CRCs categorized as MSS possess proficient DNA mismatch repair systems. These tumor cells may exhibit aneuploidy, amplifications, translocations, allelic losses, and chromosomal gains [6-9]. In contrast, MSI-high tumors frequently present with poorly differentiated mucinous histological features and marked lymphocytic infiltration. Interestingly, patients with MSI-high tumors show a more favorable prognostic clinical outcome than those with MSS tumors, but have worse survival in stage IV [10]. With the rapid development of drug types, chemotherapy, immunotherapy, and target therapy, are wildly using in various cancers. It is well known the identification of microsatellite stability status is clinically important and they respond differently to 5FU-based adjuvant chemotherapy and anti-PD1/PDL-1 depending on this status [11-14]. Observations such as these underline the importance of understanding the ways in which MSI and MSS CRC differ biologically and also have significant implications for treatment.

To deal with the DNA damage constantly occurring within them, cells have developed a constitutive response, deploying repair pathways according to the form of damage detected and the machinery available [15]. The ataxia telangiectasia mutated interactor (ATMIN) was first identified as a DNA damage response (DDR) protein involved in the base excision repair pathway of the oxidative stress response with ataxia telangiectasia mutated (ATM) [16-18]. It can be phosphorylated by ATM kinases to induce DNA repair activity and under stress conditions functions as a checkpoint regulating chromatin damage [16, 19]. Hypotonic stress

may induce it to complement the Nijmegen breakage syndrome 1 (NBS1) protein in ATM activation. However, ATMIN may be dispensable in irradiation-induced ATM signaling [16, 19-21]. Since ATM-null mice are viable, whereas ATMIN-null mice are exhibited with severe pulmonary developmental defects and are embryonic lethal [13, 14], it is apparent that ATMIN has ATM-independent functions. Indeed, it may be a critical transcription factor in the regulation of genomic stability, aging, and tumor progression [20-23].

In our previous study [24], we documented that ATMIN suppresses metastasis by altering the Wnt signaling pathway in MSI-high tumors. Here, we aim to focus on the role of ATMIN in CRC with MSS and to identify the underlying biological mechanism. This study shows that ATMIN enhances invasion ability by altering PARP1 protein expression. These findings indicate that ATMIN influences CRC progression depending on microsatellite status.

Materials and methods

Study subjects and microsatellite analyses

Institutional Review Board approval for this study was given by the Research Ethics Committee of National Taiwan University (NTU) hospital. Samples were collected from patients (n = 89) with primary colorectal adenocarcinoma who underwent surgical resection at this hospital between December 2003 and July 2005. The corresponding normal mucosae were also sampled from these patients from the margin of the distant resection which was histologically free from the tumor. All tumor samples were obtained and handled in accordance with the guidelines of the Institutional Review Board at NTU. Microsatellite status analyses were performed using five mononucleotide microsatellite markers (BAT26, BAT25, NR21, NR24, and MONO27). Tumors were designated as MSI-positive if novel allele lengths in the tumor were observed at two or more microsatellite loci when compared to the normal tissue. When no novel allele lengths were observed in the tumor, it was defined as MSS positive.

Immunoprecipitation analysis

Samples were incubated at 4°C for 2 h with no antibody, or a polyclonal antibody specific for β-catenin or TCF4. Immune complexes were recovered by blocked protein A (50% protein A-Sepharose, Amersham Bioscience, Corston, UK) for another 1 h. The sepharose beads were washed six times. The samples were validated by Western blot.

In vitro invasion assay

Transwells with 8 μm pore size were coated with 40 μg of Matrigel (BD Biosciences, Mississauga, ON, Canada) in 24-well dishes (Nucleopore Corp., Pleasanton, CA). Approximately 3×10^5 cells in 100 μL of complete medium were placed in the upper chamber, and 1 mL of the same medium was placed in the lower chamber. Following 48 h of incubation, the cells were fixed in methanol for 15 min, and the upper side filters were cleared with cotton-tipped swabs. The filters were then stained with 0.05% crystal violet for 15 min. The filters were viewed using a Leica Microsystems microscope (Type: 090-135.001, Wetzlar, Germany).

Luciferase reporter assay

A total of 2×10^5 cells of transfectants were transfected with 1.5 μg of the luciferase reporter constructs Top-Flash or Fop-Flash. Top-Flash contained three copies of the T-cell factor (Tcf)/lymphoid enhancer factor (LEF)-binding site (AAGATCAAAGGGGGT) upstream of a thymidine kinase minimal promoter. Fop-Flash contained a mutated T-cell factor/lymphoid enhancer factor Tcf/LEF-binding site (AAGGCCAAGGGGGT). Four hours after transfection, the cells were transferred and cultured in normal complete medium for another 48 h. Luciferase activity was measured using the dual-Luciferase reporter assay system (Promega Corporation).

