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The interaction between ATRIP and MCM complex is essential for ATRIP chromatin loading and its phosphorylation in mantle cell lymphoma cells

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Aim: The ATR-interacting protein (ATRIP) is responsible for the recognition of DNA damage-induced structure and regulation of cellular responses to DNA damage and replication stress. The purpose of our study was to identify the underlying mechanism with respect to chromatin loading and phosphorylation of ATRIP in mantle cell lymphoma (MCL). *Methods:* JeKo cells were used in our study. Differently tagged ATRIP (Myc-, hemaglutinin (HA) or Flag) and minichromosome maintenance (MCM) complex (MCM2, MCM3, MCM5, and MCM6) were transfected into 293T cells. After 48 h, ATRIP-interacting protein was identified by mass spectrometry (MS). Cell fractionation was done to localize proteins inside the cells. Immunoprecipitation (IP) and immunoblot (IB) analysis were used to identify immunoreactive species, and Glutathione S-transferase (GST) pull-down assays were performed to detect protein-protein interaction between ATRIP and MCM complex. After silencing the expression of MCM2 and MCM6 by short hairpin RNA (shRNA), chromatin fraction were analyzed. The expression of ATRIP phosphorylation (pS224-ATRIP) was determined after application of different doses of MCM2 shRNA (0.5 μg, 1 μg, and 2.5 μg). *Results:* ATRIP directly interacts with MCM2, MCM3, MCM6, and MCM7 in JeKo cells. Downregulation of MCM2 and MCM6 significantly reduced ATRIP chromatin fraction. Downregulation of MCM2 statistically decreased the expression of ATRIP phosphorylation. The expression levels of pS224-ATRIP were regulated by MCM2 shRNA in a dose-dependent manner. *Conclusion:* Our results suggest that interaction between ATRIP and MCM complex is required for ATRIP chromatin loading and ATRIP phosphorylation.

1. Introduction

Mantle cell lymphoma (MCL) is a well-characterized lymphoproliferative disorder, accounting for 3-10% of all non-Hodgkin's lymphomas (NHL) (Cortelazzo et al. 2012). It is characterized by rearrangement of DNA fragments interchanged between chromosomes 11 and 14 (Friedenson 2007). The incidence of MCL has been reported to be increased in many countries, particularly among the elderly (Anderson et al. 1998; Cortelazzo et al. 2012). Furthermore, the disease is associated with rapid progression and a high recurrence rate (Norton et al. 1995; Velders et al. 1996). In spite of the implementation of multimodal treatment approaches in recent years, the overall survival of MCL has only slightly been improved due to the uncertain pathological mechanisms. It has been well known that complex genomic aberrations are found in MCL (Fernandez et al. 2010; Jares et al. 2012; Perez-Galan et al. 2011). Therefore, a better understanding of MCL pathogenesis involving in genetic alteration might facilitate new targets for personalized drug therapy.

Recently, ataxia-telangiectasia mutated (ATM) and ATM and rad3-related (ATR) kinases have been paid great attention owing to their functions at the apex of cell cycle checkpoint (Abraham 2001; Shiloh 2001). It has been reported that inactivation of ATR is embryonic lethal (Brown and Baltimore 2003) and results in deleterious DNA damage during S phase (Casper et al. 2002; Syljuasen et al. 2005). ATR has been regarded as a promising target in the treatment of cancers including MCL due to its significant role in intra-S checkpoint in response to replication stress (Cimprich and Cortez 2008; Menezes et al. 2015; Toledo et al. 2011). ATR-interacting protein (ATRIP) is the regulatory partner of ATR, which binds directly to replication protein A (RPA)-coated single stranded (ss) DNA (Namiki and Zou 2006). ATRIP is independently required for the ATR-ATRIP complex that could recognize DNA damage-induced structure and regulate cellular responses to DNA damage and replication stress (Ball et al. 2005; Namiki and Zou 2006).

