

## RESEARCH ARTICLE

# High Mobility Group Box 1 Protein Is Methylated and Transported to Cytoplasm in Clear Cell Renal Cell Carcinoma

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### Abstract

**Background:** The high mobility group box 1 (HMGB1) protein is a widespread nuclear protein present in most cell types. It typically locates in the nucleus and functions as a nuclear cofactor in transcription regulation. However, HMGB1 can also localize in the cytoplasm and be released into extracellular matrix, where it plays critical roles in carcinogenesis and inflammation. However, it remains elusive whether HMGB1 is relocated to cytoplasm in clear cell renal cell carcinoma (ccRCC). **Methods:** Nuclear and cytoplasmic proteins were extracted by different protocols from 20 ccRCC samples and corresponding adjacent renal tissues. Western blotting and immunohistochemistry were used to identify the expression of HMGB1 in ccRCC. To elucidate the potential mechanism of HMGB1 cytoplasmic translocation, HMGB1 proteins were enriched by immunoprecipitation and analyzed by mass spectrometry (MS). **Results:** The HMGB1 protein was overexpressed and partially localized in cytoplasm in ccRCC samples (12/20, 60%,  $p < 0.05$ ). Immunohistochemistry results indicated that ccRCC of high nuclear grade possess more HMGB1 relocation than those with low grade ( $p < 0.05$ ). Methylation of HMGB1 at lysine 112 in ccRCC was detected by MS. Bioinformatics analysis showed that post-translational modification might affect the binding ability to DNA and mediate its translocation. **Conclusion:** Relocation of HMGB1 to cytoplasm was confirmed in ccRCC. Methylation of HMGB1 at lysine 112 might the redistribution of this cofactor protein.

**Keywords:** HMGB1 - methylation - RCC - renal cancer - transcription regulation - cytoplasmic location

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### Introduction

Renal cell carcinoma (RCC) accounts for approximately 90% of all renal malignancies and 2%-3% of all malignant tumors in adults (Rini et al., 2009). Although there are significant advances in imaging techniques and increase in incidental diagnosis, up to 30% of patients are initially diagnosed with metastatic disease. In addition, 40% of patients undergoing localized RCC treatment will have a relapse and develop into metastatic RCC (mRCC) (Motzer et al., 2007). The prognosis of patients with mRCC remain poor (Gupta et al., 2008). RCC consists of a heterogeneous group of different subtypes. Four main histological renal cancers are identified according to Heidelberg classification and the majority is clear cell renal cell carcinoma (ccRCC, 60%-80%) (Kovacs et al., 1997).

The high mobility group box 1 (HMGB1) protein was described originally as a non-histone DNA binding protein, which is highly conserved evolutionarily with high acidic and basic amino acid content (Goodwin et al., 1977). HMGB1 protein binds to minor groove of DNA and promotes the assembly of site-specific transcriptional proteins (Bonaldi et al., 2002). In addition

to its well-established nuclear functions, HMGB1 protein functions as an extracellular signalling molecule which plays a critical role in inflammation and carcinogenesis (Scaffidi et al., 2002; Ellerman et al., 2007). The HMGB1 protein, secreted actively by NK cells, monocytes and macrophage, functions as an inflammatory cytokine (Wang et al., 1999; Lotze et al., 2005). Necrotic cells, especially those derived from cancer tissues, release passively HMGB1 protein, which mediates regional inflammation and the development of cancer (Scaffidi et al., 2002; Luo et al., 2012). Extracellular HMGB1 protein interacts with several receptors including the receptors for advanced glycation end products (RAGE), Toll-like receptors (TLR) and CD24 (Tian et al., 2007). It has been reported that HMGB1 and RAGE were overexpressed in ccRCC. Besides, HMGB1 promotes the development and progression of ccRCC via ERK 1/2 activation, which is partially mediated by RAGE (Lin et al., 2012).