Real-time polymerase chain reaction analysis

Gene expression assays were performed using real-time polymerase chain reaction (RT-PCR) with the FastStart universal probe master mix (Roche, Basel, Switzerland). The reaction was amplified using an ECO sequence detector (Illumine, San Diego, California). Commercial primers specific for ATMIN (HS00739820-M1) and the control gene *B2M* (HS99999907-M1) were designed by Applied Biosystems (TaqMan® Gene Expression Assays, Foster City, CA). Relative mRNA expression levels were calculated in samples and normal control samples. Results were normalized against the *B2M* values.

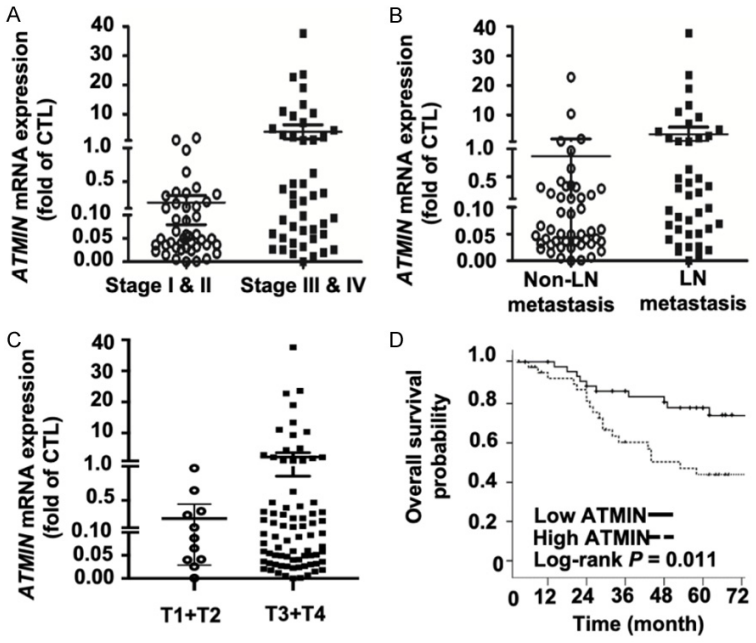

Figure 1. *ATMIN* mRNA expression is opposite in MSS CRC patients. (A-C) The correlations of *ATMIN* expression and TNM stage (A), lymph node metastasis (B), and tumor invasion level (C) in and MSS CRCs were analyzed by quantitative PCR and normalized using *B2M*. *P < .05. (D) The correlations of *ATMIN* expression and overall survival rate in MSS CRCs were calculated through ROC analysis.

Table 1. Association of ATMIN expression with clinical and pathological features in CRC patients with MSS status

Characteristics	*N*	Expression of ATMIN (mean ± SD)	*P* value
Patient number	89		
Age			0.649
< 70	50	2.672 ± 6.358	
≥ 70	39	2.042 ± 7.259	
Gender			0.592
Male	55	2.031 ± 6.265	
Female	34	2.788 ± 7.512	
T classification			0.037#
T1-T2	11	0.238 ± 0.311	
T3-T4	78	2.356 ± 6.291	
Node metastasis			0.002#
N0	46	0.900 ± 3.639	
N1-N2	43	3.372 ± 7.496	
Differentiation			0.704
Well~moderate	81	2.380 ± 7.046	
Poor	8	1.870 ± 3.845	
Clinical stage			< 0.001#
I, II	45	0.415 ± 1.565	
III, IV	44	3.813 ± 7.964	

#Statistical significance (P < 0.05).

Immunofluorescence

Cells on glass coverslips were fixed and permeated, then incubated with primary antibodies. FITC-conjugated secondary antibodies were used to visualize the location of target proteins. Cell nuclei were counterstained with DAPI and fluorescence images were captured using a Leica DMRE epifluorescence microscope.

Statistical analyses

Scale variables (expressed as the standard deviation of the mean) were compared using the Mann-Whitney test, and nominal variables were compared using the Fisher's exact test. Survival and recurrence data were analyzed using the Kaplan-Meier method. Kaplan-Meier curves were compared using a log-rank test. All statistical tests were two-sided, and the values are expressed as means with 95% confidence intervals. All results with P < .05 were considered statistically significant.

Results

ATMIN mRNA expression is positively correlated with the clinical parameters of MSS CRC patients

The study looked at eighty-nine MSS CRC patients so as to test the extent to which ATMIN expression was correlated with invasiveness. Quantitative RT-PCR assays were employed to examine the clinical relevance of ATMIN mRNA expression in the patients' tumors. The levels of ATMIN expression in MSS tumors were positively correlated with advanced clinical stage, lymph node metastases, and tumor invasion (**Figure 1A-C** and **Table 1**). We further defined

the relationship between ATMIN expression and patient postoperative survival by setting cutoff values for the ATMIN mRNA levels (fold change > 0.09 in MSS tumors) using ROC analysis to most accurately predict probability of recurrence. Patients were then assigned to the following ATMIN expression categories according to this criterion: high (fold change > cutoff value) or low (fold change ≤ cutoff value). Kaplan-Meier survival curves showed that the patients with low ATMIN expression in MSS CRC tumors (n = 47) survived significantly longer than those with high ATMIN expression (n = 42; P = 0.011; **Figure 1D**). The cumulative 5-year survival rate was 75.1% in the patients with low ATMIN expression, but only 43.5% in those with high ATMIN expression. Taken as a whole, our data suggest that ATMIN is a prognostic indicator of both stage and survival in patients with MSS.