To further understand the functional roles of ATRIP roles in MCL, we sought to identify specific ATRIP-interacting proteins in the nucleus. In the present study, we used a JeKo cell line that represents the blastoid variant of aggressive MCL. We found that minichromosome maintenance (MCM) complex interacted with the ATRIP. The interaction between ATRIP and MCM complex is required for ATRIP chromatin loading and ATRIP phosphorylation. Our data might provide new insights into the molecular mechanism of MCL.

2. Investigations and results

2.1. ATRIP interacts with MCMs

To further understand the functional role of ATRIP in MCL, we tried to identify specific ATRIP-interacting proteins in the JeKo cells. First, we produced a stable JeKo cell line expressing differently tagged ATRIP and MCM complex (MCM2, MCM3, MCM5, and MCM6). Then the nuclear extracts were immunoprecipitated with anti-Flag M2 beads, eluted with 3 × Flag peptide, and mass spectrometric analysis were carried out. Our data showed that the peptide recovery of ATRIP from the nuclear IPs was 11%, 16%, 18%, and 10%, respectively. The results indicated that ATRIP was successfully isolated. MCM complex plays significant roles in DNA replication and replication checkpoint and the complex is exclusively in the nuclear. We therefore hypothesized that the MCM complex might be the potential interactive protein. To confirm the speculation, co-immunoprecipitation was performed to determine the interaction between ATRIP and MCM complex in the cells. As shown in Fig. 1, there was an interaction between ATRIP and MCM complexes.