The mechanism have been an area of intense investigations which governing the cytoplasmic translocation and extracellular secretion of HMGB1. The active secretion of HMGB1 was first highlighted in experiments that activated macrophages secreted HMGB1 as a late mediator of lethality in sepsis of

mice (Wang et al., 1999). The question then changed, and became that what kinds of pathways regulated the release of HMGB1. Agresti and Bianchi showed that monocytes and macrophages could acetylate HMGB1 extensively upon activation by lipopolysaccharide; acetylated HMGB1 was transported from nucleus to cytoplasm, and secreted actively to blood or interstitial fluid when monocytes received appropriate signals (Bonaldi et al., 2003). Another study reported that the conformation of Cys106, whether reduced or oxidated, was critical for regulating nucleocytoplasmic shuttling of HMGB1 (Hoppe et al., 2006). In cultured hepatocytes, HMGB1 releasing was regulated by TLR4, which depended on reactive oxygen species production (Tsung et al., 2007). Later, it has been demonstrated that decreased histone deacetylases (HDACs) activity in hepatocytes, following ischemia and reperfusion, was a mechanism that promoted hyperacetylation and secretion of HMGB1 (Evankovich et al., 2010). In addition, several investigations demonstrated that HMGB1 with phosphorylation at specific serine residues of nuclear localization signals (NLS) regions was transported to cytoplasm, and subsequently secreted from cells (Youn et al., 2006; Kang et al., 2009; Lee et al., 2012). Hence, it is safe to say that post translational modifications (PTMs) play a critical role in governing the nucleocytoplasmic shuttling of HMGB1.

The purpose of this study is to investigate the subcellular distribution of HMGB1 and the possible mechanism of nucleocytoplasmic translocation in ccRCC. Our data conclusively demonstrated that HMGB1 was overexpressed and transported to cytoplasm in ccRCC. In addition, HMGB1 in ccRCC was mono-methylated at Lys 112 in ccRCC, which was barely seen in adjacent renal tissues. We further investigated the role of lysine 112 and the possible functions of Lys 112 methylation of HMGB1 in ccRCC.

## Materials and Methods

**Ethics Statement:** All procedures were consistent with the National Institutes of Health Guide and approved by institutional board with patients' written consent. This study was evaluated and approved by the Ethics Committee of Provincial Hospital Affiliated to Shandong University.

### *Tissue specimen collection and protein extraction*

Twenty cases of pathologically confirmed specimens matched with adjacent normal adjacent renal tissues were collected from patients undergoing radical nephrectomy between October 2011 and May 2012 at Department of Urology, Provincial Hospital Affiliated to Shandong University, China. None of them had any systemic anti-tumor therapy before surgery treatment. 100mg tissues was rinsed three times with ice-cold PBS before adding 1000µl lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS and protease inhibitors). The tissue was then dissected with a tissue grinder for 30 times. Lysates were kept on ice for 10min during which vibrated 30 seconds every 5 minutes.

Insoluble material was pelleted at 12,000 g for 10 minutes at 4 °C. The supernatant was stored at -80°C. Nuclear proteins were extracted following the protocol of a nuclear protein extraction kit (Sangon Biotech). Subcellular fractions of tissues were extracted by Subcellular Proteome Extraction Kit (Merck Millipore). Protein levels were measured by the Enhanced BCA Protein Assay Kit (Beyotime Biotechnology).

### *Western blotting*

100 µg total proteins or 10-20µl effluent proteins were loaded perwell and separated on 12% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred to nitrocellulose membranes followed by blocking with 5% skim milk and staining with HMGB1 antibody (1:10000; Abcam) and  $\alpha$ -tubulin antibody (1:10000; Sigma-Aldrich). Membranes were washed and incubated with fluorescent secondary antibody (1:10000; LI-COR). Then fluorescent signals were detected by Odyssey infrared imaging system (LI-COR). The gray value of bands was calculated by Odyssey 3.0 software.

