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The role of Jade-1 in DNA mismatch damage and repair in renal cancer

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Thesis

THE ROLE OF JADE-1 IN DNA MISMATCH DAMAGE AND REPAIR IN
RENAL CANCER

by

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THE ROLE OF JADE-1 IN DNA MISMATCH DAMAGE AND REPAIR IN RENAL CANCER

RUOYU TIAN

ABSTRACT

The von Hippel-Lindau (VHL) tumor suppressor pVHL is lost in 90% of clear-cell renal-cell carcinomas (ccRCCs). Jade-1 is a renal tumor suppressor that is normally stabilized by pVHL. MutS Homolog2 (MSH2) is a key initiator in DNA mismatch repair (MMR). Defects in MMR are associated with genome-wide instability and predisposition to certain types of cancer. Mass spectrometry data of immunoprecipitated Flag-tagged Jade-1 lysates showed signal for MSH2, suggesting Jade-1 may participate in MMR. Here, we confirmed an interaction between endogenous MSH2 and endogenous Jade-1 by coimmunoprecipitation. Using cell fractionation, we found that MSH2 and Jade-1 translocated to the nucleus in response to alkylating agent MNNG in kidney proximal tubule cells. We also visualized the translocation of Jade-1 by immunofluorescence. Silencing JADE1 also influenced the kinetics of MSH2 translocation. In addition, by colony forming assay, JADE1-silenced cells were resistant to mismatch damage induced by MNNG, which is a feature of cells with an MMR defect. Furthermore, reintroducing pVHL into renal cancer cells also changed the amount of translocated MSH2 and Jade-1. In contrast to wild-type mice, Jade1 heterozygous mice got spontaneous tumors, and those tumors continued to show heterozygosity for Jade1. Taken together, our results identify a mechanism for Jade-1 regulation of MMR through its nuclear translocation. pVHL may also contribute to MSH2 and Jade-1 translocation
by increasing Jade-1 abundance. These findings establish an early role for Jade-1 in MMR, provide further indication that Jade-1 helps maintain genomic stability in the kidney and support that Jade-1 is a haploinsufficient renal tumor suppressor.
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LIST OF ABBREVIATIONS

AGT .......................................................... alkylguanine-alkyltransferase
APC .......................................................... adenomatous polyposis coli
CA9 .......................................................... carbonic anhydrase IX
ccRCC ..................................................... clear-cell renal cell carcinoma
CFE ........................................................ colony forming efficiency
COSMIC ............................................... Catalogue of Somatic Mutations in Cancer
CRC ........................................................ colorectal cancer
DAPI ....................................................... 4',6-diamidino-2-phenylindole
DMEM ...................................................... Dulbecco’s Modified Eagle’s Medium
DMSO ...................................................... dimethyl sulfoxide
DNA ....................................................... deoxyribonucleic acid
E.coli ...................................................... Escherichia coli
HAT ......................................................... histone acetyltransferase
HEK 293T ............................................... human embryonic kidney 293T
HK-2 ........................................................ human kidney-2
HNPCC .................................................... hereditary non-polyposis colorectal cancer
IHC ........................................................ immunohistochemistry
IP ............................................................ immunoprecipitation
Jade-1 ..................................................... gene for apoptosis and differentiation
MDR1 ..................................................... multidrug resistance protein 1
MMR

MNNG

MSH2

MSI

NLS

NOS

O6-BG

PBS

PCR

PHD

PMSF

RCC

ROS

SDS-PAGE

SSB

TBS

TCGA

TEMED

TGF-α

VEGF

VHL

DNA mismatch repair

N-Methyl-N′-nitro-N-nitrosoguanidine

MutS Homolog2

microsatellite instability

nuclear localization signal

nitrogen species

O6-Benzylguanine

phosphate-buffered saline

polymerase chain reaction

plant homeodomain

phenylmethylsulfonyl fluoride

renal-cell carcinoma

reactive oxygen species

sodium dodecyl sulfate polyacrylamide gel electrophoresis

single strand DNA binding protein

Tris-buffered saline

The Cancer Genome Atlas

tetramethylethylenediamine

transforming growth factor alpha

vascular endothelial growth factor

von Hippel-Linda
INTRODUCTION

1.1 Renal-cell carcinoma

1.1.1 Renal-cell carcinoma

Renal-cell carcinoma (RCC), is one of the top ten most common cancers in both men and women. According to American Cancer Society data, about 61,560 new cases of renal cancer were predicted to occur in 2015, 38,270 in men and 23,290 in women, and about 14,080 people, 9,070 men and 5,010 women, would die from the disease.

Symptoms of renal cancer may include hematuria, flank pain and an abdominal mass. Initial treatment is often partial or complete removal of the affected kidney. RCC is resistant to chemotherapy and radiotherapy. Targeted therapies and immunotherapy have become common medical treatments for patients, for example, mTOR inhibitor temsirolimus, angiogenesis and tyrosine kinase inhibitors sunitinib and sorafenib and PD-1 inhibitor nivolumab.

Following the 1997 Union Internationale Contre le Cancer and American Joint Committee on Cancer guidelines, all malignant RCC tumors were subclassified as clear-cell renal-cell carcinoma (ccRCC), papillary renal-cell carcinoma, chromophobe renal-cell carcinoma, collecting duct carcinoma and oncocytoma (Kovacs, Akhtar et al. 1997).

1.1.2 Clear-cell renal -cell carcinoma

Among those five types of RCC, ccRCC is the most common, comprising 70~75% of all cases. ccRCC is a renal cortical tumor, which has the following features. The cytoplasm of malignant epithelial cells is clear. The tumor has a compact-alveolar or acinar growth pattern, and intricate, arborizing vasculatures intersperse the tumor tissue.
(Figure 1). Historically, ccRCC is typically characterized by epithelial cells with transparent cytoplasm and a clearly distinguishable cell membrane, interlaced with a highly vascularized stroma (Figure 2). The translucency of the cytoplasm is effected by accumulation of droplets of phospholipids, glycogen and neutral lipids, in particular, cholesterol ester, which wash out on histologic preparation and leave an empty cytoplasm. Pathophysiologically, ccRCC is thought to originate from kidney proximal tubules, so proximal tubule cells are likely to be the precursor cells of this common malignancy.

There are several molecular alterations that are observed in ccRCC: frequent alterations of the VHL gene and protein pVHL and its interactive partners, mutations of genes in the PI(3)K/AKT pathway and the SETD2, PBRM1 and BAP1 genes (Network 2013). pVHL is involved in cellular oxygen sensing, which will be discussed further. The PI(3)K/AKT pathway regulates the cell cycle and cell survival and is found to be highly activated in cancer. Histone-lysine N-methyltransferase SETD2 methylates DNA, and its mutation leads to global hypomethylation of DNA promoters and vast changes in gene expression.