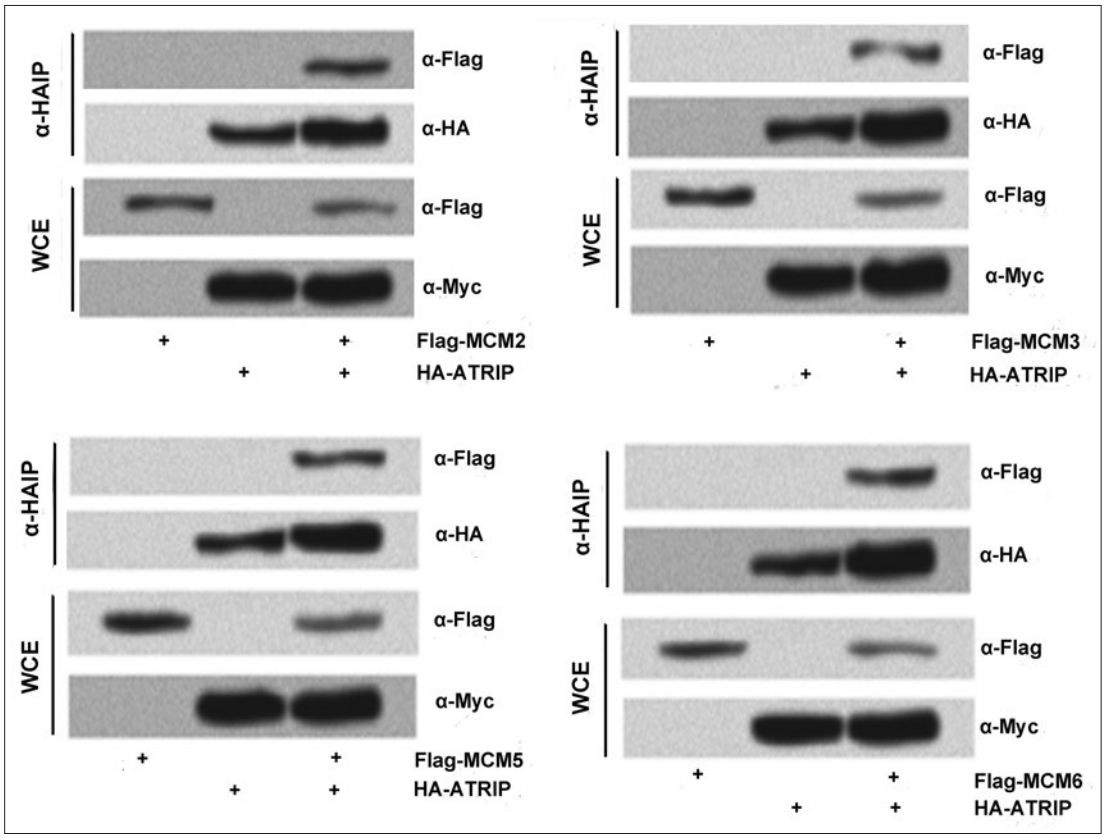

Fig. 1: ATRIP interacts with MCMs. Differently tagged ATRIP and MCM complex (MCM2, MCM3, MCM5, and MCM6) are transfected into 293T cells for 48 h. The cells are immunoprecipitated with the antibody against the tag of ATRIP, and immunoblotted with antibodies against the tag for each individual MCM.
ATRIP, ATR-interacting protein; MCM, minichromosome maintenance complex; WCE, whole cell extracts; HA, hemaglutinin

2.2. ATRIP directly interacts with MCMs

To test whether ATRIP interacts with MCMs directly or not, we performed a GST pull down assay. GST-ATRIP bound on glutathione-agarose beads were pulled down to MCM proteins individually. As indicated in Fig. 2, there were interactions between ATRIP and MCM2, MCM3, MCM 5 and MCM6 *in vitro*, indicating a multi-interface association between ATRIP and the MCM complex.

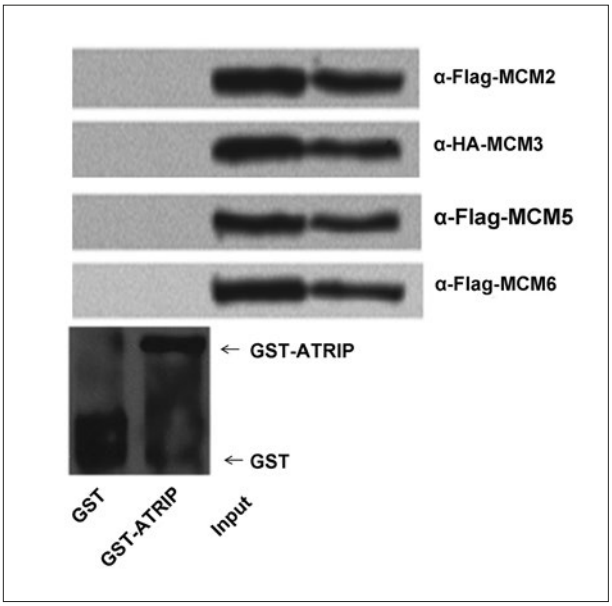

Fig. 2: ATRIP directly interacts with MCMs. GST or GST-ATRIP proteins bound on glutathione-agarose beads are purified and used to pull down MCM proteins. MCM2, MCM3, MCM6, and MCM7 were detected by GST-ATRIP beads.
ATRIP, ATR-interacting protein; MCM, minichromosome maintenance complex; GST, Glutathione S-transferase; HA, hemaglutinin

2.3. Downregulation of MCMs reduces ATRIP chromatin fraction

To test whether MCMs are essential for ATRIP chromatin loading, the expression of MCM2 and MCM6 was downregulated by using shRNA in JeKo cells and chromatin fraction was carried out. As demonstrated in Fig. 3, after suppression of MCM2 and MCM6 expression, the ATRIP chromatin fraction was significantly reduced by MCM2 shRNA and MCM6 shRNA compared to the non-chromatin. Orc2 and PLCγ-1 are respectively marker proteins for chromatin and non-chromatin fractions.