ATMIN increases MSS cell invasion abilities in vitro

In order to ascertain whether there was a direct relationship between ATMIN and MSS CRC invasion, we employed four MSS CRC cell lines to demonstrate a greater invasion ability for higher endogenous ATMIN expression than for lower ATMIN expression (**Figure 2A**). Next, we transiently knocked-down ATMIN expression in SW620 cells and overexpressed ATMIN expression in SW620 and SW480 cells to assess the motility of the cells. Downregulation of ATMIN in SW620 cells significantly decreased invasion abilities (**Figure 2B**). The gain-of-function, which transiently expressed ATMIN-expressing plasmids in SW620 and SW480, exhibited markedly enhanced invasion abilities (**Figure 2C** and **2D**). Similarly, stable downregulation of ATMIN in SW620 cells or ATMIN overexpression in SW480 cells also demonstrated reduced or enhanced cell invasion, respectively (**Figure 2E** and **2F**). Collectively, these *in vitro* data suggested that ATMIN expression enhances the invasion in MSS tumor cells.

To explore the roles of ATM, an important interactor protein of ATMIN in the mechanism of ATMIN-enhanced MSS CRC cell invasiveness, we transduced silenced-ATM expression plasmids (shATM#1 and #2) into SW480 and ATMIN stable transfectants. The results showed that ATMIN increased the MSS CRC cell invasion in an ATM-independent manner (**Figure 2G** and **2H**).

ATMIN mediates the activity of the Wnt signaling pathway in MSS cancer cells

ATMIN's unique function in the MSS system prompted us to perform a high-throughput microarray assay in stable shATMIN SW620 transfectants to discover the possible downstream genes (**Figure 3A**, GSE58550). An ingenuity pathway analysis (IPA) showed that *β*-catenin is a central pivot in the ATMIN-regulated MSS CRC system (**Figure 3B**). Through a gene set enrichment analysis (GSEA), the entire sets of *adenomatous polyposis coli (APC)* target genes were markedly downregulated as a group after ATMIN knockdown in MSS SW620 cells (**Figure 3C**).

Transfection of the TCF4 luciferase reporter (Top-Flash) and its control vector (Fop-Flash) were used to further investigate the effects of ATMIN on β-catenin/TCF transcriptional activity in MSS transfectants. ATMIN knockdown decreased Wnt signaling transcriptional activity in SW620 transfectants (**Figure 3D**, left panel). As expected, ATMIN overexpression increased Wnt signaling transcriptional activity in SW480 transfectants (**Figure 3D**, right panel). In immunofluorescent staining, β-catenin protein was shown to be markedly concentrated in the cell nucleus of SW480/ATMIN cells (**Figure 3E**), but not in the SW480/neo clone.

A crucial factor in the regulation of CRC tumorigenesis and progression is played by *Wnt*/β-catenin signaling. Indeed, *Wnt* pathway activity may be controlled by protein-protein interactions, including β-catenin and TCF4 [25]. To further dissect the β-catenin/TCF4 binding complex in the ATMIN transfectants, immunoprecipitation and Western blot analysis was performed. As shown in **Figure 3F**, ATMIN transfectants yielded more abundant TCF4/β-catenin protein-protein complexes than SW480/neo clones (**Figure 3F**, left panel). Furthermore, silenced-ATMIN clones yielded a lesser quantity of the TCF4/β-catenin protein complex than SW620/neo control cells (**Figure 3F**, right panel). These data showed that ATMIN was able to modulate the binding affinity of TCF4/β-catenin protein-protein complex.

PARP1 expression attributes invasion ability in MSS tumors

In our previous study [24], PARP1 was the target protein of ATMIN in MSI-high tumors. In this