1.2 Function of pVHL and VHL associated diseases

1.2.1 VHL and pVHL

The von Hippel-Lindau gene VHL is located in chromosome 3p25.3, with a size of 12.7 kb, and it encodes a tumor suppressor protein called pVHL. pVHL contains 213 amino acids and its molecular weight is roughly 24-30 KDa. pVHL has two domains: a
beta domain, a roughly 100-residue N-terminal domain rich in beta sheet, and an alpha domain, a smaller alpha-helical domain including the C terminus.

1.2.2 The Function of pVHL

pVHL functions as a tumor suppressor in ccRCC by inhibiting hypoxia-inducible gene expression. Those genes encode proteins involved in angiogenesis (e.g., vascular endothelial growth factor, VEGF), cell growth (e.g., transforming growth factor alpha, TGF-α), glucose uptake (e.g., GLUT-1 glucose transporter) and acid-base balance (e.g., carbonic anhydrase IX, CA9). In normoxia, pVHL, with elongin proteins C and B, binds cul2 protein and serves as the receptor subunit of a ubiquitin ligase complex that promotes the ubiquitination and destruction of HIF-α transcription factors (Cohen and McGovern 2005). In hypoxia, key proline residues of HIF-α cannot undergo hydroxylation. The oxygen-dependent hydroxylation of HIF-α is required for pVHL binding, and asparagine hydroxylation can also block the interaction of HIF-α with transcription coactivator p300 (Cohen and McGovern 2005). In hypoxia, HIF-α is not degraded and translocates into the nucleus to bind to p300 to activate downstream hypoxia-inducible gene transcription (shown in Figure 3) (Cohen and McGovern 2005).

1.2.3 von Hippel-Lindau disease

von Hippel-Lindau (VHL) disease is a rare, autosomal dominant hereditary cancer syndrome associated with hemangioblastomas, ccRCC and pheochromocytoma. Other features of VHL disease include kidney cysts, pancreatic cysts, epididymal cystadenomas, and endolymphatic sac tumors.
The VHL tumor suppressor gene was identified in 1993 (Latif, Tory et al. 1993). In this disease (Figure 4), one VHL allele is inherited with a mutation. Associated focal lesions, such as RCC, arise from the inactivation or silencing of the remaining wild-type VHL allele. With accumulation of mutations in additional genes, a ccRCC is formed. Defects in VHL account for 90% of ccRCC cases (Kim and Kaelin 2004), which represents a high portion of all cases of RCC.

1.3 Jade-1

1.3.1 JADE1 gene and proteins

JADE1, Gene for Apoptosis and Differentiation in Epithelia, is also called jade family PHD finger 1. JADE1 is located at chromosome 4q28.2, with a size of 65.6 kb. JADE1 encodes multiple isoforms of protein, but the two major ones are Jade-1 and Jade-1L. Jade-1 (also called Jade-1S), the short form of Jade-1, contains 509 amino acids, and Jade-1L, the long isoform of Jade-1, contains 842 amino acid residues.

Jade-1 was identified from a human kidney cDNA library using a yeast two-hybrid screen against human pVHL p30 (Zhou, Wang et al. 2002). Jade-1 contains 509 amino acid residues and its molecular weight is 64 KDa. Jade-1 is a short-lived protein and contains a candidate destabilizing PEST motif (a peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T)). As shown in Figure 5, it also contains two plant homeodomains (PHD). The PHD zinc-finger domain is about 50-80 amino acids in length and is found in proteins involved in chromatin-mediated gene
regulation. The known post-translational modifications of Jade-1 are phosphorylation and ubiquitination (Zhou, Wang et al. 2002).

Jade-1 is abundant in kidney tissue and kidney proximal tubule cells (Zhou, Wang et al. 2002), which are the precursor cells of ccRCC. Jade-1 is located both in cytoplasm and the nucleus diffusely and in speckles. Jade-1 is degraded by the proteasome pathway. Strikingly, proteasome inhibitor treatment can increase endogenous Jade-1 protein levels up to 10 fold (Zhou, Foy et al. 2005).

1.3.2 Functions of Jade-1

Jade-1 has multiple roles in the cell cycle, apoptosis, gene regulation and cell signaling. Jade-1 is a short-lived protein and is stabilized by pVHL. Jade-1 partly colocalizes with pVHL. The reintroduction of pVHL into renal cancer cells increases Jade-1 abundance and increased its half life up to 3 fold (Zhou, Wang et al. 2002).


Jade-1 regulates the Wnt signaling pathway by directly binding and ubiquitylating the oncoprotein β-catenin. The well-established β-catenin E3 ubiquitin ligase component β-TrCP only ubiquitylates phosphorylated β-catenin. Jade-1
ubiquitylates both phosphorylated and non-phosphorylated β-catenin, but exhibits preference for the phosphorylated form. Moreover, pVHL downregulates β-catenin in a Jade-1-dependent manner and inhibits Wnt signaling. Therefore, the pVHL tumor suppressor and Wnt tumorigenesis signaling are directly linked via Jade-1 (Chitalia, Foy et al. 2008).

Activation of PI3K/AKT pathway is another common feature in renal cancer, with PTEN loss occurring in 30% of cases. JADE1-silenced renal proximal tubule cells show increased AKT activity, transformation and AKT-dependent anchorage-independent growth. Jade-1 inhibits AKT kinase activity by direct binding of the N-terminus of Jade-1 to both the catalytic domain and C-terminal regulatory tail of AKT. Moreover, reintroducing pVHL in renal cancer cells increased endogenous Jade-1 and hence reduced the level of phospho-AKT (Zeng, Bai et al. 2013). These observations suggest a new mechanism of superactive action of AKT with PTEN inactivation, pVHL loss and decreased Jade-1 levels in RCC.

Jade-1 is also associated with histone acetyltransferase (HAT) activity. Both Jade-1 and Jade-1L positively regulate HBO1 complex HAT activity. Co-expression of Jade-1 and HBO1 increase acetylation of endogenous histone H4 in epithelial cells in a synergistic manner. Jade-1 functions by direct binding with HBO1 via a Jade-1 PHD domain. Deletion of a Jade-1 PHD finger not only completely affected the binding, but also abolished the synergistic Jade-1/1L- and HBO1-mediated histone H4 acetylation (Foy, Song et al. 2008).
In summary, Jade-1 is a novel tumor suppressor in ccRCC. It has multiple roles in signal transduction through direct protein-protein interaction with other functional targets. For example, Jade-1 binds and is stabilized by pVHL. Jade-1 acts as an E3 ligase of β-catenin in the regulation of Wnt pathway. Jade-1 also binds to the catalytic domain and regulatory tail of AKT and inhibit its kinase activity. Jade-1 upregulates the HAT activity of the HBO1 complex. Jade-1L may have some different roles from Jade-1, such as transcription elongation (Saksouk, Avvakumov et al. 2009) and DNA replication (Miotto and Struhl 2010).