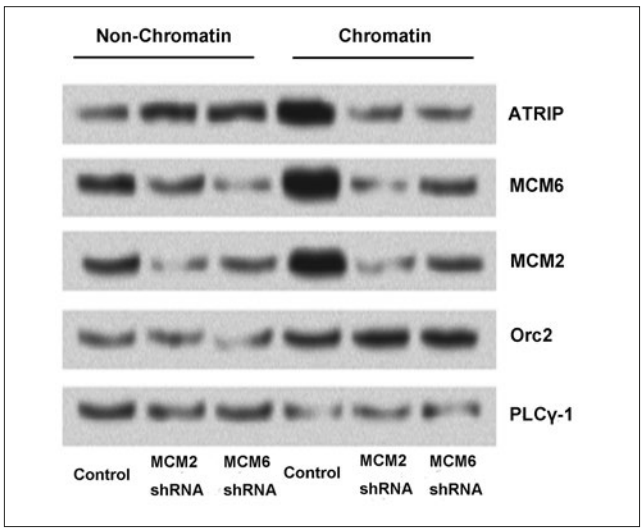

Fig. 3: Downregulation of MCMs reduces ATRIP chromatin fraction. The expression of MCM2 and MCM6 was down-regulated by using shRNA in JeKo cells and chromatin fraction is performed. Orc2 and PLCγ-1 are respectively marker proteins for chromatin and non-chromatin fractions.
ATRIP, ATR-interacting protein; MCM, minichromosome maintenance complex; shRNA, short hairpin RNA

2.4. Downregulation of MCMs reduces ATRIP phosphorylation

To test whether MCMs are essential for ATRIP phosphorylation, the expression of MCM2 was downregulated by using shRNA in JeKo cells and ATRIP phosphorylation was analyzed. As shown in Fig. 4, we observed that the expression levels of pS224-ATRIP were significantly decreased by MCM2 compared to the control group. Besides, the levels were statistically reduced in a dose-dependent manner in JeKo cells. The expression levels of pS224-ATRIP were at the lowest levels by transfection with 2.5 μg MCM2 shRNA. Our data suggested that downregulation of MCMs reduced ATRIP phosphorylation in a dose-dependent manner.

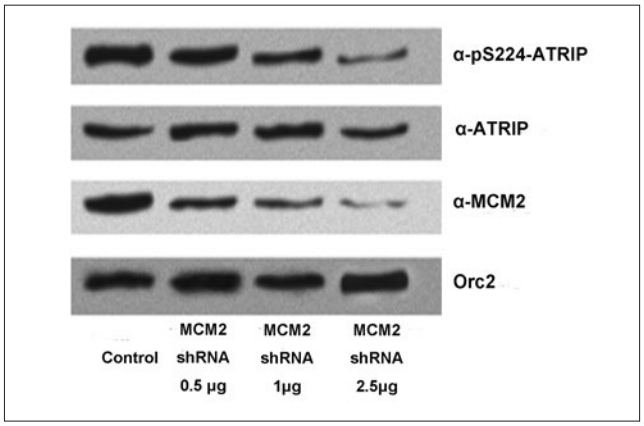

Fig. 4: Downregulation of MCMs reduces ATRIP phosphorylation. The expression of MCM2 is down-regulated by using shRNA in JeKo cells and the expression levels of pS224-ATRIP are analyzed. Orc2 is a marker protein for chromatin.
ATRIP, ATR-interacting protein; MCM, minichromosome maintenance complex; shRNA, short hairpin RNA

3. Discussion

In the present study, we provide new evidence for the interaction between MCM complex and ATRIP. Our data demonstrate that ATRIP directly interacts with MCM2, MCM3, MCM6, and MCM7 in JeKo cells. Suppression the expression of MCM2 and MCM6 markedly reduces ATRIP chromatin fraction, and suppression the expression of MCM2 statistically decreases the expression of ATRIP phosphorylation in a dose-dependent manner. These results indicate that interaction between ATRIP and MCM complex is essential for ATRIP chromatin loading and ATRIP phosphorylation.

MCL is a distinct lymphoid malignancy characterized by low-grade lymphoproliferative disorders derived from mature B-cells. The disease is particularly aggressive with short responses to treatment, and few patients could be cured with the current therapies. Recent studies have focused on genetic and molecular mechanisms (Jares et al. 2007). It has been suggested that the cell cycle and the DNA damage response are involved in the pathogenesis mechanism of MCL (Fernandez et al. 2005; Jares et al. 2007). The deletion of chromosomal region 11q22-23 is the most common secondary cytogenetic alterations in MCL, including ATM (Stilgenbauer et al. 1999). In addition to ATM, Checkpoint Kinase (CHK) 1 and CHK2 have been reported to be responsible for the DNA damage response pathway in MCL (Tort et al. 2002, 2005). CHK1 and CHK2, two kinases of ATM downstream, play critical roles in prevention of cell cycle progression with respect to ATM and ATR activation following DNA damage (Zhou and Elledge 2000).