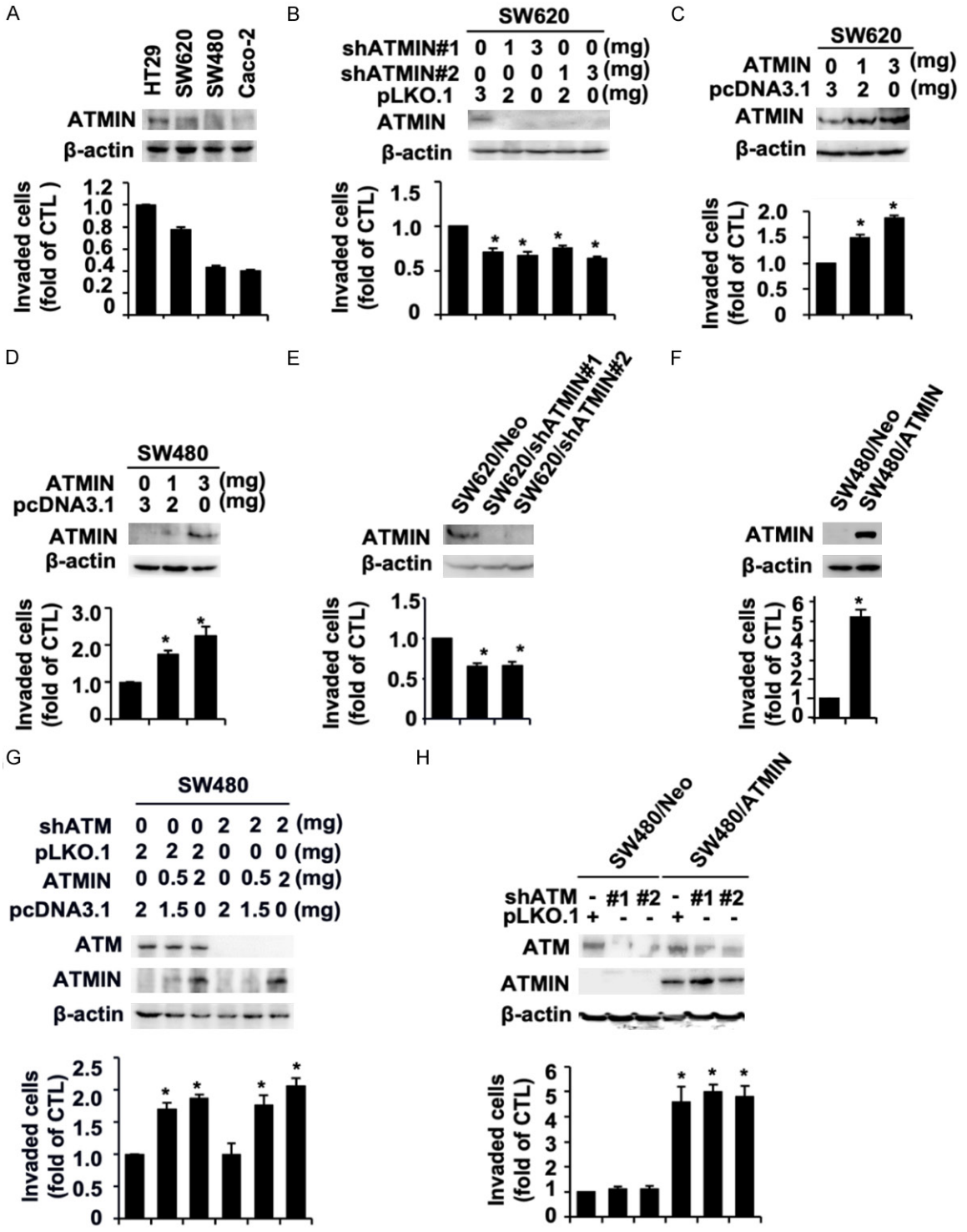

Figure 2. ATMIN positively regulates invasion ability in MSS cells. A. ATMIN protein expression in MSS cells was analyzed through Western blotting. B. SW620 were transiently transfected with shATMIN plasmids and subjected to invasion assays. *P < .005. C, D. SW620 cells and SW480 cells were transiently transfected with ATMIN-expressing plasmids and subjected to invasion assays. *P < .005. E, F. SW620 cells and SW480 cells were stably transfected with shATMIN or ATMIN plasmids and subjected to invasion assays. *P < .005. G, H. SW480 were Co-transfected with shATM and ATMIN plasmids and subjected to invasion assays. Invasion assays were performed using 8-μm pore trans-wells. After incubating for 48 hours, the cells were stained with crystal violet and counted.