1.4 DNA Mismatch Repair

DNA mismatch repair (MMR) is a highly conserved biological pathway that plays a key role in maintaining genomic stability. Defects in MMR are associated with genome-wide instability and predisposition to certain types of cancer, including Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC).

1.4.1 DNA mismatch damage

DNA damage accumulates in cells over time as a result of both exogenous and endogenous stimuli. The exogenous stimuli include chemical and physical agents that the cells may be exposed to, for example, polychlorinated biphenyls, dioxin, cigarette smoke and UV light. The endogenous reactive metabolites include reactive oxygen and nitrogen species (ROS and NOS) (Iyer, Pluciennik et al. 2006). Another source of DNA damage are the errors occurring during DNA replication and recombination, including the errors
made by DNA polymerases. The specificity of MMR is primarily for base-base mismatches and insertion/deletion mispairs.

Methylnitronitrosoguanidine, or N-Methyl-N’-nitro-N-nitrosoguanidine (MNNG) is a carcinogen and mutagen that acts by adding alkyl groups to the O₆ of guanine and O⁴ of thymine, which can lead to transition mutations between GC and AT, shown in Figure 6. MNNG has been widely used as a chemical tool to induce mismatch damage in tissue culture cells (Andrabi, Umanah et al. 2014) and animal models (O'Connell, Klein-Szanto et al. 1986).

1.4.2 The DNA mismatch repair pathway

The *E. coli* MMR system includes the following proteins: MutS, MutL, MutH, DNA helicase II (MutU/UvrD), four exonucleases (ExoI, ExoVII, ExoX, and RecJ), single stranded DNA binding protein (SSB), DNA polymerase III, and DNA ligase (Lamers, Perrakis et al. 2000). MMR is highly conserved between human and *E. coli*, shown in Table 1.

In mammalian cells, the MutS heterodimeric complexes initiate MMR. MutS contains two complexes, MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ). MutSα and MutSβ recognizes different mispairs: MutSα recognizes base-base mispairs and single base insertion/deletions, while the MutSβ complex is primarily responsible for larger insertion/deletions up to 13 nucleotides long (Kolodner and Marsischky 1999). The MutS complexes undergo ATP-hydrolysis-dependent conformational change, and the ADP-bound form disassociates from the recognized mispair following the recruitment of a MutL complex and binding to the MSH2-containing complex. The interaction between
MutS and MutL requires ATP, although it is not clear that the ATP-binding site of MutL or MutS or both are involved (Kolodner and Marsischky 1999). MutL (MLH1-PMS2 heterodimer) is an endonuclease, which incise of mismatched DNA and is an important component in MMR (Li 2008). The endonuclease activity of MutL requires the presence of the mismatch recognition factor MutS, a DNA mismatch, a pre-existing strand break, the PCNA clamp, the RFC clamp loader and ATP (Li 2008). Incisions produced by MutL serve as entry sites for MutS-activated exonuclease I to excise the mismatch DNA in a 5’-3’ direction (Li 2008). The generated gap is removed by the DNA polymerase delta holoenzyme (Li 2008).

The later steps in MMR are called the formation of incision, postincision and DNA resynthesis/ligation complexes, shown in Figure 8. Many other protein components that participate in various DNA processing pathways are also involved in MMR. The participating proteins and their functions are shown in Table 1.

1.4.3 MSH2 and HNPCC

*MutS Homolog 2 (MSH2)*, also called *HNPCC*, is homolog to the bacterial *MutS* gene and is located at chromosome 2p21, with a size of 159.3 kb. It encodes a 943 amino acid protein with 104.7 KDa mass. MSH2 contains a DNA binding domain and two protein interaction domains, one for MSH3 or MSH6 and the other for MutL homologs, shown in Figure 9. It is located both in the cytoplasm and nucleus.

Lynch syndrome, also called hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominant genetic condition. Lynch syndrome increases the risk of many types of cancer, particularly colorectal cancer, which are cancers of the colon and rectum.
Lynch syndrome patients also have an increased risk of cancers of the endometrium, ovaries, stomach, small intestine, liver, gallbladder duct, kidney and upper urinary tract, and brain (Vasen, Watson et al. 1999).

Inherited mutations in the *MSH2* gene are found in about 40 percent of all cases of Lynch syndrome with an identified gene mutation (Fishel, Lescoe et al. 1994). The mutated *MSH2* may cause the translation of an unusually short or inactive MSH2 protein that cannot function properly. The dysfunction or the absent of MSH2 leads to the increasing number of DNA mistakes, which should be repaired during cell proliferation. The mistakes accumulate as the cells keep dividing, which may cause the cells to function aberrantly, increasing the risk of tumor formation in the colon or other parts of the body.

The association between MSH2 expression and the incidence of ccRCC is not clear yet. But there is evidence that MSH2 may be a useful marker for predicting TNM stage and prognosis in ccRCC (Yoo, Won et al. 2014). Tumor stage was significantly higher in the MSH2-negative patients than in the MSH2-positive patients. It was not significantly different in terms of N stage and M stage (Yoo, Won et al. 2014). The MSH2-negative group presented decreased rates of survival without recurrence, without progression and overall survival, even though it did not achieve statistical significance results, as shown in Figure 10 (Yoo, Won et al. 2014).

1.5 Current Study
In order to identify proteins that interact with Jade-1, a PhD student, Ms. Delia Lopez, in our laboratory immunoprecipitated Flag-Jade-1 and Flag-5’UTR-Jade-1 and excised coimmunoprecipitated protein bands appearing at various molecular weight regions that are not visible in the control IgG IP. These bands were sent for mass spectrometry at the University of Massachusetts Medical School, shown in Figure 11. Interestingly, there was an overrepresentation of DNA repair proteins. There are approximately 165 proteins involved in the DNA damage response and 14 of these were identified, including MSH2, which I have pursued on my own. We hope that this study can provide insight into how the DNA mismatch repair pathway is regulated in ccRCC and identify novel targets for renal cancer treatment.
Figure 1: Clear-cell renal-cell carcinoma.

Typical gross presentation of a clear-cell renal-cell carcinoma (lower left) with a golden color due to intracellular lipid accumulation. White areas are foci of sarcomatoid differentiation (http://emedicine.medscape.com/article/1612043-overview).
Figure 2: Histologic slide of clear-cell renal-cell carcinoma.

