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THE TUMOR SUPPRESSOR NDRG2 DISRUPTS THE ONCOGENICITY OF THE CYTOPLASMIC PRMT5 IN ATL LEUKEMIA

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Received: 12 March 2017; **Accepted:** 28 March 2017

ABSTRACT

Adult T-cell leukemia (ATL) is an oncogenic disease derived from the HTLV-1-infected T cells and there is no effective therapy known yet. We previously reported that down-regulation of N-myc downstream-regulated gene-2 (NDRG2) expression by DNA Methylation and genetic deletion presents one of the most common alterations in adult T-cell leukemia (ATL) and other various kinds of cancers. A stress-induced NDRG2 suppresses important signaling pathways (PI3K and NF-κB) through the de-phosphorylation of PTEN and NIK as a PP2A recruiter. In this manuscript, we identified protein arginine methyltransferase 5 (PRMT5) as a NDRG2/PP2A binding partner. A NDRG2/PP2A complex down-regulated arginine methyltransferase activity of PRMT5 through de-phosphorylation of the serine and threonine residues and changing its co-localization to the nucleus of ATL cell lines increasing the histone arginine methylation; however, PRMT5 was highly phosphorylated and localized in cytoplasm in NDRG2-deficient ATL.

Key words: *NDRG2, PRMT5, PP2A, ATL, Leukemia.*

INTRODUCTION

Adult T-cell leukaemia-lymphoma (ATLL) is a malignant disease caused by the oncogenic retrovirus Human T-cell leukemia virus type 1 (HTLV-1) which aggressively infect CD4+ T-cells. The manifestations of this disease appear after a long clinical latency period up to 3 decades. The Genetic, epigenetic cellular changes and the molecular mechanism of leukemogenesis that occur in HTLV-1-infected cells, which contribute to the disease development, is not completely understood. ATLL is develops in 6% during the lifetime of an infected individual (Hinuma *et al.*, 1981; Proietti *et al.*, 2005; Yoshida *et al.*, 1984). N-myc downstream-regulated gene 2 (NDRG2) was identified as a novel PTEN- associated protein that recruits protein Phosphatase 2A (PP2A) to modify the PTEN phosphorylation at the Ser380, Thr382 and Thr383 residues in its C-terminal domain and the NIK-Thr559 modification (Ichikawa *et al.*, 2015; Nakahata *et al.*, 2014).

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The expression of the N-myc downstream-regulated gene 2 (NDRG2) was significantly down-regulated in ATLL through the DNA Methylation and genetic deletion which followed by its inactivation which is reported in many types of cancers. N-myc downstream-regulated gene 2 (NDRG2) has a crucial role in suppression of the phosphorylation of many pivotal signalling molecules in the most important signalling pathways through recruitment of PP2Ac which resulting in their dysregulation. Protein arginine methylation, catalyzed by members of the protein arginine methyltransferase (PRMT) family, which existed in the nucleus and cytoplasm (Pahlich *et al.*, 2006). According to the methylation products, PRMTs are classified into three types (Baldwin *et al.*, 2015). Type II PRMT that generates symmetric di-methyl arginine (SDMA) modification, PRMT5 is involved in tumorigenesis via both epigenetic silencing and organelle biogenesis (Karkhanis *et al.*, 2011).

In the mammalian cells, PRMT5 localizes to both the cytoplasm and the nucleus and it methylates multiple histone and non-histone proteins (Bedford and Clarke, 2009). In the cytoplasm, PRMT5 forms a 20S protein arginine complex called the methylosome, consisting of PRMT5, WD repeat protein (MEP50),

spliceosomal snRNP Sm proteins and pICln to function as a master regulator of splicing (Meister *et al.,* 2001). PRMT5 in the cytoplasm is required for proliferation of prostate epithelial cells, while PRMT5 in the nucleus in function with the androgen receptor to drive prostate epithelial cell differentiation and function (Gao and Wang, 2012; Gu *et al.,* 2012). Translocation of PRMT5 from the nucleus to the cytoplasm is associated with prostate tumorigenesis indicating that the Cytoplasmic PRMT5 is required for the growth of prostate cancer (Gu *et al.,* 2012). PRMT5 over-expression was found in different types of cancer, and the PRMT5 was considered as a significant target for cancer therapy (Ibrahim *et al.,* 2014; Powers *et al.,* 2011).

In this study, we identified new molecular mechanisms of targeting the leukemogenesis ATL, suggesting that the Cytoplasmic PRMT5 has a crucial role in the proliferation and growth of the ATL cell lines, while the nuclear PRMT5 may suppressing the growth of ATL.

MATERIALS AND METHODS

Reagents

Cell proliferation/cell toxicity Cell Counting Kit-8 was purchased from DOJINDO (Kumamoto, Japan). Most of the antibodies we used in this experiment were purchased from the companies listed in **Table 1.**

Antibody against.	Manufacturer.	Catalog no.	Clone no.	Dilution factor.
PRMT5	SANTA CRUZ	Sc-22132	C-20	1/1000
NDRG2	SANTA CRUZ	Sc-19468	E20	1/1000
Iκ Bα	SANTA CRUZ	Sc-371	C-21	1/1000
NEMO (IKK γ)	SANTA CRUZ	Sc-8330	FL-419	1/1000
Histone H1	SANTA CRUZ	Sc-8030	AE-4	1/1000
NF-kB P52	SANTA CRUZ	Sc-7386	C5	1/1000
AKT	Cell signaling	#9272		1/1000
P-AKT (Ser473)	Cell signaling	#4060	D9E	1/1000
PTEN	Cell signaling	#9559	138G6	1/1000
P-PTEN (Ser380/Thr382/Thr383)	Cell signaling	#9554		1/1000
ERK1/2	Cell signaling	#4695	137F5	1/1000
P-ERK1/2(T202-Y204)	Cell signaling	#4094	D13.14.4E	1/1000
Caspase 3	Cell signaling	#9665	8G10	1/1000
Cleaved-Caspase3 (Asp175)	Cell signaling	#9661		1/1000
PP2Ac subunit	Cell signaling	#2259	52F8	1/1000
P-Iκ Bα (Ser32/36) (5A5)	Cell signaling	#9246	5A5	1/1000
Myc-Tag	Cell signaling	#2276	9B11	1/1000
β -actin	Sigma-Aldrich	A5441	AC- 15	1/1000
Flag	Sigma-Aldrich	F3165	M2	1/1000
HA	Roche	11867423001	3F10	1/1000
P-Tyrosine	Millpore	05-321	4G10	1/1000
P-Threonine	QIAGEN	37420	Q7	1/1000
P-Serine	QIAGEN	37430	Q5	1/1000
H3R8	Abcam	Ab130740		1/1000
H4R3	Abcam	AB5823		1/1000
TAX	Kyoto University	MI73		1/1000
Alexa Fluor-488 donkey anti-goat	Molecular Probes	A11055		1/400
Alexa Fluor-647 donkey anti-mouse	Molecular Probes	A31571		1/400
Polyclonal Rabbit anti-Mouse IgG/HPR	Dako	P0260		1/1000
Polyclonal Swine anti-Rabbit IgG/HPR	Dako	P0399		1/1000
Polyclonal Rabbit anti-Goat IgG/HPR	Dako	P0449		1/1000
Polyclonal Rabbit anti-Rat IgG/HPR	Dako	P0450		1/1000