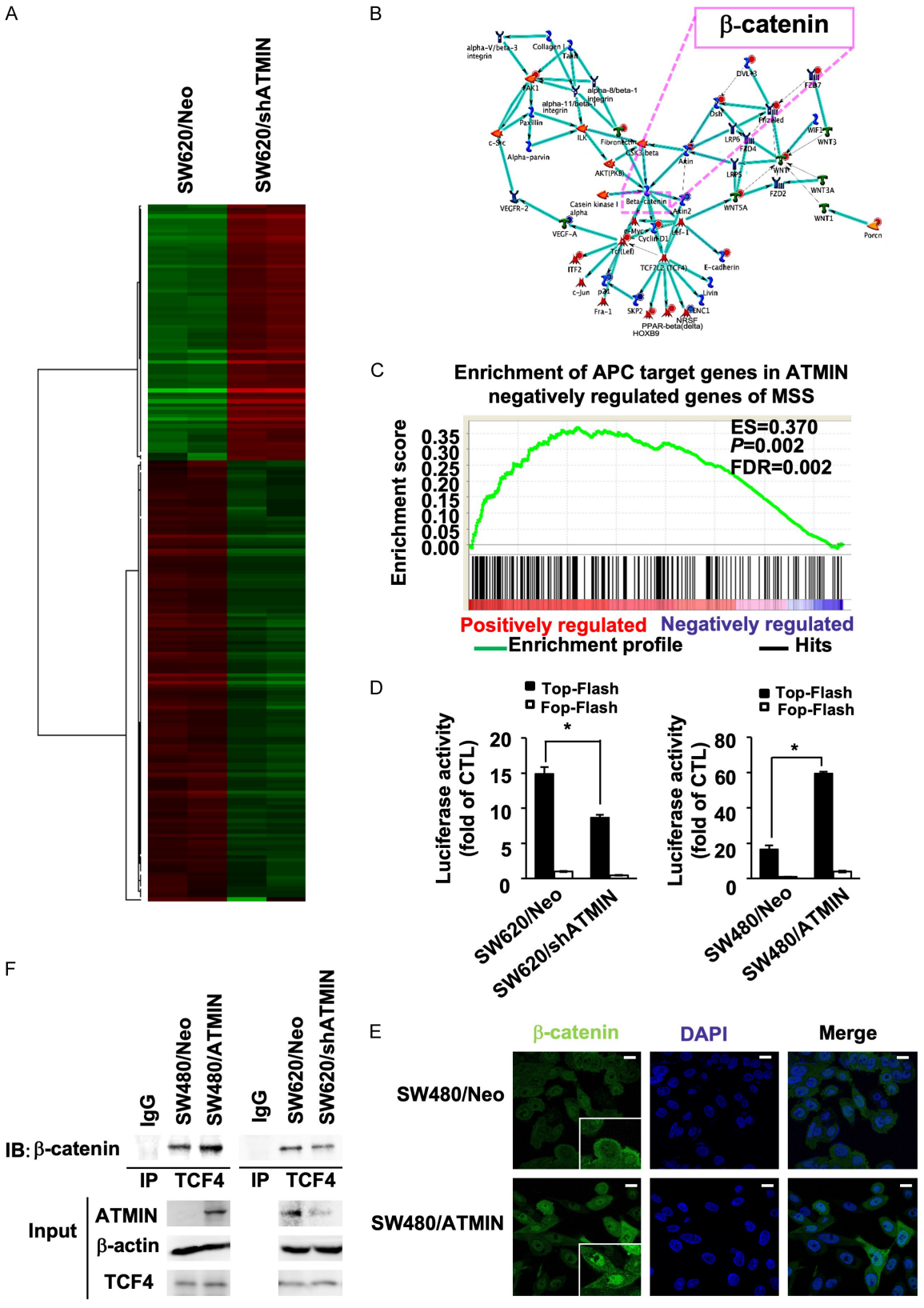

Figure 3. ATMIN activates canonical Wnt signaling genes in MSS cells. A. The high-throughput analysis was performed in shATMIN stable transfectants of SW620 transfectants. B. The IPA indicated that β-catenin was critical for ATMIN manipulated in MSS cells. C. GSEA of the shATMIN profiles in MSS cells showed that the APC target genes were significantly enriched. D. Luciferase activity assays of SW620 and SW480 stable transfectants were performed

with the plasmid Top-Flash or Fop-Flash (n = 4). *P < .05. E. Immunofluorescence of β-catenin and DAPI in SW480 transfectants. Scale bar 10 μM. F. The binding affinities of β-catenin/TCF4 in SW480 and SW620 transfectants were investigated using immunoprecipitation analyses.