### *Immunohistochemistry*

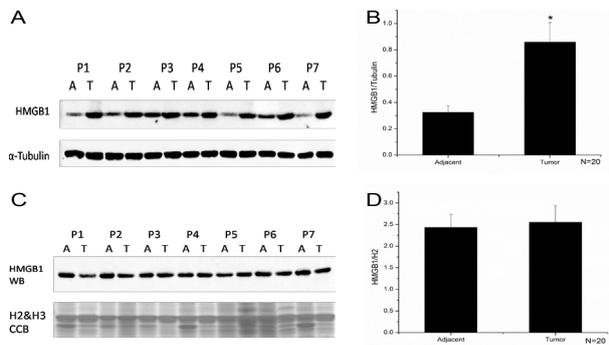
Kidney tissue was fixed in formalin overnight and embedded in paraffin with standard techniques. Sections (4 µm) were cut and stained with Hematoxylin-Eosin (HE). The immunohistochemistry detection of HMGB1 was performed with the SP HRP kit (Cowin Bioscience). As described in protocols of the kit, antigen retrieval was performed in a water bath using citrate-EDTA buffer (10 mM citric acid, 2 mM EDTA, 0.05% Tween 20, pH 6.2). Hydrogen peroxide was used to deactivate intrinsic peroxidase. Sections were incubated with diluted monoclonal rabbit HMGB1 antibody (1:500; Abcam) overnight at 4°C. Add biotin labeled secondary antibody to the slides followed by adding horseradish peroxidase (HRP) labeled streptavidin. After staining with DAB and counterstaining with hematoxylin, the slides were recorded using a digital camera (Olympus).

### *Immunoprecipitation*

Lysates were precleared for nonspecific binding to beads by incubation with normal serum IgG-bound agarose beads (Beyotime Biotechnology) for 1 h at 4°C. After 5 min of centrifugation at 3000 rpm, the supernatants were transferred to fresh tubes for further analysis. Then combined 100 µl lysate and 1µg HMGB1 antibody (Abcam) and incubated overnight at 4°C with gentle rotation. Added 20 µl homogeneous protein G agarose (Roche Applied Science) and allowed to rotate for 3 hours at 4°C. The beads were then washed four times with the PBS. Added 25µl protein loading buffer to the pellet. Boiled the solution for 5 minutes for elution and denaturing protein. Centrifuged at 3,000 rpm and transferred supernatant to a fresh tube. Resulting lysates were then loaded and fractionated on a 12% SDS-polyacrylamide gel.

### *In gel digestion*

HMGB1 was separated by SDS-PAGE followed by

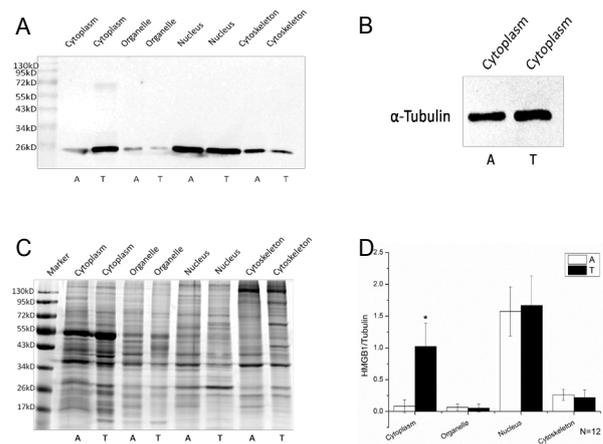


**Figure 1. Expression of HMGB1 in Whole Tissue Extracts.** A. Western blotting of HMGB1 and  $\alpha$ -tubulin in whole cell lysates from tumor tissue(T) and adjacent tissue(A). B. The statistic results of gray values ratios analyzed by Odyssey 3.0 ( $P < 0.001$ ). C. Western blotting of HMGB1 in whole nuclear extracts and coomassie blue staining of histone H2 and H3. D. The statistic results of gray values ratios analyzed by Odyssey 3.0 ( $P > 0.05$ )