Plasmids

The Flag-tagged NDRG2 expression vector (Flag-NDRG2) has been described in (Nakahata *et al.*, 2014). The full-length complementary DNA (cDNA) sequence of PRMT5 was subcloned into the *Eco*RI - *Bam*HI site of the p3xFlag-myc-CMV26 expression vector (Sigma-Aldrich) (Flag-PRMT5). A DNA-based short hairpin RNA (shRNA) against PRMT5 was cloned into the *Bam*HI–*Eco*RI site of the RNAi-Ready-pSIREN-RetroQ-ZnGreen vector (Clontech) (shPRMT5). An shluc plasmid containing a shRNA against luciferase (Clontech) was used as a control. The sense and antisense shRNA sequences against PRMT5 and mutagenic primers are listed in **Table 2.**

Name		Sequence (5' to 3')
shPRMT5-3	sense	GAGGGAGTTCATTCAGGAA
	anti-sense	TTCCTGAATGAACTCCCTC
shPRMT5-4	sense	GGCCATCTATAAATGTCTG
	anti-sense	CAGACATTTATAGATGGCC

Cell culture

Jurkat and MOLT4 are HTLV-1-negative human T-ALL cell lines. KOB and KK1 are IL2-dependent ATL cell lines. ED and Su9T-01 and S1T are IL2-independent ATLL cell lines. MT2 and HUT102 are human T-cell lines transformed by HTLV-1 infection. The human embryonic kidney cell line 293T. IL2-dependent ATL cell lines were maintained in RPMI 1640 medium (Wako) supplemented with 10% fetal bovine serum and 50 JRU per ml recombinant human IL2 (Takeda). HTLV-1-negative cell lines, cell lines transformed with HTLV-1 and IL2-independent ATL cell lines were maintained in the same medium without IL2. The other cell lines were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM, Wako) supplemented with 10% fetal bovine serum.

Establishment of stable knockdown of PRMT5 expression in ATL cell lines

shRNA vectors were co-transfected into 293GP cells along with the envelope plasmid pVSV-G using HilyMax reagent according to the manufacturer's protocol. After 6 h of transfection, the medium was changed, and the cells were incubated for 48 h in DMEM with 10% FBS and 10 μM Forskolin (Sigma-Aldrich). The supernatant containing retrovirus was collected by polyethylene glycol (PEG, Wako) purification. After two days of retroviral infection in ATL cell lines (HUT102 and KOB), EGFP-positive cells were sorted with a JSAN cell sorter (Bay Bioscience).

Western blot

Cells were harvested for the extraction of proteins by homogenization in NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40) supplemented with a proteinase inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor tablet (PhosStop, Roche). The lysate was centrifuged for 10 min at 15,000 x g (maximum) at 4 °C, and the supernatant was then collected. Equal amounts of protein samples were loaded, separated by SDS-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore). The membranes were blocked in PBS–Tween (0.1%) with 1% BSA or 5% nonfat dried milk and were then probed with primary antibodies diluted in PBST-BSA or 5% nonfat dried milk. The bands were detected using a Lumi-light Plus kit (Roche) and LAS-3000. Band intensities were quantified with the NIH Image J software. All primary antibodies were used at a dilution of 1:1000. For subcellular fractionation, cytoplasmic and nuclear protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The efficiency of fractionation was confirmed by western blot analysis for β-actin (cytoplasm) and histone H1 (nucleus).

Immunoprecipitation

The lysates were incubated with 1 μg of the indicated antibodies or normal IgG with constant rotation at 4 °C overnight and were then incubated with Protein G Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden) for 2 h. The immunoprecipitates were washed 3 times with PBS, and the bound proteins were denatured in SDS sample buffer. Each sample was subjected to western blot analysis.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed with TBS 0.1 M glycine, treated with 0.1% NP-40 and rewashed with TBS 0.1 M glycine. After blocking with 1% BSA in TBS, the cells were incubated with primary antibodies (1:200) overnight at 4 °C. The cells were then washed three times with TBST and incubated with secondary antibodies (1:400) at room temperature for 2 h. The coverslips were washed three times with TBST and then mounted on glass slides using an antifade reagent (Invitrogen). Nuclei were counterstained with DAPI. The proteins were visualized using a confocal laser-scanning microscope (Leica Microsystems).

Cell proliferation assay
Cells were seed into 96-well plates at a density of $5x10^3$ cells per well and incubated for the indicated time period. Viable cells were counted by a methyl thiazolyl tetrazolium assay using a cell counting kit-8.

Trypan blue assay
Cell growth was evaluated with a Trypan blue exclusion assay. The living cells were examined by light microscopy at low magnification after Trypan blue staining. The cell viability percentage was calculated with the following formula: (% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100). This counting was repeated 3 times after 2 days in sub-culture.

TUNEL assay
For the TUNEL assay, 5 x 10^6 ATL cells were fixed with 1% paraformaldehyde, and the in situ detection of cells with DNA-strand breaks was performed by the TUNEL labeling method using an ApopTag Peroxidase In Situ Apoptosis Detection Kit, which detects apoptotic cells in situ (Millipore), according to the manufacturer's instructions. Standard deviations of three independent experiments were indicated.

Cell cycle assay
Ethanol-fixed ATL cells were stained with DAPI and incubated at 37 °C for 20 min. At least 30,000 cell events were collected and analyzed by flow cytometry (BD FACSCalibur). Cellular DNA histograms were examined for cell cycle analysis.

Statistical analysis
The data, bars and markers in the figures represent the mean ± s.d. We used the ANOVA for multiple comparisons and to compare each of a number of treatments with a single control. Differences were considered statistically significant when the *P* value was <0.05.