Typical histologic appearance of clear-cell renal-cell carcinoma on hematoxylin and eosin stain, showing nests of epithelial cells with clear cytoplasm and a distinct cell membrane, separated by a delicate branching network of vascular tissue (http://emedicine.medscape.com/article/1612043-overview).
Figure 3: The regulation of HIF-α by pVHL in normoxia and hypoxia.

In normoxic conditions HIF-α is hydroxylated on two proline residues by a proline hydroxylase and on an asparagine residue by an asparagine hydroxylase. Hydroxylation of HIF-α permits binding of HIF-α to pVHL, which promotes the ubiquitination and destruction of HIF-α by the proteasome pathway. In hypoxic conditions, HIF-α is not hydroxylated and so it cannot bind pVHL and is stabilized. HIF-α and HIF-β form a heterodimer, binding with p300 in the absence of oxygen to activate downstream hypoxia-inducible gene expression (Cohen and McGovern 2005).
Figure 4: The development of clear-cell renal-cell carcinoma.

In contrast to sporadic renal-cell carcinoma (A), fewer steps are required for the development of renal-cell carcinoma in the inherited forms of the disease (B). As a result, the renal cancers associated with VHL disease occur earlier and are often multifocal. A plus sign represents the wild-type allele, a minus sign represents a null allele (Cohen and McGovern 2005).
**Figure 5: Protein structure of Jade-1.**

Jade-1 contains a candidate PEST domain and two zinc-binding PHD domains (Zhou, Wang et al. 2002).

---

**Figure 6: The alkylation of guanine.**

The alkylation product of the $O^6$ of guanine prefers thymine, instead of cytosine, with which to form hydrogen bonds during DNA replication. With DNA replication, guanine alkylation can cause GC to AT transitions.
Figure 7: The damage targets of MutS and MutL complexes.

MSH2-MSH6 recognizes base-base mispair, while MSH2-MSH3 detects insertion/deletion mispairs up to 13 nucleotides long (Kolodner and Marsischky 1999).
Figure 8: DNA mismatch repair pathway in *E.coli*.

MutS starts the initiation process: while MutSα preferentially recognizes base-base mismatch, MutSβ recognizes larger mispairs. MutLα possesses an ATPase activity and uses ATP to bind with MutS and form a preincision complex. MutH, a very weak endonuclease, is activated once bound to MutL. MutH nicks unmethylated DNA and the unmethylated strand of hemimethylated DNA. By adding MutH into the complex, the incision complex is formed. UvrD is a DNA helicase and facilitates the process. Single-strand DNA-binding protein (SSB) and exonucleases are added to the complex to excise the mispairs, following by DNA resynthesis (http://www.mm2m.eu/general/perspectives.html).

Figure 9: Diagram of the MSH2 in scale.

Numbers inside the grey boxes indicate the exon from which each part of the protein is translated. The boxes inside represent the DNA binding domain (red), the hMSH3/hMSH6 interaction domain (yellow) and the MutL homologs interaction domain (green); C: Carboxy terminus; N: Amino terminus (http://atlasgeneticsoncology.org/Genes/GC_MSH2.html).
Figure 10: The association of MSH2 negative status versus the positive group with recurrence-free survival (A), progression-free survival (B) and overall survival (C) in 129 ccRCC patients.

The 129 ccRCC patients were analyzed according to the degree of MSH2 expression by immunohistochemistry. The MSH2-negative group showed trends for decreased rates of recurrence-free survival, progression-free survival and overall survival, without achieving statistical significance, compared to MSH2-positives (Yoo, Won et al. 2014).
Figure 11: Silver staining of coimmunoprecipitated proteins with Flag-Jade-1 and Flag-5'UTR-Jade-1 immunoprecipitations.

Intense bands marked with blue arrows are not visible in the control IgG IP, and they were cut out and sent for mass spectrometry, yielding samples at around 125, 100, 85, 70 and 37 KDa. The black and red markers show the molecular weight of protein markers (from Ms. Delia Lopez, PhD student in Dr. Cohen’s laboratory).
<table>
<thead>
<tr>
<th>E. coli</th>
<th>Human</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MutS)₂</td>
<td>hMutSα (MSH2-MSH6)⁺</td>
<td>DNA mismatch/damage recognition</td>
</tr>
<tr>
<td></td>
<td>hMutSβ (MSH2-MSH3)</td>
<td></td>
</tr>
<tr>
<td>(MutL)₂</td>
<td>hMutLα (MLH1-PMS2)⁺</td>
<td>Molecular matchmaker; endonuclease, termination of mismatch-provoked excision</td>
</tr>
<tr>
<td></td>
<td>hMutLβ (MLH1-PMS1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hMutLγ (MLH1-MLH3)</td>
<td></td>
</tr>
<tr>
<td>MutH</td>
<td></td>
<td>Strand discrimination</td>
</tr>
<tr>
<td>UvrD</td>
<td></td>
<td>DNA helicase</td>
</tr>
<tr>
<td>ExoI, ExoVII, ExoX, RecJ</td>
<td>ExoI</td>
<td>DNA excision; mismatch excision</td>
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<tr>
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<td>RPA</td>
<td>ssDNA binding; protection; stimulating mismatch excision; termination of DNA excision; promoting DNA resynthesis</td>
</tr>
<tr>
<td>SSB</td>
<td>HMGB1</td>
<td>Mismatch-provoked excision</td>
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<td></td>
<td>RFC</td>
<td>PCNA loading; 3' nick-directed repair, activation of MutLα endonuclease</td>
</tr>
<tr>
<td>DNA Ligase</td>
<td>DNA ligase I</td>
<td>Nick ligation</td>
</tr>
</tbody>
</table>

³Major components in cells.

⁴Not yet identified.

Table 1. MMR components and their functions.

a, Major components in cells; b, Not yet identified. (Iyer, Pluciennik et al. 2006)
METHODS

2.1 Materials

2.1.1 Cell lines

HEK293T and HK-2 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 4.5g/L glucose and L-glutamine, 10% fetal bovine serum, and penicillin-streptomycin. Renal cancer 786-O cells were grown in RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum, and penicillin-streptomycin.

HK-2 nonsi, JADE1 sh1 and JADE1 sh2 stable cell lines were maintained with 2 µg/ml puromycin. The 786-O Flag-VHL and Flag-ctrl renal cancer cell lines were maintained with 0.2 µg/ml G418. All cell lines were grown at 37°C in 5% CO₂.