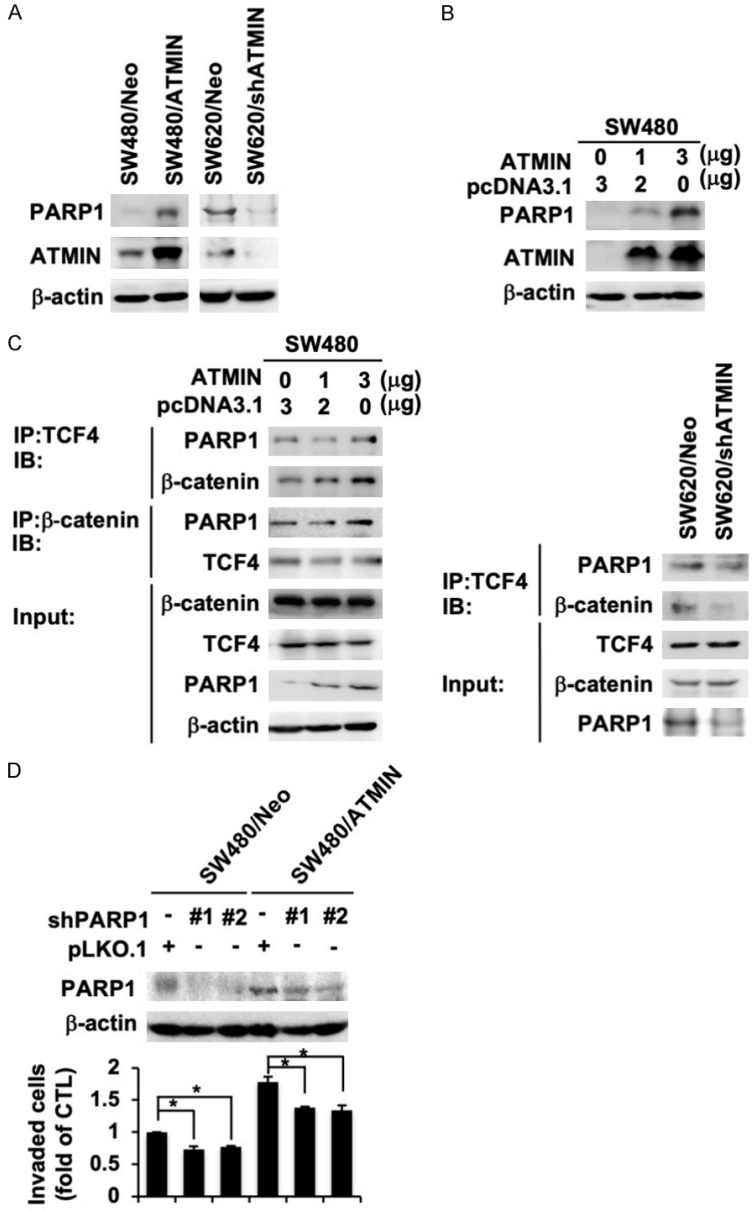

Figure 4. ATMIN manipulated PARP1 expression to regulate the binding affinity of β-catenin/TCF4 complex. A, B. The expression of β-catenin/TCF4 enhancer, PARP1, was measured by Western blotting in SW480 and SW620 transfectants or transiently transfected cells. C. The expression of the β-catenin/TCF4/PARP1 complex was performed using immunoprecipitation/Western blot analysis in SW480 transiently transfected cells and SW620 transfectants. D. SW480 stable transfectants were transiently transfected with shPARP1 plasmid. Invasion assays were performed using 8-μm pore trans-wells. *P < .05.

study, we used RT-PCR and WB analysis to ascertain the part played by PARP1 in MSS tumors. We found that PARP1 mRNA and protein levels were significantly increased in ATMIN overexpressed clones, and decreased in shATMIN-transfected cells, respectively (Supplementary Figure 1 and **Figure 4A**). To rule out the effects of adaptation in stable clones, we then overexpressed ATMIN in SW480 cells. In accordance with our previous findings, ATMIN overexpression significantly increased the expression of the PARP1 protein and the β-catenin/TCF4 complex in a dose-dependent manner (**Figure 4B** and **4C**). Overexpression of ATMIN did not change total β-catenin expression (**Figure 4C**). Thus, ATMIN may induce β-catenin nuclear translocation (**Figure 3E**). For confirmation of the key roles played by PARP-1 as regards ATMIN-regulated TCF-4/β-catenin signaling in MSS cell progression, ATMIN transfectants were transfected with PARP-1 knockdown plasmids (shPARP1#1 and #2), and their cell invasion abilities were investigated. Unsurprisingly, as shown in **Figure 4D**, PARP1 knockdown inhibited ATMIN-induced cell invasiveness more than pLKO.1 control plasmid transfection.

ATMIN is positively correlated with β-catenin regulators, CREBBP and p300

It was well known that β-catenin and PARP1 both interacted with nuclear transcriptional regulators, CREBBP and p300. Importantly, CREBBP and p300 not just acted on β-catenin but also on chromatin. Interestingly, the high-throughput microar-

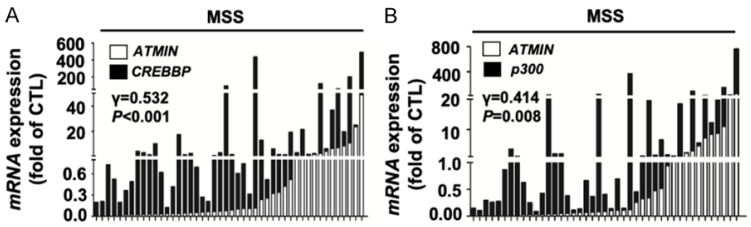

Figure 5. The nuclear transcriptional regulators, CREBBP and p300 were positively correlated with ATMIN. A, B. The correlation of *ATMIN* and *CREBBP* or *p300* in MSS. The mRNA of patients' samples was measured by RT-PCR. *P < 0.05.*