Recently, the ATR-ATRIP complex has been reported to play a significant role in DNA damage and replication stress. ATRIP and its homologues have multiply regulatory roles in the respective kinase complexes (Ball et al. 2005; Namiki and Zou 2006). ATRIP has at least three functional domains: an N-terminal domain, a coiled-coil domain, and an ATR-interaction domain. The N-terminal domain is required for its stable association with replication protein A (RPA)-ssDNA and facilitates recruitment of ATR-ATRIP to damaged lesions of nuclear foci (Ball et al. 2005; Kim et al. 2005). The coiled-coil domain regulates the dimerization of ATRIP and is of importance to ATR signaling pathway (Ball and Cortez 2005; Itakura et al. 2005). The ATR-interaction domain that is localized in the C-terminus of ATRIP is of great importance for the stability of both proteins (Ball et al. 2005; Falck et al. 2005). These two proteins are mutually dependent partners in cell cycle checkpoint signaling pathways. It has been reported that loss of ATRIP by using specific small interfering RNA (siRNA) results in destabilization of ATR (Cortez et al. 2001; Ogi et al. 2012). Suppression of both ATRIP and ATR leads to the loss of checkpoint responses to DNA damage (Cortez et al. 2001). In addition, previous studies have confirmed that phosphorylation of ATRIP regulates the G2 checkpoint, while the underlying mechanism has not yet defined (Myers et al. 2007; Venere et al. 2007). Therefore, a better understanding of the ATRIP function might develop new target therapeutics for treatment human diseases.

Han et al. (2014) have found that MCM complex facilitates the recruitment of CHK1 onto chromatin and the interaction between CHK1 and MCM complex is essential for DNA damage-induced phosphorylation of CHK1. The MCM complex is a well-known component of the replicative DNA helicase in eukaryotic cells and has been considered as a critical player in ATM and ATR checkpoint kinases (Cortez et al. 2004). Therefore, we speculated that there might be an interaction between ATRIP and MCM complex. To confirm the results, we first produced a stable JeKo cell line expressing differently tagged ATRIP (Myc-, HA or Flag) and MCM complex (MCM2, MCM3, MCM5, and MCM6). After transfection for 48 h, we identified ATRIP-interacting protein by using MS. We observed that ATRIP interacts with MCM2, MCM3, MCM6, and MCM7 in JeKo cells. GST pull-down assay was performed to confirm the direct interaction between ATRIP and MCM complex. In addition, to test whether MCMs is essential for ATRIP chromatin loading, we silenced the expression of MCM2 and MCM6. The data showed that ATRIP chromatin fraction was significantly reduced by suppression of MCM2 and MCM6 compared to the non-chromatin. We further tested whether MCMs was essential for ATRIP phosphorylation. MCM2 expression was downregulated and different doses of MCM2 shRNA were added, and then the levels of ATRIP were analyzed. The results showed that the expression levels of pS224-ATRIP were significantly decreased by MCM2 shRNA compared to the control group in a dose manner.

In conclusion, we suggest that interaction between ATRIP and MCM complex is required for ATRIP chromatin loading and ATRIP phosphorylation.

4. Experimental

4.1. Cell culture

JeKo cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). All the cells were cultured RPMI-1640 medium or Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) at 37 °C in a humidified atmosphere of 5% CO_2.

4.2. Plasmid construction and cell infection

Myc-, or Flag-tagged vectors expressing ATRIP wide type (WT) or mutants were transiently transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. MCMs were produced by using standard polymerase chain reactions (PCR) with lentiviral vectors for each MCM (Tsuji, Ficarro et al. 2006). In addition, small hairpin RNA (shRNA) against MCM2 and MCM6 were transiently transfected by using Lipofectamine 2000 (Invitrogen). After transfection, the supernatants were prepared and JeKo cells were infected with retroviral medium with polybrene.