2.1.2 Antibodies

Jade-1 rabbit polyclonal antiserum was produced previously as described [3]. MSH2 mouse and rabbit monoclonal antibodies and fibrillarin, β-actin and α-tubulin mouse monoclonal antibodies were from Cell Signaling Technology. pVHL antibody was from BD Pharmingen. Normal rabbit and mouse IgG were from Santa Cruz Biotechnology. Horseradish peroxidase-linked anti-rabbit and anti-mouse IgG secondary antibodies for immunoblotting were from Bio-Rad. Oregon Green 488 goat anti-rabbit IgG secondary antibody was from Molecular Probes. Alexa Fluor 594 donkey anti-mouse IgG secondary antibody was from Invitrogen.
2.1.3 Chemicals

Dimethyl sulfoxide (DMSO) and O⁶-Benzylguanine were from Sigma. MNNG was from TCI America.

2.2 Methods

2.2.1 Extraction of whole cell lysates

Media was removed, and residual media was removed and cells were washed with 1X PBS. Proper volume of cold whole cell lysis buffer (1X TBS, 3 mM EDTA, 1% Triton X-100, 1mM PMSF and proteasome inhibitor tablet) was added onto the plate to lyse cells. The volume depended on the cell confluence and cell type. Cells were incubated with lysis buffer on ice for 5 minutes, then were scraped into a 1.5 ml eppendorf tube. Cells were lysed on ice for 10 minutes. Meanwhile, the tube was vortexed every 3 minutes. The extract was centrifuged for 15 minutes at 14,000 rpm in a cold centrifuge. The supernatant was collected into a new cold eppendorf tube for further use. The protein lysates were stored at -80°C.

2.2.2 Cytoplasmic and nuclear fractions

Cold cytoplasmic, nuclear lysis buffer and wash buffer were made with following ingredients:

1) Cytoplasmic lysis buffer: 20 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂, 0.5% NP-40, protease inhibitor tablet and 1 mM PMSF.
2) Nuclear lysis buffer: 20 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂, 0.5% NP-40, 0.5 M NaCl, protease inhibitor tablet and 1 mM PMSF.

3) Wash buffer: 20 mM HEPES pH 7.4, 10 mM KCl, 2 mM MgCl₂, protease inhibitor tablet and 1 mM PMSF.

Media was suctioned out of each plate, and residual media was removed. And cells were washed with 1X PBS. The 0.2 ml cytoplasmic lysis buffer was added onto each 10 cm plate to lyse cells. Cells were scraped into a 1.5 ml eppendorf tube and were resuspended to mix thoroughly. Cells were lysed for 10 minutes on ice and were spun down at 2000 rpm for 3 minutes in a cold centrifuge. The supernatant was collected in a new pre-chilled tube to get the pre-cytoplasmic fraction. The nuclear pellet was washed with 500 ml wash buffer three times. The tube was tapped and inverted a couple of times and was spun down at 4000 rpm for 5 minutes. The 40-60 µl nuclear lysis buffer was added to resuspend and lyse the nuclear pellet for 10 minutes on ice. The pellet was vortexed at a low speed every 2 minutes to lyse the pellet completely. The nuclear pellet and pre-cytoplasmic fraction were spun down at 14000 rpm for 20 minutes in cold room. The supernatants were collected into new cold eppendorf tubes for further use. The protein lysates were stored at -80 °C.

2.2.3 Bradford protein assay and sample preparation

1 ml Bradford (Bio-rad) was added into 2 µl protein lysate and was mixed with protein lysate. 900 µl of the sample was added into spectrometer to measure the absorption at 595 nm. The standard curve was used to calculate the protein concentration.
6X protein sample buffer was added into protein lysate to denature the protein at 95 °C for 5 minutes. Then the samples were stored at -20 °C if those were used within 10 days. For longer storage, protein samples were stored at -80 °C.

2.2.4 Immunoblot

Protein samples were melted on ice from fridge. Samples were mixed with pipette and were span down at 3000 rpm for 1 minute. SDS-PAGE gels were prepared as following: 10 ml 7.5% separating gel: 5 ml dH₂O, 2.5 ml 1.5 M Tris pH 8.8, 2.5 ml 30% Acrylamide/Bis, 50 μl 10% APS, 10 μl TEMED; 5 ml 4% stacking gel: 3.1 ml dH₂O, 1.25 ml 1.5 M Tris pH 6.8, 0.65 ml 30% Acrylamide/Bis, 25 μl 10% APS, 5 μl TEMED. The protein samples were loaded into wells of the gel. The maximum volume for each well was 60 μl. The gels were run at 20 mA per gel for about 2 hours. The protein was transferred from the gels to nitrocellulose membrane at 100V for 1 hour in the cold room. Transfer buffer can be used twice. The nitrocellulose membranes were blocked with 5% non-fat milk in PBST (1X PBS, 0.1% Tween 20) for 45 minutes to 1 hour at room temperature. The membranes were incubated with primary antibodies (diluted with 1% milk in PBST) in the cold room overnight. Jade-1 antiserum was diluted 1: 250. The primary antibodies were stored at -20 °C and were used multiple times. The membranes were washed with PBST for 10 minutes, 3 times, with shaking. Then, they were incubated with secondary antibodies (1: 5000 dilute in 1% milk in PBST) at room temperature for 1 hour or at 4 °C overnight. The membranes were washed again with PBST for 10 minutes, 3 times, with shaking. The protein signals from the membranes
were detected by enhanced chemiluminescence (ECL) and autoradiography. Finally, films were scanned and the signals were quantified by Image J software.

2.2.5 Immunoprecipitation (IP)

Whole cell lysates were prepared as in 2.2.1 by using IP lysis buffer (1X TBS, 3mM EDTA, 1% NP-40). The 1.25 mg protein with 0.5 ml whole cell lysis buffer was used as one IP group in a 1.5 ml eppendorf tube. The proteins were pre-cleared with washed 20 µl protein A/G beads at 4 °C for 1 hour. Beads were spun down at 3500 rpm for 5 minutes in a cold centrifuge. The supernants were pre-cleared protein, that did not non-specifically bind to agarose beads. The pre-cleared protein lysates were incubated with primary antibodies on a 4 °C rotator overnight. The dilution of primary antibodies was performed according to their data sheets. Normal rabbit or mouse IgG was used as negative control. The next day, IP samples were incubated with 25 µl washed beads in a 4 °C rotator for 3.5 hours. The antibody, precipitated and co-precipitated protein complexes were attached on the beads. The beads were spun down at 3500 rpm for 3 minutes in a cold centrifuge and then, were washed with 500 µl IP lysis buffer for 3 times to wash away those weak binding proteins. The beads were washed with 1X PBS again. The 40 µl 2X sample buffer was added to resuspend the beads for immunoblotting. IP samples were stored at -80 °C or -20 °C for 10 days.