a Coomassie Blue staining. Rinsed the gel, excised the band and destained the gel completely with 100mM  $\text{NH}_4\text{HCO}_3$  and 30% acetonitrile (Merck Millipore); vacuum centrifuged to dryness. Then added 100mM DTT to reduce proteins. Then alkylation reaction was processed by adding 200mM iodoacetamide (Sigma-Aldrich) and incubating for 20 minutes in the dark; vacuum centrifuged to dryness, added 50 ng trypsin and 40 $\mu\text{l}$  50 mM  $\text{NH}_4\text{HCO}_3$  buffer, incubated 20 hours at 37°C. Withdrawed trypsin lysis to a fresh tube and added 100 $\mu\text{l}$  60% acetonitrile /0.1% trifluoroacetic acid; incubated the tube for 15 minutes in the sonication bath; withdrew supernatant and mixed with the lysis; repeated twice; vacuum centrifuged to dryness. Added 10  $\mu\text{l}$  0.1% formic acid for LC-MS analysis.

#### Mass spectrometry analysis

Peptides were fractionated by reversed phase high pressure liquid chromatography (HPLC) using Easy-nano-LC-II system (Thermo Fisher Scientific). The column was a 75  $\mu\text{m}$ -inner diameter 150mm self-packed column with 5  $\mu\text{m}$  C18 resin (Reprosil-Pur). The flow rate was 250 nL/min; solvents were water, 0.1% formic acid (Phase A) and acetonitrile, 0.1% formic acid (Phase B); the gradient was 2-10% in 10 minutes; 10%-35% B over 85 minutes; 35%-50% B in 10 min; 50%-90% B in 10 minutes; 90% B in 5 min. The HPLC system was coupled to a LTQ-Orbitrap Elite (Thermo Fisher Scientific) mass spectrometer. The MS parameters were as follows. Full scan by Orbitrap at a resolution of 24,000 and followed by 20 LTQ MS/MS depended scans for top 20 intense ions from the full scan. Dynamic exclusion was used in global data dependent settings: repeat count was 1; repeat duration was 20s; exclusion list size was 500; exclusion duration was 60s. Every sample was run repeatedly for three times with the same methods. The softwares for database searching were SEQUEST and Mascot v2.4 (in house version). Oxidation of methionine, methylation of lysine and arginine were set as dynamic modification; carbamidomethylation of cysteines was static modification. And for determination of HMGB1 modification, manual spectrum interpretation was used.



**Figure 2. Expression of HMGB1 in Subcellular Tissue Extracts.** A. Western blotting of HMGB1 in subcellular fraction from tumor tissue(T) and adjacent tissue(A). B. Coomassie blue staining of subcellular fraction from tumor tissue(T) and adjacent tissue(A). C. Western blotting of  $\alpha$ -tubulin in cytoplasmic extracts from tumor tissue(T) and adjacent tissue(A). D. The statistic results of gray values ratios analyzed by Odyssey3.0. Differences with  $P < 0.05$  were considered significant

#### Statistical analysis

Data was presented as the mean  $\pm$  standard deviation (SD). Student's t test and chi-square test were used for statistical analysis. All statistical analyses were done with SPSS v13.0. A  $P$  value  $< 0.05$  was considered statistically significant. The three dimension structural drawing was built using UCSF Chimera (<http://www.cgl.ucsf.edu/chimera>) and PDB file 2YRQ (<http://www.rcsb.org/pdb/home/home.do>) (Pettersen et al., 2004).