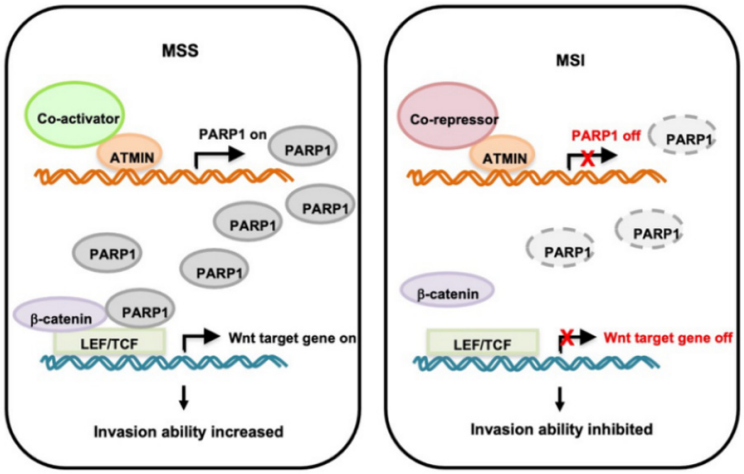

Figure 6. The working model of ATMIN-regulated CRC invasion and progression in MSI and MSS systems.

ray assay which performed by altering the chromatin damage checkpoint, ATMIN, indicated CREBBP and p300 significantly changed (**Figure 3A**, GSE58550). Therefore, we demonstrated that endogenous ATMIN mRNA expression was also significantly positively correlated with p300 and CREBBP mRNA endogenous expressions in clinical MSS patient samples (**Figure 5A** and **5B**). The regulated mechanisms still need further investigation.

Taken together, the results collectively demonstrated that ATMIN enhanced PARP1 mRNA and protein expression in MSS CRC cells to increase TCF-4/β-catenin complex formation, resulted in the subsequent activation of *Wnt* signaling, which further improved CRC invasion and progression.

Discussion

The role of ATMIN in conjunction with ATM signaling is already fairly well understood as regards double-strand breaks (DSBs) of the DDR, whereby efficient DNA repair can be triggered in the DDR by a reduction in ATMIN expression [15]. One example is that ATM activation following chloroquine treatment and hypotonic stress is mediated by ATMIN [19, 20]. Likewise, perturbation of pulmonary arterial endothelial cells in primary arterial hypertension patients was found by Li et al, as well as a relationship between high ATMIN levels and impaired DNA damage sensing and repair [26]. Interestingly, Wnt signaling is important for kidney development and perturbation of Wnt signaling pathways can result in cystic, and other renal abnormalities. Atmin^{Gpg6} homozygous kidneys exhibited altered cytoskeletal organization and modulation of Wnt signaling pathway molecules, including β-catenin and non-canonical Wnt/planar cell polarity (PCP) pathway factors, such as Daam2 and Vangl2 [27]. Anjos-Afonso et al. showed that absence of ATMIN results in perturbed hematopoiesis, especially during stress conditions and aging and these functions are both ATM-dependent and -independent [28]. In addition, ATMIN is frequently altered in lung adenocarcinoma and reduced expression correlates with poor survival [29]. Inhibiting ATMIN-dependent ATM activation led to oncogenic chromosomal translocations followed by tumor development in B-cell lymphoma [20]. Conversely, ATMIN blockage reduced invasion, migration and metastasis abilities both *in vitro* and *in vivo* of hand and neck squamous cell carcinoma [30]. ATMIN, therefore, has multiple roles in different cell types and we at first considered that those results might reflect a marked difference in various tissue backgrounds and the hypothesis that interactions of ATM/ATMIN vary under different stress conditions, genetic backgrounds, and ectopic microenvironments. However, our studies show that increased ATMIN expression in MSS cells increased Wnt transcriptional activity whereas

the opposite result was observed in MSI-high cells in vitro [24]. We also noticed that the in vitro effects of ATMIN to regulate CRC cell invasion were found to be ATM-independent. The mechanism underlying the dual role of ATMIN in MSI and MSS status is possibly explained, in part at least, by the fact that MSI-high and MSS cells are essentially different and that they cannot be considered to be the same in CRC. The other possible cause is the multiplicity of roles ATMIN plays. Not only has it been identified as a transcription factor for the dynein light chain (DYNLL1) but further possible interaction partners (co-activators or co-repressors) are being found in increasing numbers [31-33]. The microarray results in shATMIN stable transfectants had demonstrated several co-activators, such as CREBBP, NCOA5, and p300, were decreased in the SW620/shATMIN MSS system, indicating that shATMIN in MSS cells may inhibit co-activators, resulting in downregulating gene repression (**Figure 3A**). By contrast, a crucial co-activator gene, the NCOA3, was upregulated and several corepressors, including TRIM59, TRIM7, TRIM14, were downregulated in the HCT116/shATMIN MSI system [24]. Therefore, the dynamic changes between ATMIN and co-activator or co-repressor might be the reason why ATMIN had opposite roles in regulating PARP1-WNT-signaling between MSS and MSI colorectal cancer (**Figure 6**). These results might indicate that discriminating the functions of ATMIN by defining its transcriptional targets or interacting partners would provide a better understanding of ATMIN biology [34].