2.2.6 Immunofluorescence

Sterilized coverslips were plated into wells of 12-well plates before passaging cells. Cells were grown on coverslips 18 h to 24 h before fixation. Cells were treated or
transfected if needed. Media was removed and cells were washed with cold PBS. The samples were fixed with 400 µl 3.7 % paraformaldehyde (PFA) in PBS, pH 7.4 for 15 minutes at room temperature. Fixed samples were washed twice with cold PBS. If the target protein was an intracellular protein, then samples were permeabilized with 400 µl 0.25% Triton X-100 in PBS for 10 minutes at room temperature. Samples were washed with PBS three times, 5 minutes each. Samples were blocked with 1% BSA in PBST (1X PBS, 0.5 % Triton X-100) for 30 minutes at room temperature. Samples were incubated with 200 µl diluted primary antibodies with blocking buffer for 1 h at room temperature or 4 °C overnight. The dilution factor for primary antibodies was from their data sheets. The dilution factor of Jade-1 antiserum was 1:250 and incubation time was 1 h at room temperature. Samples were washed with PBS for 4 times, 5 minutes each time, then were incubated with 200 µl 1:1000 diluted fluorescein linked secondary antibodies for 1 h at room temperature in the dark. Samples were washed again with PBS for 4 times, 5 minutes each time. The coverslip was mounted with one drop of mounting medium with DAPI and sealed with nail polish. The slides were checked in a confocal or fluorescent microscope and were stored at 4 °C in the dark.

2.2.7 Colony forming assay

Cells were trypsinized and counted, then seeded into 6-well plates. Cells in the control groups were less than the treatment groups. After 18 h to 24 h later, cells were treated or transfected if necessary. After treatment, cells were washed with fresh media and were fed with 2 ml fresh media per well. Cells were grown at 37 °C in 5% CO2 for about 2 weeks. Cells were checked under the microscope frequently. Fresh media were
added during the 2 weeks if needed. When most of the single cells grew to colonies (more than 50 cells), cells were collected and washed with cold PBS. Then, 1 ml 0.5% crystal violet was added into each well to stain the cells for at least 30 minutes. Crystal violet was removed, and the plates were washed with tap water 3 times. Plates were dried on the bench, and colonies were counted manually with the aid of a marker pen.

2.2.8 DNA isolation

Mouse tissue samples were incubated with 200 µl 50mM NaOH at 95 °C for 30 minutes, then were vortexed to destroy the tissue structure. Then, 50 µl 1M Tris (pH 8.0) to NaOH was added into each sample. Samples were span down at 12000 rpm for 6 minutes. The supernant contained the genomic DNA. DNA samples were stored at -80 °C or 4 °C for short.

2.2.9 Genotyping (PCR)

Forward primer: 5’-GATGTTAAGAGTGGCATCCTGG-3’
Reverse primer for wild-type band: 5’-ACATCTAGGAGTGGAACACTAG-3’
Reverse primer for mutant band: 5’-CCACCACGGGTTCTTCTGGTAG-3’

PCR reagents were added and mixed in eppendorf tubes as follows: 5 µl ddH2O, 10 µl 2X PCR mixture, 2 µl 10 pmol/µl forward primer, 2 µl 10 pmol/µl reverse primer, 1 µl DNA.

PCR cycles were run as follows:
30 cycles

\[
\begin{align*}
94 \, ^\circ C, & \, 2 \, \text{min}; \\
94 \, ^\circ C, & \, 1 \, \text{min}; \\
54 \, ^\circ C, & \, 45 \, \text{s}; \\
72 \, ^\circ C, & \, 1 \, \text{min}; \\
72 \, ^\circ C, & \, 1 \, \text{min}; \\
4 \, ^\circ C, & \, \text{forever.}
\end{align*}
\]

The PCR products were loaded into 1% agarose gel. The gel was run at 100V for 30 minutes. The mutant bands appeared at 800 bp and the wild-type ones were at 620 bp.
RESULTS

3.1 Endogenous Jade-1 protein binds endogenous MSH2 protein

When human embryonic kidney cells HEK 293T cells were grown to 50% - 60% confluence, Flag-JADE1 expression vector was transfected. After 2 days expression, cell lysates were collected and immunoprecipitated with Flag antibody linked beads or MSH2 antibody. Immunoprecipitated samples were immunoblotted with MSH2 or Flag antibody, respectively. Figure 12 shows that in the Flag IP sample, MSH2 was co-immunoprecipitated, however, in MSH2 IP sample, Flag-tagged Jade-1 was not co-immunoprecipitated. It suggests that MSH2 and Jade-1 may interact together.

Further, we used the same technique to examine the interaction between endogenous Jade-1 and endogenous MSH2. Human kidney-2 (HK-2) proximal tubule cells, which represent a model of clear-cell renal-cell carcinoma (ccRCC) precursor cells, and human embryonic kidney cells HEK 293T cells were grown to 80% confluence, and cell lysates were collected and immunoprecipitated with Jade-1 polyclonal antiserum or MSH2 monoclonal antibody. Immunoprecipitated lysates were then immunoblotted with MSH2 or Jade-1 antibody, respectively. The whole cell lysates were also immunoprecipitated with normal rabbit IgG as negative control, and the coimmunoprecipitated samples were immunoblotted with Jade-1 and MSH2 antibodies.

Figure 13 shows that an interaction between endogenous Jade-1 and endogenous MSH2 proteins was detected in both directions in HK-2 cells as well as 293T cells. Moreover, the intensity of coimmunoprecipitated MSH2 and Jade-1 was stronger in HK-2 cells, the ccRCC precursor cells, than 293T cells.
These experiments further provide evidence and exclude the possibility that MSH2 interacts with Flag tag in the previous transfection experiment. They also verify the mass spectrometry data, shown in Figure 11, and also suggest that Jade-1 may participate in DNA mismatch repair through interaction with MSH2, one of its key initiators.

3.2 Jade-1 translocates to the nucleus following DNA mismatch damage

3.2.1 Jade-1 does not regulate the abundance of MSH2

In order to test the hypothesis that Jade-1 regulates MSH2 protein abundance by protein binding, we silenced JADE1 in HK-2 cells to check for changes in MSH2 protein level by immunoblot. As shown in Figure 14, with the Jade-1 protein abundance knocked down, the MSH2 protein level is unchanged. There is also no difference between the JADE1 (JADE1 sh2) alone knock down or JADE1 and JADE1L knock down (JADE1 sh1). Therefore, Jade-1 does not alter the abundance of MSH2 protein. There may be other mechanisms by which Jade-1 regulates MSH2 and participates in MMR.