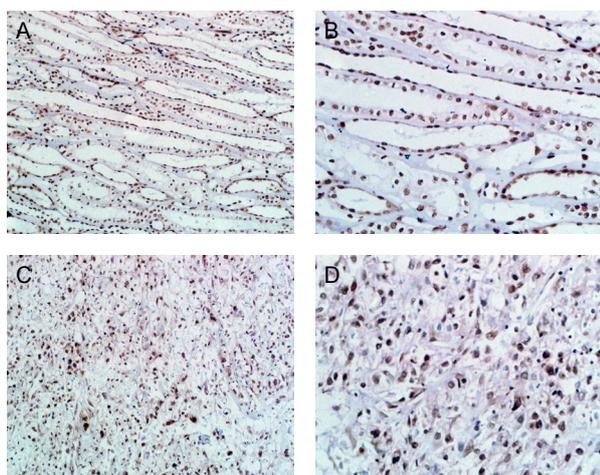
## Results

#### HMGB1 protein is overexpressed in whole tissue extracts

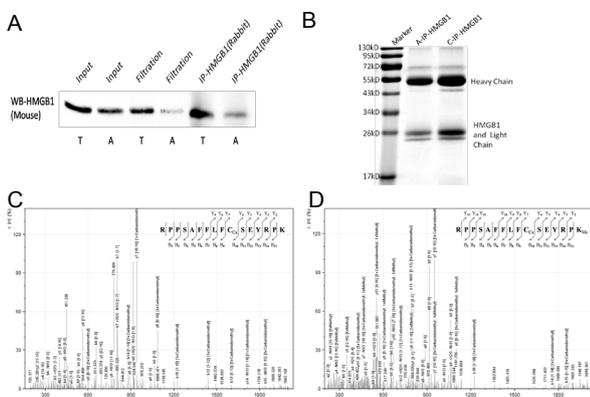
It has been reported that HMGB1 is overexpressed in renal cell carcinoma. To evaluate the expression level of HMGB1 in our samples, we collected tumor tissue specimens with matched adjacent renal tissues from 20 patients undergoing radical nephrectomy. Whole tissue lysates and nuclear lysates were extracted and undergone western blotting respectively. In whole tissue extracts, the expression of HMGB1 protein was up-regulated as previous studies reported (Figure 1A). The expression level of 20 cases was evaluated by densitometry, and the expression level of tumors was 2.64 fold higher than adjacent tissues ( $P < 0.001$ ; Figure 1B). However, significant difference of the HMGB1 expression level couldn't be identified in nuclear extracts from cancer tissues and adjacent tissues ( $P > 0.05$ ). These data implicated that HMGB1 expressed stably in nuclear extracts in ccRCC (Figure 1C&D).

#### HMGB1 protein is overexpressed in cytoplasm during subcellular fractionation

It is hypothesized that redundant HMGB1 was transported to cytoplasm while the nuclear maintained a stable amount of HMGB1 to operated normally in ccRCC. To demonstrate that overexpression of HMGB1 mainly exists in cytoplasmic compartment, the subcellular

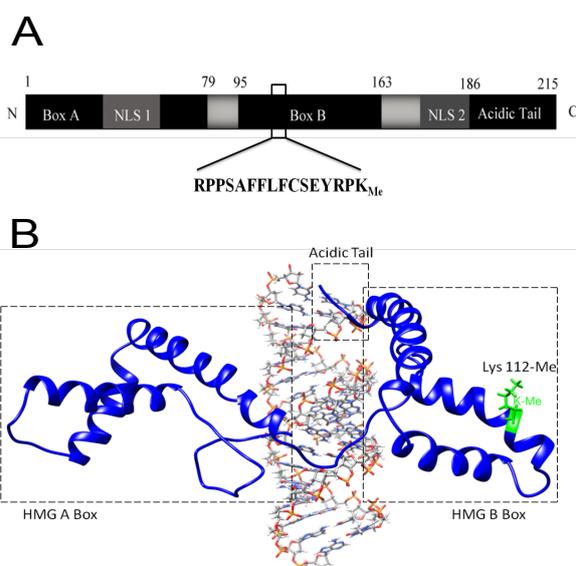


**Figure 3. Expression and Localization of HMGB1 in Immunohistochemistry Assay.** A. B. Expression and localization of HMGB1 in normal renal tissue(A, 1:200; B, 1:400). C. D. Expression and localization of HMGB1 in ccRCC tissue (C, 1:200; D, 1:400)