RESULTS

The expression and co-localization of PRMT5 in ATL cell lines.
NDRG2 was identified as a novel PTEN binding protein for recruiting PP2A, resulting in regulating PTEN Phosphatase activity by PTEN C-term phosphorylation status (Nakahata *et al.,* 2014). We confirmed later that the PRMT5 is an NDRG2 binding protein.

We examined the expression levels of PRMT5 and NDRG2 proteins in ATL-related cell lines. As it compared with two T-ALL cell lines (Jurkat and MOLT4) as a control, the protein expression levels of PRMT5 was sustained in the ATL-related cell lines, although with the loss of NDRG2 expression (Fig. 1A). It means that, the function of PRMT5 maybe differs from normal to cancer cells. Since the oncogenic function of PRMT5 was found its localization in cytoplasm of prostate cancer cells (Gu *et al.,* 2012), and as mentioned before in our results that the PRMT5 protein not differ between normal and cancer cells, we next investigate the sub-cellular localization of PRMT5 by western blot analysis after separation of nucleus and cytoplasm and immune-fluorescence staining to the ATL-related cells with or without forced-expression of NDRG2. In ATL-related cells with low expression of NDRG2, most of the PRMT5 protein was localized in cytoplasm along with PTEN, AKT, and beta-actin moreover there is a high level of the phosphorylated AKT and PTEN; however, after introduction of NDRG2 expression, a part of PRMT5 was moved into nucleus. Interestingly, although the majority of NDRG2 protein was detected in the cytoplasm, PTEN and AKT were also moved to nucleus with PRMT5 and we detected lower phosphorylated levels of AKT and PTEN which still remaining in the cytoplasm (Fig, 1 B). To confirm the localization of PRMT5 in these ATL-related cell lines with or without NDRG2 expression, immunofluorescence staining of PRMT5 and NDRG2 was done to these cell lines by the specific antibody of Alexa-488-labeled PRMT5 (green) and Alexa Fluor-647-labeled NDRG2 (red), respectively. In ATL-related cells (HUT102 and KOB) with low expression of NDRG2, the majority of the green-labeled PRMT5 was detected in the cytoplasm without merge with DAPI-stained nucleus (blue). On the other hand, after introduction of NDRG2, PRMT5 was detected both in the cytoplasm and the nucleus (Fig. 1C). Furthermore the histone modifications (histone arginine methylatin which is a target for PRMT5) in the cell expressing the exogenous NDRG2 were confirmed by the western blot analysis (Fig. 1D).

PRMT5 in ATL-related cells loss it's activity for enhancing the cell growth after NDRG2 expression.
To investigate whether the function of PRMT5 in ATL-related cells is dependent on the loss of NDRG2 expression, we established ATL-related cells with forced expression of NDRG2 and/or the knockdown expression vector for PRMT5 (shPRMT5-3/NDRG2 and shPRMT5-4/NDRG2 in HUT102 and KOB). After confirmation of the expression levels of NDRG2 and PRMT5 by western blot analysis (Figs 2A and 2C), we determined the cell growth rates (Figs 2B &2D), where the transformat cells with high expression of NDRG2 showed reduction of cell growth with enhanced apoptosis with or without PRMT5 knockdown. The apoptosis of the transformant cells with NDRG2 expression was detected by presence of the cleaved caspase3 as shown in (Fig 2E &2F). Otherwise in the NDRG2 transformant (HUT102 and KOB) cells with or without PRMT5 KD most of these signals not

significantly changed (Figure 2G), in compare to the low NDRG2 expression in the parental lane in both HUT102 and KOB.

PRMT5 regulates the cell cycle and apoptosis in ATL:

The cell cycle checked by flow cytometry showing increased population of sub-G1 fraction in the NDRG2 transformant cells whatever the PRMT5 down-regulated or not (Fig 2A). Moreover the DNA staining using the TUNEL assay showing the damage of the DNA in the NDRG2 transformant cells which enhancing the apoptotic pathways (Fig 2B), also the cell viability was decrease in comparison with the NDRG2 negative cells (Fig 2C) by the trypan blue exclusion assay, suggesting that high expression of NDRG2 abrogated the effect of PRMT5 knockdown. Therefore, the cell growth of NDRG2low cell lines is mostly dependent on the expression of PRMT5 and the function of PRMT5 in NDRG2low cell lines is possible dependent on modified phosphorylation status of PRMT5 by the loss of NDRG2 expression.

PRMT5 is a novel binding protein to the NDRG2/PP2Ac complex which regulates its phosphorylation status:

To confirm the PRMT5 as a new binding protein of NDRG2, Flag-tagged NDRG2 expression vector was transfected to HUT102 or KOB/ATL-related cell lines and then this exogenous NDRG2 and the PP2Ac (SERINE and THREONINE Phosphatase) complex could specifically bind to endogenous PRMT5 in both ATL-related cell lines (Figs 3A &3B). Then we checked the phosphorylation status of the PRMT5 in both HUT102 and KOB cell lines with or without NDRG2 expression by immunoprecipitation, interestingly we found that in the cell line with low NDRG2 expression showing highly phosphorylated PRMT5, while those with high expression of NDRG2 showing low phosphorylated PRMT5 (Figs 3C &3D). Hence the NDRG2/PP2Ac complex is a serine and threonine Phosphatase; we noticed that also the tyrosine residue phosphorylation was decreased. To confirm whether PP2A could de-phosphorylate the PRMT5, we transfected the HEK293T cells with MYC-PP1c, PP2Ac or PP5c and FLAG-PRMT5, by immunoprecipitation by using the indicated antibodies we detected that the PP2Ac is strongly bind to the FLAG-PRMT5 than the PP1c or the PP5c which is weekly bind to the complex (Fig. 4F), suggesting that the PP2Ac is the most important Phosphatase bind to the PRMT5 and regulating its phosphorylation and moreover its oncogenic activity.