The PAPR family comprises a group of nuclear proteins that are activated upon binding to damaged DNA and which have crucial roles in various aspects of the DDR. The main function of these proteins is to detect single strand breaks and DSBs, recruit the DNA repair machinery and stabilize replication forks during repair [34]. Interestingly, other studies are emerging which demonstrate the additional functions of PARP1, including regulation of inflammatory mediators, cellular energetics and death pathways, gene transcription, sex hormone- and ERK-mediated signaling, and mitosis [35]. These PARP1-mediated processes play a part in oncogenesis, cancer progression, and the development of therapeutic resistance. In CRC, PARP1 acts as a co-activator of the β-catenin and TCF-4 complex and PARP1 expression is associated with the undifferentiated status of intestinal epithelial cells and crypt cells, which are reduced along the axis of cell differentiation [36]. In our current and previous study [24], increased expression of PARP1 in CRC cooperatively enhances TCF-4/β-catenin-mediated gene transactivation and may contribute to colorectal carcinogenesis. The relatively specific areas in cancer cells targeted by genetic instability, as discussed above, provide a potential route to increasing the efficacy of anticancer treatments. This is clear from two phase 2 nonrandomized clinical trials which indicate olaparib monotherapy may have benefits for previously treated pancreatic cancer patients with DNA damage repair-related genetic alterations other than germline BRCA variants [15, 37, 38]. Even more promising is the recent approval of PARP inhibitors for BRCA-mutated ovarian, pancreatic and breast cancer therapies. Studies have supported the association of a high tumor mutation burden (TMB) and dynamic neoantigen renewal with neoepitope-specific T-cell responses against mismatch repair-deficient (MSI-high) tumors treated with immune-checkpoint inhibitors [39-42]. However, direct evidence that targeting of DSB repair protein with DDR inhibitors causes an increased TMB in DNA repair proficient tumors through a variety of mechanisms is only beginning to emerge [35, 43]. It provides the rationale for combining PAPR inhibitors with immunotherapies in MSS CRC.

In conclusion, our study revealed novel insights into the dual roles of ATMIN in CRC progression, and indicated that ATMIN expression may allow stratification of prognostic outcomes for MSS CRC patients. We expect that these novel, CRC-specific prognostic factors may stimulate further research studies into the development of more effective therapeutic strategies in the future.

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Disclosure of conflict of interest

None.

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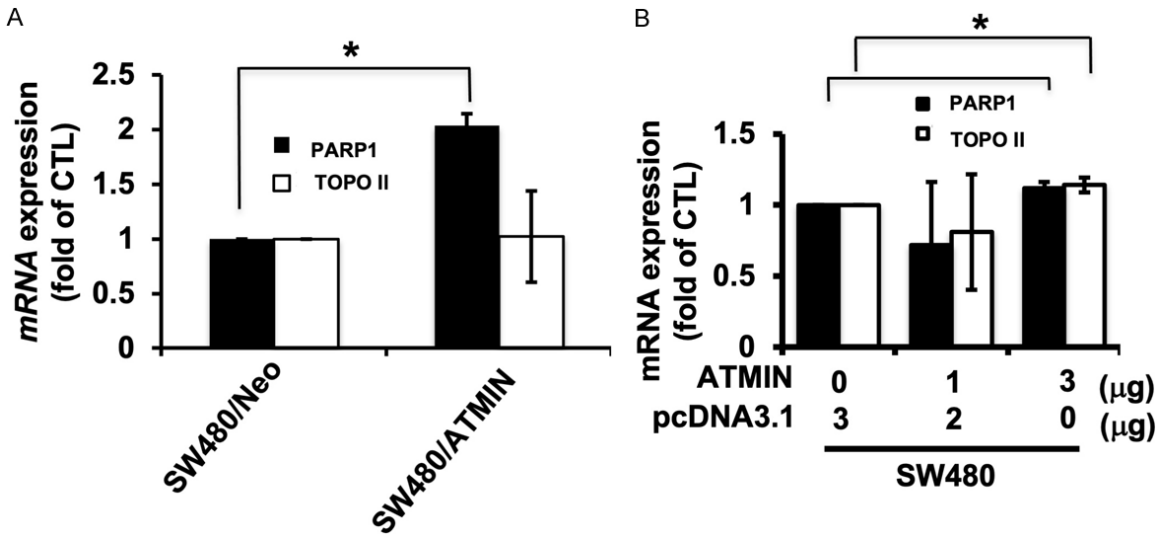

Supplementary Figure 1. A, B. β-catenin/TCF4 binding enhancer, *PARP1* and *TOPO II* mRNA expression were validated through RT-PCR in SW480 transfectants and transiently transfected cells. **P* < .05.