**Figure 4. Immunoprecipitation of HMGB1 and LC-MS/MS Analysis of HMGB1.** A. Immunoprecipitation and Western blotting of HMGB1 from tumor tissue(T) and adjacent tissue(A). B. SDS-PAGE and coomassie blue staining of immunoprecipitated HMGB1 protein from adjacent (A-IP-HMGB1) and cancer tissue (C-IP-HMGB1). C. The MS/MS spectrum of the peptide covering unmodified K112. D. The MS/MS spectrum of the peptide covering methylated K112

fractions were extracted from 12 specimens which had HMGB1 overexpression in whole cell lysates. Cytosolic, membrane, organelle, nuclear and cytoskeletal proteins were extracted by the kit mentioned above. The efficiency of the subcellular proteome extraction kit was well established (Sabio et al., 2005; Alao et al., 2006). Since there was not a qualified internal reference expressed in all four fractions, coomassie brilliant blue staining was used as a reference of protein concentration (Figure 2B). Western blotting was applied to detect the expression of HMGB1 in all four subcellular compartments (Figure 2A). For densitometry evaluation, the  $\alpha$ -tubulin in cytoplasm was consider as an internal standard (Figure 2C). Statistical results showed that HMGB1protein was overexpressed significantly in cytoplasm (Figure 2D). While in nuclear protein fraction, the expression level was relatively steady. The HMGB1 bands appeared in organelle protein and cytoskeletal fractions were considered as the contamination during protein extraction.



**Figure 5. Structure of HMGB1 Protein.** A. HMGB1 protein has 215 amino acids and a molecular weight of 25 kDa. HMGB1 protein is composed of three domains: two positively charged domains (A box and B box) and a negatively charged carboxyl terminus (Acidic tail). A and B boxes are responsible for its DNA-binding ability. B. The three dimension structure of HMGB1 protein. The schematic of HMGB1 was built using UCSF chimera (<http://www.cgl.ucsf.edu/chimera>) and PDB file 2YRQ (<http://www.rcsb.org/pdb/home/home.do>). The Lys 112 was in a  $\alpha$ -helix structure of HMGB1 B box

Thus, it is quite possible that HMGB1 is overexpressed in ccRCC, while redundant HMGB1 protein is transported to cytoplasm rather than staying in nucleus.

*HMGB1 is transported to cytoplasm in immunohistochemistry assay*

Twenty RCC tissue slides with matched adjacent tissues were analyzed by immunohistochemistry assay. The amount of nuclear HMGB1 in adjacent tissue and well differentiated ccRCC (G1, G2) were not significantly different. However, HMGB1 was mainly located in nucleus and transported to cytoplasm at a low level in adjacent tissues of ccRCC (Figure 3A&B), whereas cytoplasmic HMGB1 was found at a high level in most tumor tissues. For poorly differentiated ccRCC (G3, G4), HMGB1 was overexpressed both in nucleus and cytoplasm, as shown in Figure 3C&D.

*HMGB1 is methylated at lysine 112 in cancer tissue*

To investigate the mechanism of HMGB1 relocation in ccRCC, we hypothesized that some post translational modifications governed this nucleocytoplasm shuttling. Immunoprecipitation was used to enrich HMGB1 protein. The whole tissue extracts from 12 cases were mixed whose HMGB1 protein were overexpressed, and so were the matched adjacent tissues. Since the molecular weight of HMGB1 is almost the same with the light chain of IgG, rabbit HMGB1 antibodies were used for immunoprecipitation and mouse HMGB1 antibodies were used for western blotting. Immunoblotting assay following immunoprecipitation indicated that the HMGB1 protein was pulled down by antibodies successfully (Figure 4A).

HMGB1 and light chain of IgG were excised from the gel and digested by trypsin together, followed by mass spectrometry analysis (Figure 4B). The raw data was searched by a series of database searching softwares. SEQUEST and MASCOT were used first to evaluate the quality of the raw file and 75.48% of peptides were identified in HMGB1 protein sequence. ProteinProspect and MODa were used for unrestricted modification searching, and the coverage was 86.0% by blind searching (Na et al., 2012). In the results of unrestricted modification searching, methylation appeared four times in cancer group and was absent in adjacent group. The four methylated spectra were conservatively the same peptide, RPPSAFFLFCSEYRPKMe (Figure 5A). And the methylation site of HMGB1 was at lysine 112, which was confirmed by manual spectrum annotation (Figure 4C&D).