Figure 1

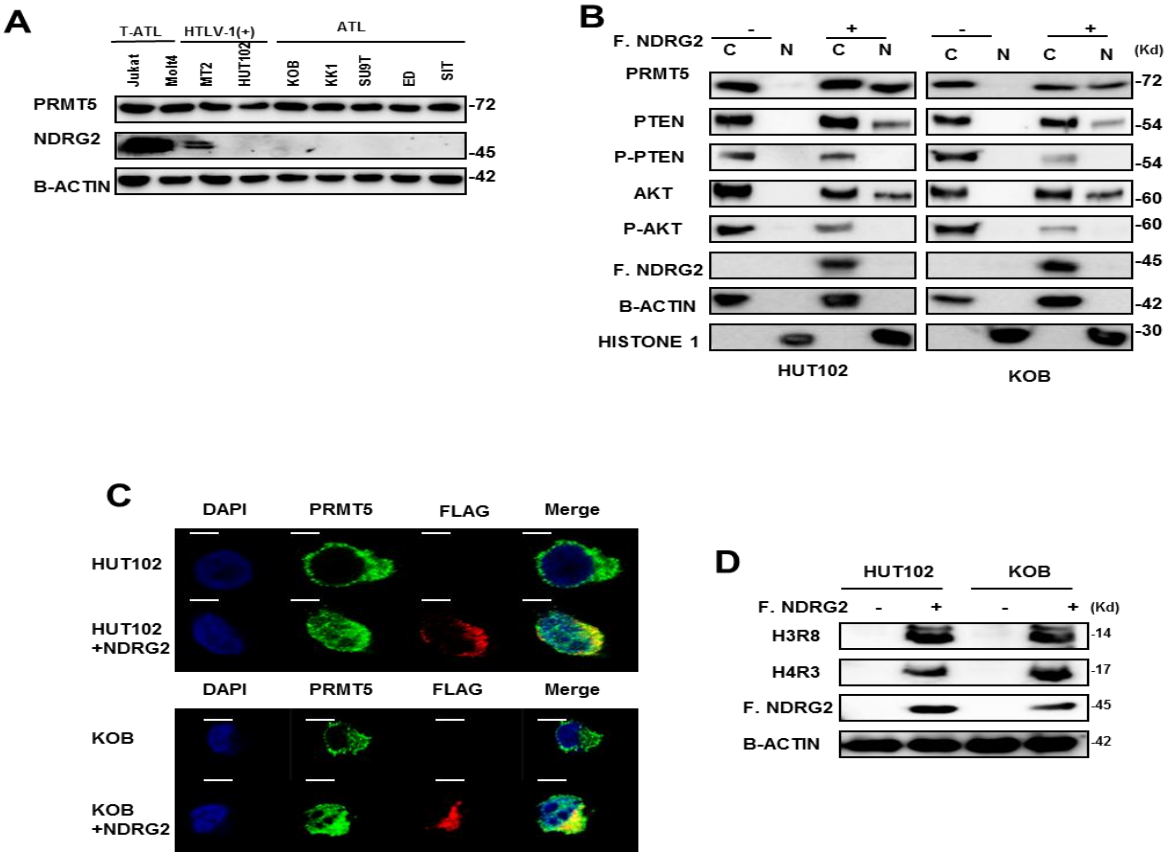
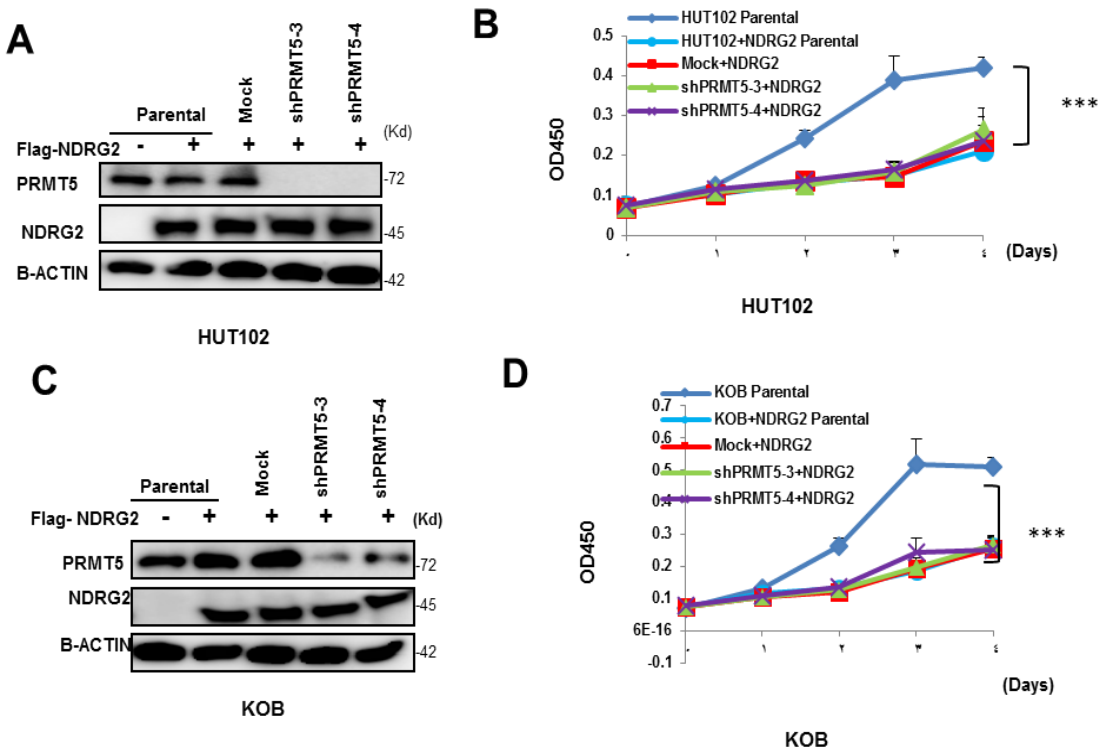