3.2.2 Jade-1 and MSH2 translocate into the nucleus with MNNG treatment

Alkylguanine-alkyltransferase (AGT) repairs DNA by transferring the alkyl group from the O-6 position of guanine to a cysteine. O⁶-Benzylguanine (O⁶-BG) is an inhibitor of AGT that acts as false substrate for AGT (Berg, Murry et al. 1998). O⁶-BG increases the cell sensitivity to agents that alkylate the O-6 position of guanine, such as MNNG. Therefore, we used O⁶-BG to pretreat cells to inhibit AGT activity and then induced mismatch damage with alkylating agent MNNG.
As shown in immunoblots in Figure 16A, Jade-1 and MSH2 abundance in the nucleus increased in HK-2 cells in response to MNNG. The accumulation of MSH2 and Jade-1 started at 0.5 h. MSH2 and Jade-1 continued increasing until 4 h and Jade-1 increased 12.5 fold. Plus, the Jade-1 and MSH2 showed similar timing of movement, gradually increasing to the 4 h time point. Moreover, we have done immuocytochemistry to visualize the translocation of Jade-1 with MNNG treatment. We chose a 1 h time point for treatment since the immunoblot result shows Jade-1 accumulated in the nucleus at 1 h. As shown in Figure 16B, the difference of fluorescence intensity of nucleus and cytoplasm in control group is less significant as the one in treatment group. It shows that the movement of Jade-1 and MSH2 from cytoplasm to nucleus exists. Moreover, there were more nuclear speckles with MNNG treatment than control. Those speckles likely represent some functional regions in nucleus where Jade-1 may localize, but the specific function of proteins which gather in these speckles is not clear yet. Taken together, MSH2 and Jade-1 may bind and move together into the nucleus when cells are damaged.

It has been reported already that in response to alkylating agents, MSH2 translocates from the cytoplasm to nucleus (Christmann and Kaina 2000). Our experiments provide new insight into Jade-1 in nuclear translocation in response to DNA mismatch damage.

### 3.3 JADE1 silencing may change the timing of MSH2 nuclear translocation

As mentioned in the introduction, our laboratory has focused primarily on the physiological and pathological functions of Jade-1 short form, with a molecular weight of 64 KDa. The biological roles of Jade-1 and Jade-1L may differ. So, it should be
interesting to see the roles of both Jade-1 and Jade-1L in DNA mismatch repair. We have established JADE1-silenced HK-2 cell lines HK-2 sh1 and HK2 sh2 (Zeng, Bai et al. 2013). When JADE1 and JADE1L were both knocked down, as shown in Figure 17A, MSH2 started translocating at 0.5 h and accumulated to the highest level at 4 h. However, in HK-2 nonsi cells (Figure 17B), MSH2 increased rapidly in the nucleus to 2.4 fold at 0.5 h and reached a peak at 1 h. So we propose that changes in Jade-1 levels alter the kinetics of MSH2 translocation with treatment.

To further test the hypothesis, the HK-2 JADE1 sh2 cell line, which knocks down Jade-1 p64 alone, was used to perform the same experiment. The same experiments were repeated twice (Figure 17B, a, b), however, the results were different. The average amount of MSH2 translocation is presented in Figure 17C. Similar as HK-2 nonsi cells, 2.5 fold more MSH2 started translocating at the early time point, 0.5 h, and the translocation amount reached peak at 1 h and the amount dropped slightly at 4 h. We need more experiments to verify this conclusion. JADE1 alone knockdown cells show kinetics similar to nonsi cells.

In conclusion, these experiments suggest that knockdown of both Jade-1 p64 and Jade-1L is required to influence the kinetics of MSH2 translocation with MNNG treatment.

3.4 pVHL may change the amount of MSH2 and Jade-1 nuclear translocation

pVHL is a renal tumor suppressor that also regulates Jade-1 protein abundance. The expression of endogenous pVHL is absent in most ccRCC cell lines, such as the 786-O
cell line. Overexpression of pVHL increases the amount of endogenous Jade-1 by stabilizing it (Zhou, Wang et al. 2002). We wished to examine whether pVHL plays a regulatory role in DNA mismatch damage through Jade-1.

We examined the effect of pVHL on MSH2 nuclear translocation in 786-O stable cell lines. Pooled 786-O stable cell lines were established that either overexpress Flag-tagged pVHL or Flag-tagged empty vector. The same experiments have been repeated twice in Figure 18 A and B, however the results were not conclusive. It is hard to draw a conclusion whether the introduction of pVHL changes the kinetics of Jade-1 or MSH2 translocation. In Figure 18 C-E, we can see that the fluctuation of both Jade-1 and MSH2 are stronger in empty vector cells than in pVHL overexpressed cells. In renal cancer cells, pVHL may play an inhibitive role in Jade-1 and MSH2 translocation in MMR. Moreover, as shown in Figure 18A, pVHL existed exclusively in the cytoplasm and did not move into the nucleus. So pVHL is not involved in MMR by nuclear translocation. It is may be an upstream regulator of MMR initiation by regulating the abundance of Jade-1. This new finding may provide insights into the potential role of Jade-1 in MMR in renal cancer, but more experiments are needed.

3.5 JADE1 knockdown cells are resistant to MNNG

The colony forming assay was first described in 1956 by Puck and Marcus (Puck and Marcus 1956). It is a method to determine cell viability to produce a full colony after drug treatment. The cell survival is based on the ability of a single cell to grow to a colony. A colony is defined as containing at least 50 cells. On a plate, any colony larger
or similar in size to the tip of a marker pen is regarded as containing at least 50 cells. It usually takes two to three weeks for the cells to form a colony. Colonies are assessed after fixing and staining with crystal violet, as shown in Figure 19.

More colonies were formed in JADE1-silenced cell lines with or without treatment in Table 2A. This observation supports the generalization that Jade-1 inhibits cell growth (Zhou, Foy et al. 2005). Colony forming efficiency (CFE) is defined as the percentage of seeded cells that go on to form a colony. While CFE reflects a cell’s ability to recover and grow after treatment, the ratio of nonsilenced cells CFE and silenced cells CFE shows this relative ability with silencing of JADE1, shown in Table 2B. These ratios vary with each cell line and treatment group, but they are all greater than 1, which reflects a higher survival and growth rate of all JADE1-silenced cells.