## Discussion

In 2000, Weinberg and Hanahan proposed the six essential hallmarks of cancer. They were self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan et al., 2000). With rapid advances in cancer researching in the past decade, it is well accepted that HMGB1 is associated with each of the six hallmarks (Tang et al., 2010). Despite its critical roles in nucleus, such as DNA repair, interaction with transcriptional factors like p53, p73 and Rb protein, the functions of extracellular HMGB1 receive a great deal of attention recently (Stros et al., 2002; Banerjee et al., 2003; Krynetski et al., 2003; Jiao et al., 2007). It is noteworthy that HMGB1 protein released from cancer cells is a double-edged sword: it promotes tumor neoangiogenesis; it triggers protective anti-neoplastic T-cell responses (Campana et al., 2008). HMGB1 protein which is secreted by dying cancer cells interacts with TLR4 expressed by dendritic cells (DCs) and activates anti-tumor T-cell immunity (Apetoh et al., 2007). However, HMGB1 protein mediates the activation of TLR2 and thereby the progression of brain cancer (Curtin et al., 2009). Interaction with another important HMGB1 receptor RAGE causes activation and leukocyte recruitment (Lotze et al., 2003). And inhibition of HMGB1-RAGE pathway suppresses tumor progression (Taguchi et al., 2000). In addition, HMGB1-RAGE signalling is activated in ccRCC which promotes the development and progression of tumor via ERK1/2 activation (Lin et al., 2012). Although a great deal of studies have reported roles of HMGB1 in oncogenesis, few reported the mechanisms of its cytoplasmic translocation and extracellular secretion. There is now a general consensus that cytoplasmic translocation is the precondition of extracellular secretion (Gauley et al., 2009). And it is safe to say that post translational modifications play an important role in the nucleocytoplasmic redistribution. As mentioned above, oxidation, hyperacetylation and phosphorylation of HMGB1 contribute to its cytoplasmic relocation in inflammatory and cancer cells (Hoppe et al., 2006; Youn et al., 2006; Tsung et al., 2007; Kang et al., 2009; Evankovich

et al., 2010; Lee et al., 2012). Experiments in *Schistosoma mansoni* conclusively demonstrated that acetylation and phosphorylation played roles in cellular trafficking, culminating with its secretion to the extracellular milieu (Carneiro et al., 2009; de Abreu da Silva et al., 2011). In addition, it has been reported that single post translational methylation at lysine 42 of HMGB1 in neutrophil altered its conformation and weakened its DNA binding activity, causing it translocated to cytoplasm by passively diffusion out of nucleus (Ito et al., 2007).

Our data implicate that the cytoplasmic distribution of HMGB1 is associated with Lys112 post translational methylation in ccRCC. To our knowledge, this is the first report to indicate that HMGB1 is mono-methylated at Lys112 in ccRCC. As shown in Figure 5A, HMGB1 protein comprises three domains: two tandem positively charged HMG-box (termed A box and B box) connected by a basic linker to a carboxyl terminus (termed acidic tail) (Sessa et al., 2007). Lys112 lies in the B box of HMGB1 and its methylation may affect the affinity of HMGB1 to DNA apparently. Functionally, A box and B box are DNA-binding motifs. Extensive work has been done in the area of molecular interaction between HMGB1 and DNA (Yang et al., 2013). The solution structure of A box and B box have been solved respectively by NMR spectroscopy (Figure 5B) (Read et al., 1993; Weir et al., 1993). Each box consists three  $\alpha$ -helices that form an L shape. The A box or B box binds DNA in the minor groove despite of DNA sequence (Bonaldi et al., 2002). HMG boxes cause a significant bend in DNA and bind to the distorted DNA, such as four-way DNA junctions, cisplatin-damaged DNA and DNA mini-circles (Bianchi et al., 1989; Pil et al., 1992). Further studies indicate that A box is more effective than B box at binding to distorted DNA while B box has a higher preference for bending DNA and generating distorted DNA (Teo et al., 1995; Webb et al., 1999). However, B box is able to substitute for the non-functional A box without the presence of acidic tail (Assenberg et al., 2008). The general function of acidic tail remains elusive despite its negatively regulations on HMG boxes. It down-regulates the binding and bending ability of HMG boxes for linear DNA and distorted DNA although the binding affinity to DNA minicircles is unaffected (Bianchi et al., 1989; Pil et al., 1992; Stros et al., 1994; Pasheva et al., 1998; Lee et al., 2000). Several investigations have shown that the acidic tail binds to HMG boxes in vitro (Ramstein et al., 1999; Jung et al., 2003). In another study, separated acidic tail peptide has been shown to contacts with HMG boxes and the two linkers (Knapp et al., 2004). Recently, it has been found that the acidic tail has a higher preference for the B box, which may have important implications for HMGB1 in vivo (Stott et al., 2010).