Figure 2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shPRMT5-4
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B-ACTIN
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-45
-42
HUT102
B
HUT102 Parental
HUT102+NDRG2 Parental
Mock+NDRG2
shPRMT5-3+NDRG2
shPRMT5-4+NDRG2
OD450
0.5
0.4
0.3
0.2
0.1
0
***
(Days)
HUT102
C
Parental
Mock
shPRMT5-3
shPRMT5-4
Flag- NDRG2 - + + + +
(Kd)
PRMT5
NDRG2
B-ACTIN
-72
-45
-42
KOB
D
KOB Parental
KOB+NDRG2 Parental
Mock+NDRG2
shPRMT5-3+NDRG2
shPRMT5-4+NDRG2
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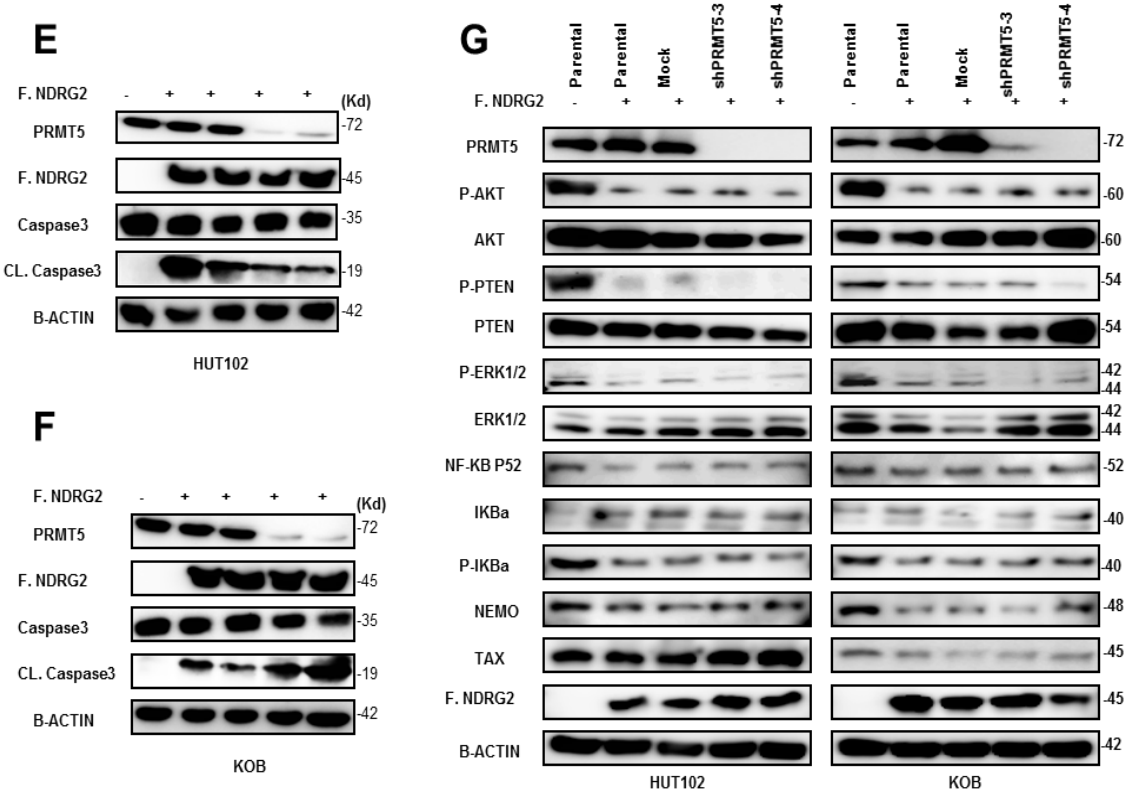
(Days)
KOB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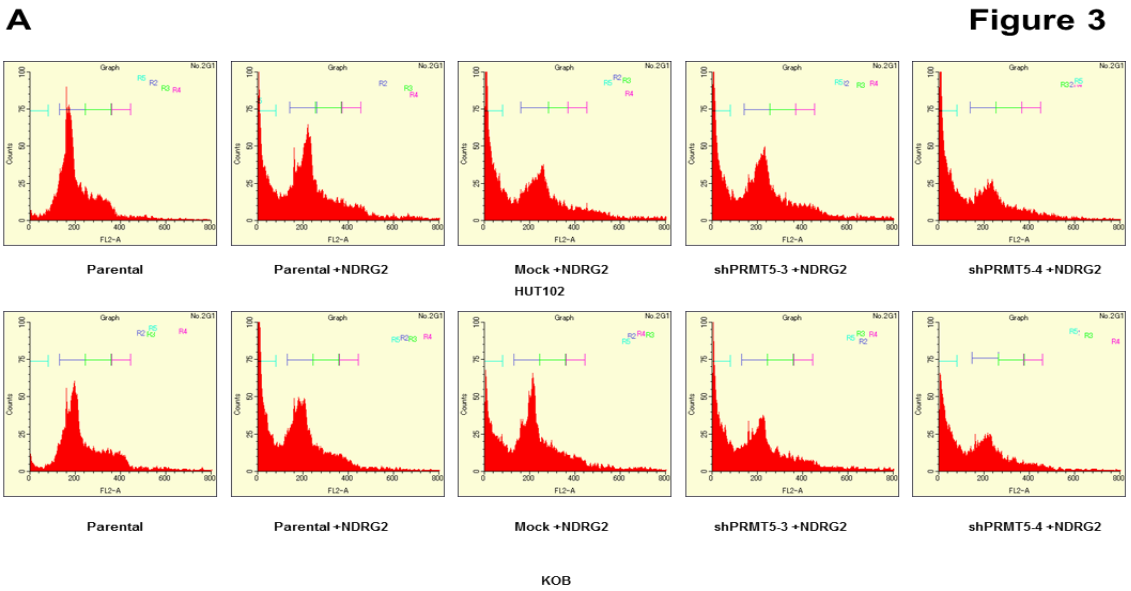
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Figure 3
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Mock +NDRG2
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shPRMT5-4 +NDRG2
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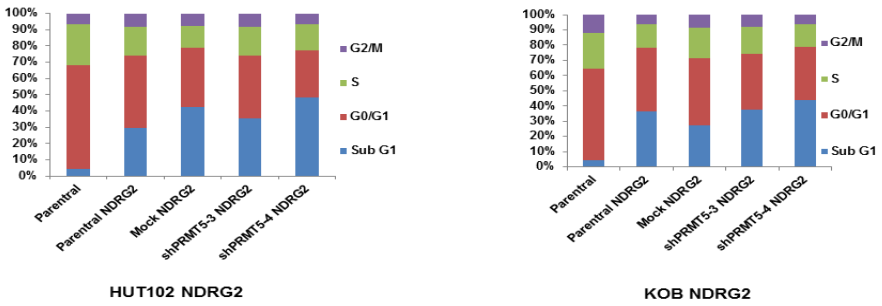
Parental +NDRG2
Mock +NDRG2
shPRMT5-3 +NDRG2
shPRMT5-4 +NDRG2
KOB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G0/G1
Sub G1
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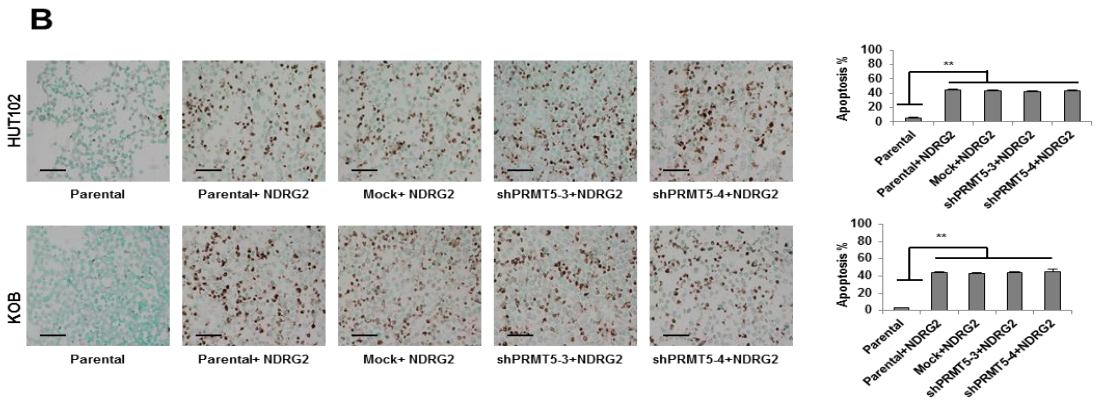
HUT102 NDRG2
KOB NDRG2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HUT102
KOB
Parental
Parental+ NDRG2
Mock+ NDRG2
shPRMT5-3+NDRG2
shPRMT5-4+NDRG2
Apoptosis %
**
Parental
Parental+NDRG2
Mock+NDRG2
shPRMT5-3+NDRG2
shPRMT5-4+NDRG2