4.3. Chromatin fractionation

Cell fractionation was performed as previously described (Guo et al. 2006; Mendez and Stillman 2000). Briefly, JeKo cells were suspended in buffer A (150 mM HEPES, pH 7.9, 150 mM KCl, 1.5 mM MgCl2, 0.2% NP-40, 5 mM NaF, 1 mM dithiothreitol, and 10% glycerol). The cell suspension that contains cytoplasmic fraction was then

centrifuged, re-suspended in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT), and centrifuged again. The cell suspension that contains cytoplasmic fraction and non-cytoplasmic fraction was re-suspended in buffer C (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% NP-40, 1 mM DTT, and protease inhibitors) and centrifuged. The proteins in these fractions were analyzed by using western blotting.

4.4. Identification of ATRIP-interacting protein by mass spectrometry (MS)

The ATRIP-interacting protein was identified by MS as previously described (Han et al. 2014). Briefly, JeKo cells stably expressing differently tagged ATRIP fractionated into cytoplasmic and nuclear compartments. ATRIP-interacting proteins were isolated, eluted, and denatured in urea. The ATRIP-interacting proteins were subsequently reduced with tris-(2-carboxyethyl)-phosphine hydrochloride (Molecular Probes, Eugene, OR), alkylated with iodoacetamide (Sigma-Aldrich), digested overnight with trypsin (Invitrogen), and immobilized to monolithic silica capillary columns (Restek, USA). A quaternary high performance liquid chromatography (HPLC) pump (Agilent Technologies) was connected to flush the columns and a linear ion trap (LTQ) mass spectrometer (Thermo-Fisher, San Jose, CA) was applied to detect ions. MS raw data were extracted to obtain MS/MS files using RawXtract (version 1.9.9) (McDonald et al. 2004).

4.5. Immunoprecipitation (IP) and immunoblot (IB) analysis

Cells were lysed in 1% Nonidet P-40 lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM $CaCl_2$, 1 mM Na_3VO_3, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture), incubated on ice, and centrifuged. The cell lysates were immunoprecipitated with anti-Flag, anti-HA, anti-GFP, or anti-Myc and immunoblotted with the indicated antibodies: anti-MCM2 antibody (ab4461, Abcam Inc., Cambridge, MA, USA), anti-MCM3 antibody (ab4460, Abcam), anti-MCM5 antibody (ab17967, Abcam), and anti-MCM6 antibody (ab201683, Abcam).The immunoprecipitates were then washed three times with 0.1% Nonidet P-40 lysis buffer, subjected to immunoblot analysis, and separated by sodium dodecyl sulfonate (SDS)-polyacrylamide gel electrophoresis (PAGE). The membranes were then transferred to polyvinylidene difluoride (PVDF) membranes and incubated with the above antibodies, and were visualized using chemiluminescence (MicroSpin GST Purification Module; GE Healthcare).

4.6. Expression and purification of glutathione S-transferase (GST)-tagged proteins

GST fusion proteins were transformed into *Escherichia coli* BL21 using glutathione agarose (GE Healthcare) affinity chromatography. The cells were then inoculated into Luria Bertani (LB) (Sigma-Aldrich) (pH 7.0) at 37 °C.The recombinant protein was induced by addition of 1 mM isopropyl 1-thio-β-d-galactopyranoside (IPTG, Thermo Scientific, Waltham, MA). Four hours later, the cells were lysed and centrifuged, and the supernatants were collected. Then the supernatants were purified with a Glutathione Sepharose 4B column (GE Healthcare). The recombinant GST or GST-tagged proteins were eluted by an elution buffer containing 50 mM Tris-HCl (pH 9.5), 150 mM NaCl, 0.1 mM EDTA, 0.1mM $ZnCl_2$, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol and 10 mM reduced glutathione), and detected with SDS-PAGE.

4.7. Statistical analysis

All experiments were carried out in triplicate and the results were represented as the mean±standard deviation (SD). Differences comparison between groups was performed by one-way analysis of variance and/or t test with SPSS 16 (IBM, New York, NY). $P < 0.05$ was considered with statistically significant differences.

Conflicts of interest: Authors declare that there is no conflict of interests.

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