A previous report has shown that methylation at Lys42 alters the conformation of HMGB1 and reduces its affinity to DNA, causing it to become largely distributed in the cytoplasm by passive diffusion out of nucleus (Ito et al., 2007). Thus Lys112 methylation may facilitate the translocation in a similar way. The HMG box is L shaped and comprises three  $\alpha$ -helices that contain many of the conserved basic and aromatic residues that define the HMG box. The structure is stabilized by

profuse hydrophobic interaction formed by conserved aromatic residues (Weir et al., 1993). Methylation at Lys112 affects the formation of the hydrophobic core by altering the trajectory of  $\alpha$ -helix I. Besides, this Lys112 is highly conserved in vertebrate HMGB1 and HMGB2, so the residue may be important for the conformation of HMG box. It is probably that the DNA binding ability of HMGB1 is significantly weakened when methylated. Then HMGB1 with a molecular weight of 25kDa shuttles quickly between the nucleus and the cytoplasm, which mainly localized in nucleus in renal cells. The nuclear pore, with a diameter of 10nm, allows passive diffusion of proteins less than 40~60kDa (Peters et al., 1986). Thus the methylated HMGB1 becomes distributed in the cytoplasm through passive diffusion. Cytoplasmic HMGB1 may be released to extracellular matrix with specific signals or cell necrosis, causing the activation of HMGB1/ERK axis which promotes the development of ccRCC (Lin et al., 2012). In addition, the B box also has cytokine-like activity which can induce macrophage secretion of excess proinflammatory cytokines (Li et al., 2003). It is speculative that Lys112 methylation might affect its cytokine-like activity. Coincidentally, it has been reported that Lys112 of HMGB1 has post translational ubiquitination in normal hepatocyte (Tan et al., 2008). However, the precise functions of Lys 112 ubiquitination have yet to be fully defined. A previous report had shown ubiquitination of histone H2BK123 enhanced its binding activity and stabilized nucleosome (Chandrasekharan et al., 2010). It is implicated that methylation of HMGB1 at Lys112 reduces its DNA-binding ability through the substitution of Lys112 ubiquitination. Viewed in toto, the DNA-binding activity may be reduced after Lys112 methylation, and subsequently HMGB1 is transported to cytoplasm in ccRCC.

In conclusion, it is reported for the first time that HMGB1 protein is mono-methylated at Lys112, which might mediate its cytoplasmic translocation. We have demonstrated the overexpression of HMGB1 in whole ccRCC tissue lysates and its stable expression in nuclear protein extracts in ccRCC. HMGB1 is mainly overexpressed in cytoplasm instead of other subcellular fractions. We further investigated the differences in post-translational modifications of HMGB1 between renal cancers and matched adjacent tissues. Methylation at Lys112 was identified and confirmed. Lys112 is a conserved residue lying in B box of HMGB1. Its methylation causes the conformational change of HMG box and weakens the binding ability of HMGB1 to DNA. Thus the methylated HMGB1 becomes distributed in the cytoplasm through passive diffusion. These data collectively demonstrate that methylation at Lys112 is associated with the cytoplasmic transportation of HMGB1 in ccRCC.

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