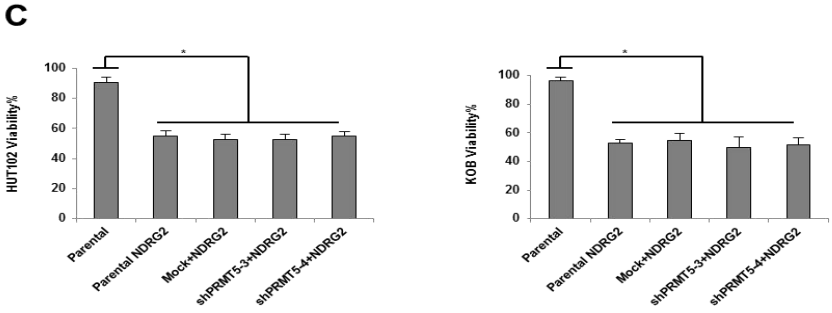
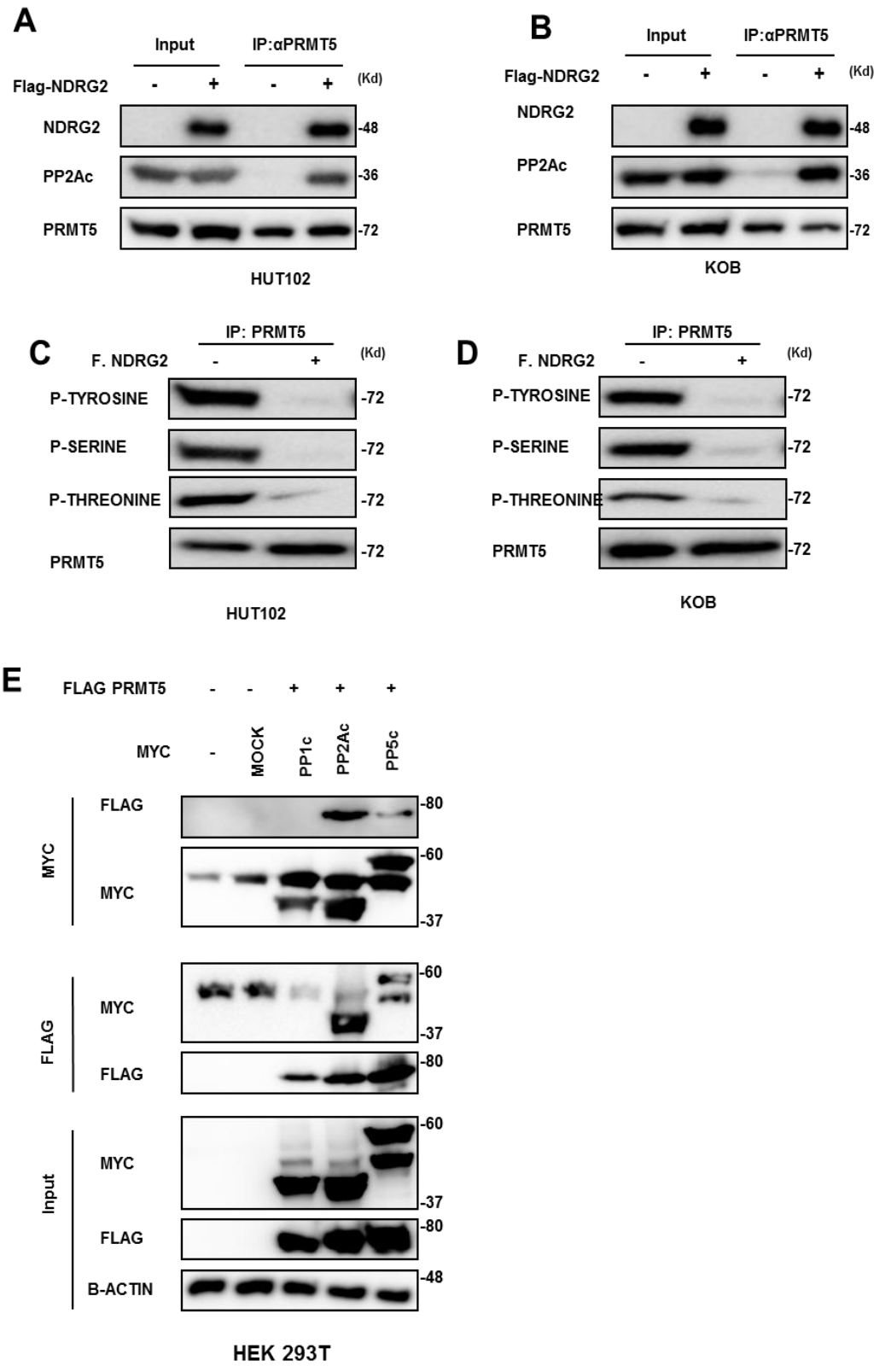

C
HUT102 Viability%
KOB Viability%
*
Parental
Parental NDRG2
Mock+NDRG2
shPRMT5-3+NDRG2
shPRMT5-4+NDRG2

Figure4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-72
HUT102
B
Input
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- + - +
(Kd)
NDRG2
PP2Ac
PRMT5
-48
-36
-72
KOB
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- +
(Kd)
P-TYROSINE
P-SERINE
P-THREONINE
PRMT5
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HUT102
D
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- +
(Kd)
P-TYROSINE
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P-THREONINE
PRMT5
-72
KOB
E
FLAG PRMT5 - - + + +
MYC - MOCK PP1c PP2Ac PP5c
MYC
FLAG
MYC
-80
-60
-37
FLAG
MYC
FLAG
-60
-37
-80
Input
MYC
FLAG
B-ACTIN
-60
-37
-80
-48
HEK 293T

Figures ligands.

Figure 1: The expression and co-localization of PRMT5 in ATL cell lines.

(***A***) Expression of PRMT5 and NDRG2 proteins were determined by immunoblot analysis using specific antibodies in 7 ATL-related cell lines and two T-ALL cell lines as indicated in the figure.

(***B***) After separating the nuclear and cytoplasmic fractions, the lysates of ATL-related cells were immunoblotted with anti-PRMT5, anti-NDRG2 and other important oncogenic proteins. To confirm the subcellular localization, an anti-histone H1 antibody was used for the nucleus and an anti-β-actin antibody for the cytoplasm.

(*C*) Immunofluorescence staining of HUT102 and KOB cells that were mock transfected or transfected with an NDRG2 expression vector was performed with anti-human PRMT5 antibody with Alexa Fluor-488-conjugated anti-goat IgG antibody (green) and anti-FLAG with Alexa Fluor-647-conjugated anti-mouse IgG antibody (red), along with DAPI staining for the nucleus. Scale bar, 10 μm.

(***D***) The lysates of ATL-related cells were immunoblotted with anti-histone H3R8me2 or H4R3me2 and anti-NDRG2. Anti β-actin antibody for the control.

Figure 2: PRMT5 in ATL-related cells loss it's activity for enhancing the cell growth after NDRG2 expression.

(***A***) And (***C***) Immunoblot analysis of PRMT5 was performed in the NDRG2 transfected/ATL-related cell lines HUT102 or KOB cells expressing shPRMT5 and also the expression of NDRG2 was shown in ATL cell lines, β-actin as a control.

(***B***) And (***D***) Cell growth curves of the NDRG2 transfected/ATL-related cell line HUT102 over four days, including parental, NDRG2 parental, mock-transfected, and shPRMT5-transfected cells. (***D***) The same experiment was performed in the NDRG2 transfected/ATL-related cell line KOB. The statistical analysis of the growth of the control cells versus the shPRMT5-transfected cells indicated that P<0.001 (ANOVA).

(***E***) And (***F***) Cleaved caspase 3, as an indicator for apoptosis, was identified in NDRG2 transfected/HUT102 or KOB cell lines with NDRG2 expression by an immunoblot analysis, along with PRMT5, total caspase 3 and β-actin as a control.

(G) Expression of various types of cellular proteins, such as signal transduction or cell cycle regulators, was analyzed in NDRG2 transfected/ATL-related cell line HUT102 or KOB cell lines (parental, NDRG2 parental, mock- or shPRMT5 transfected cells) by specific antibodies.

Figure 3: PRMT5 regulates the cell cycle and apoptosis in ATL.

(*A*) Cell cycle profiles in the NDRG2 transfected/ATL-related cell lines HUT102 or KOB cells expressing shPRMT5 and also the expression of NDRG2 were assessed by FACS analysis after DAPI staining.

(*B*) Cell apoptosis was evaluated by a TUNEL assay in the same cells as in (***A***). Apoptotic cells were stained brown (left panel). The bar graph represents the percentages of apoptotic cells (right panel). Scale bar, 100 μm. The mean ± s.d. is shown, P<0.001 (ANOVA).

(*C*) The cell viability detected by TRYBAN BLUE exclusion test the same as in (***A***). The data are representative of three experiments. The mean ± s.d. is shown, P<0.001 (ANOVA).

Figure 4: PRMT5 is a novel binding protein to the NDRG2/PP2Ac complex which regulates its phosphorylation status;

(***A***) And (***B***) After Flag-tagged NDRG2 was transfected into the HUT102 and KOB /ATL-related cells and the cell lysates were precipitated by an anti-PRMT5antibody, the precipitated proteins were immunoblotted with specific antibodies (anti-PRMT5, anti-PP2Ac or anti-NDRG2).

(***C***) And (***D***) Co-immunoprecipitation using PRMT5 antibody with the lysates of HUT102 and KOB /ATL-related cells and blotted against the antibodies of anti-tyrosine, anti-serine and anti-threonine respectively and the PRMT5 as a control.

(***E***) HEK293T cells transfected with MYC-PP1c, PP2Ac or PP5c and FLAG-PRMT5, and then the interaction was checked by immunoprecipitation by using the indicated antibodies.

DISCUSSION

The expression of N-myc downstream-regulated gene 2 (NDRG2) was significantly downregulated in ATL through DNA methylation and genetic modifications (Nakahata *et al.,* 2014). The downregulation of NDRG2 associated with tumor growth, progression and metastasis as the deletion of NDRG2 was reported in a wide variety of cancers, including pancreatic cancers, oral cancers and gastric cancers (Furuta *et al.,* 2010; Hu *et al.,* 2016; Yao *et al.,* 2008). NDRG2, is a stress-responsive gene that have a role in suppressing the phosphorylation of many important signaling molecules in several signaling pathways through the recruitment of the protein

phosphatase PP2A, which results in de-phosphorylation and the maintenance of cellular homeostasis after the over-activation of stress response factors (Nakahata *et al.,* 2014). NDRG2 is a novel PTEN-binding protein that activates PTEN phosphatase activity by recruiting PP2A, which dephosphorylates PTEN at Ser380, Thr382 and Thr383 (STT) in its C-terminal domain (C-term). In most ATL cells and oral cancers, the expression of wild-type PTEN is sustained with low phosphatase activity by maintaining the highly phosphorylated status of the PTEN C-term (STT), resulting in the constitutive activation of the PI3K/AKT signaling pathway, Moreover, we identified that NF-κB inducible kinase (NIK) is a novel binding partner of NDRG2 (Ichikawa *et al.,* 2015; Nakahata *et al.,* 2014). Since NDRG2, as a tumor suppressor, is downregulated or undetectable in many human cancers, identifying the molecular mechanism of NDRG2 is important to the development of therapeutic interventions. Down-regulation of NDRG2 has been identified in some types of solid tumors, including 80.4% of OSCC and 73.9% of pancreatic cancers (Furuta *et al.,* 2010; Yamamura *et al.,* 2013). Here in ATL the NDRG2 not expressed and we identified the PRMT5 as a new binding partner of the NDRG2/PP2Ac complex.

The methyltransferase activity of PRMT5 is disrupted by its phosphorylation at Y297, Y304, and Y307 by a mutated JAK kinase (V617F) (Liu *et al.,* 2011). In embryonic stem cells, cytoplasmic PRMT5 has a role in maintenance of pleuripotency (Tee *et al.,* 2010). The HTLV-1-transformed T cells display constitutive activation of STAT3 and STAT5 (Migone *et al.,* 1995), so the activation of STAT signaling pathway might be involved to the cytoplasmic PRMT5 function in ATL. The tyrosine phosphorylation of PRMT5 by JAK2-V617K inhibits its arginine methyltransferase activity on histone proteins.

Among the known substrates of PRMT5 are histones H4R3 and H3R8. It has been shown that symmetric dimethylation (me2) of H4R3 and H3R8 lead to transcription repression, whereas that of H3R8 has also been associated with transcriptional activation (Fabbrizio *et al.,* 2002; Richard *et al.,* 2005).

In this study the arginine methylation status of histones H3 and H4 was determined in ATL-related cell lines using a specific antibody for H3R8me2 and H4R3me2 before and after NDRG2 transfection. The arginine methylation of histones H3 and H4 could not be detected in the ATL-related cells (Figure 1D); however, after the over-expression of NDRG2 in the ATL-related cell lines HUT102 or KOB, the PRMT5 translocated to the nucleus, and enhanced arginine methylation of histone H3 (H3R8me2) and H4 (H4R3me2) (Figure 1D). In our study the relocalized PRMT5 in the nucleus might modulate the arginine methylation of histone H3 ariginine 8 (H3R8) and of histone H4 arginine 3 (H4R3) through nuclear transport signal by unknown modification of PRMT5. Therefore, the cytoplasmic PRMT5 accelerated their cell growth of ATL-related cells and the oncogenic function of PRMT5 might be dependent on the loss of NDRG2 expression in ATL-related cells. Here we confirmed that the PRMT5 function is related to the sub-cellular localization, hence the Cytoplasmic PRMT5 work as oncogenic factor with a high phosphorylation status, while that in the nucleus may be work as a tumor suppressor due to the histone modification which in turn down-regulates the genes which responsible for the cell growth after losing its phosphorylation by the NDRG2/PP2Ac complex. Overall as a part of the dephosphorylated PRMT5 translocated to the nucleus, resulting in enhanced histone arginine methylation.

Interestingly, STAT signaling pathway with high metastatic potential is inhibited by NDRG2 expression in several types of tumors (Wang *et al.,* 2014). The loss of NDRG2 expression might enhance the cytoplasmic PRMT5 expression through activation of the STAT signaling pathway. Moreover, the cytoplasmic PRMT5 expression is correlated with poor prognosis.

So far the activity of PRMT5 in ATL cell lines depends on its Cytoplasmic co-localization and the phosphorylation status in which enhances the cell growth and the metastasis through the activation of some oncogenic signaling pathways and inhibition of histone arginine methylation, while the forced transfection of NDRG2 to the ATL cell lines recruited the protein Phosphatase PP2Ac to the PRMT5 resulting in its de-phosphorylation, moreover nuclear localization and histone arginine modifications, which inactivates the genes that responsible for the cell growth.

In conclusion, our in vitro data indicated that the oncogenic activity of PRMT5 was depends on its cytoplasmic localization and the phosphorylation status which is essential for ATL development. Moreover it is a critical activator for the regulatory pathways affecting cell growth, survival, migration and tumor cells activity. Furthermore, the PRMT5 can bind with the NDRG2/PP2Ac complex which responsible for its de-phosphorylation and changing its co-localization from the cytoplasm to the nucleus which in turn modify the histone H3 and H4 through its arginine methylation and lowering the growth of ATL.

So cytoplasmic PRMT5 is considered as a novel target gene in the ATL, as targeting of PRMT5 with a drugs that regulates its phosphorylation status maybe used as a novel therapeutic pattern in ATL leukemia.

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ال ان دى ار جى الثانى المثبط للأورام يحدث خللاً فى قدرة ال بى ار ام تى الخامس المتواجد فى السيتوبلازم على احداث التورم فى مرض الابيضاض التائى الخلايا.

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مرض الابيضاض التائى الخلايا من الأمراض الخبيثة التى تحدث للانسان بسبب الاصابة بفيروس تى الليمفاوى البشرى واحد وحتى الان لم يتوصل الى أى علاج فعال له حيث انه تم التوصل قبل ذلك أن الجين المثبط للتورم ال ان دى ار جى ٢ قد حدث له بعض التعديلات الوراثية مثل المثيله مما أدى الى الحذف الوراثى له فى مرض الابيضاض التائى الخلايا وبعض الأنواع الأخرى من الأورام. حيث أن هذا الجين يقوم بقمع بعض المسارات الحيوية الخاصة بنمو الأورام عن طريق نزع مجموعات الفسفرة من الحمضين الأمينين السيرين والثريونين فى بعض البروتينات مثل البروتين المماثل الفوسفاتاز والتنسين عن طريق الانزيم النازع للفسفره الثانى فى البروتين. فى هذه الدراسة اكتشفنا أن بروتين ال بى ار ام تى الخامس له القدره على الارتباط بالمركب السابق حيث أن هذا المركب له القدرة على تقليل وتثبيط وظيفه ال بى ار ام تى الخامس المتواجد فى سيتوبلازم الخلايا السرطانية عن طريق نزع مجموعات الفسفرة من حمضى السيرين والثريونين الخاصين به والذى ينتج منه انتقال جزء من ال بى ار ام تى الخامس من السيتوبلازم الى النواة وعمل ميثله للحمض الأمينى الأرجينين فى بروتينات الهستون ومن ثم تثبيط بعض الجينات الخاصة بنمو الأورام حيث أن ال بى ار ام تى الخامس يوجد فى السيتوبلازم الخاص بالخلايا السرطانية وتركيز مجموعت الفسفره به عالى جداً.