With the treatment of alkylating agent, MNNG, cells with lower expression of Jade-1 shows higher survival rate (Figure 20). The survival and growth ability of all JADE1-silenced cells increased 3.5 fold with MNNG treatment, compared to DMSO control (Figure 20, JADE1 sh). The survival and growth ability of cells with low expression of the short form of Jade-1 increased 6.9 fold, while those with low long and short form of Jade-1 increased 1.9 fold. Intriguingly, MSH2-deficient cells also show resistance to MNNG (de Wind, Dekker et al. 1995). In the presence of MSH2, when thymidine incorporates to methylated guanine, it triggers excision and resynthesis, and results in the presence of single-stranded regions. The single-stranded region will lead to double-stranded gaps when DNA replicates in S phase. This “double-stranded break” is a signal for cell death. On the other hand, loss of MSH2 will prevent this process, thus conferring
tolerance to methylating agents. So it is possible that Jade-1 short form may contribute to normal MSH2 function in the DNA mismatch repair pathway. Cells with low expression of Jade-1 and Jade-1L may bypass the methylation in tolerance, as occurs with loss of *MSH2* (de Wind, Dekker et al. 1995). Alternatively, it is possible that Jade-1 is required for the proper initiation of MMR.

### 3.6 Heterozygosity for *Jade1* is found in most of mouse tumors

In a gene trap screen for genes expressed in the primitive streak and tail bud similar to *brachyvry* during mouse embryogenesis, *Jade1* was isolated. *Jade1* E148 mice were generated by gene trap: *β*-galactosidase (*β*-Gal) was inserted into *Jade1* to generate a null or hypomorphic allele of *Jade1* (Tzouanacou, Tweedie et al. 2003).

Five of ten *Jade1* heterozygous mice had metastatic tumors, while none of wild-type ones got tumors. This suggests that Jade-1 is a tumor suppressor. We resected some of those tumors, abnormal tissues and some normal tissues, then isolated genomic DNA. We performed PCR and X-gal staining for genotyping. Three out of four tumors maintained heterozygous for *Jade1* (Figure 21). Only the liver tumor did not show a mutant band signal. We suspect that probably this is because the total amount of genome DNA is not enough to amplify the mutant band since the density of wild type band is lower than the others.

The Knudson hypothesis, also called the two-hit hypothesis, supposes that one mutation occurs in each allele of a tumor suppressor gene, two mutations total, are required to cause a tumor (Knudson 1971). However, this hypothesis has been modified
recently. Some candidate tumor suppressors, particularly haploinsufficient ones, require inactivation of only one allele, which supports the motion that heterozygosity for tumor suppressor genes can cause cancer (Paige 2003). *JADE1* appears to be a haploinsufficient tumor suppressor: deletion of one allele of *JADE1* can lead to tumor formation. These results further indicate that Jade-1 is a haploinsufficient tumor suppressor.
Figure 12: Flag-tagged Jade-1 protein binds to endogenous MSH2 protein.

*Flag-JADE1* expression vector was transfected into HEK 293T cells. After 48 hours, immunoprecipitations were performed with either Flag antibody linked agarose beads or normal rabbit IgG as negative control. Coimmunoprecipitated MSH2 was detected by immunoblotting with MSH2 rabbit monoclonal antibody. Likewise, MSH2 was immunoprecipitated with MSH2 antibody and then the coimmunoprecipitated lysate was immunoblotted with Flag antibody. Five percent of whole cell lysate was used to show the expression of Flag-tagged Jade-1 and endogenous MSH2 by immunoblot. These figures represent one of two experiments with similar results.
Figure 13: Endogenous Jade-1 protein binds endogenous MSH2 protein.

Coimmunoprecipitation of endogenous Jade-1 with endogenous MSH2 in HK-2 cells (A) and HEK 293T cells (B). Immunoprecipitations were performed with either Jade-1 rabbit polyclonal antiserum or normal rabbit IgG as negative control. Coimmunoprecipitated MSH2 was detected by immunoblotting with MSH2 rabbit monoclonal antibody. Likewise, MSH2 was immunoprecipitated with MSH2 antibody and then the coimmunoprecipitated lysate was immunoblotted with Jade-1 antiserum. Five percent of whole cell lysate was used to show the expression of endogenous Jade-1 and endogenous MSH2 by immunoblot. These figures each represent one of two experiments with similar results.
Figure 14: *JADE1* silencing does not affect the abundance of endogenous MSH2 protein.

Whole cell lysates were prepared from HK-2 parental control, HK-2 stable cell lines nonsilencing (nonsi) control, *JADE1* sh1, *JADE1* sh2, then the protein abundance of MSH2 was assessed by immunoblot. β-actin was used as a loading control. These figures represent one of two experiments with similar results.

![Blot images](image)

Figure 15: Chemical structures of O⁶-Benzylguanine (A) and MNNG (B).
Figure 16: Nuclear translocation of Jade-1 in cells treated with MNNG.

(A) HK-2 parental cells were pretreated with 4 µg/ml O6-BG for an hour (h) to inhibit O6-alkylguanine-DNA alkyltransferase activity, then treated with 50 µM MNNG for 0.5, 1 and 4 h; DMSO served as vehicle control (veh). MNNG treatment induces formation of DNA mismatches. After treatment, cells were collected and the nuclear fraction was extracted. The nuclear fraction were assessed by immunoblot with Jade-1 antiserum, and MSH2, fibrillarin and α-tubulin antibodies. Fibrillarin was used as nuclear fraction marker and α-tubulin as cytoplasmic marker. (B) HK-2 parental cells were pretreated with O6-BG for 1 h and then treated with MNNG for 1 h. Cells were treated with DMSO (veh) for 2 h as negative control. Cells were fixed and permeabilized by Triton X-100, and incubated with Jade-1 antiserum and fluorescent secondary antibody. (a), (b) and (c) show the cells treated with DMSO control, while (d), (e) and (f) show cells treated with MNNG. (a),(d), Jade-1 fluorescence; (b), (e), DAPI; (c), merged image of (a) and (b); (f), merged image of (d) and (e). Figure 16A represents one of two experiments with similar results. Figure 16B was done once.
Figure 17: *JADE1* silencing may change the timing of MSH2 nuclear translocation.

HK-2 *JADE1* nonsi, sh1 and sh2 cells were treated with MNNG, and nuclear fractions were obtained as in Figure 15A. (A) The nuclear fraction of HK-2 *JADE1* sh1 cells was assessed by immunoblot with MSH2 antibody, Jade-1 antiserum and fibrillarin, α-tubulin antibodies. (B) The abundance of MSH2 and fibrillarin was also assessed in HK-2 nonsi and *JADE1* sh2 cells. HK-2 *JADE1* sh2 cells were assessed twice, a and b, with different results. (C) Taken (A) and (B) together, the relative abundance of MSH2, which is the density shown in (A) and (B) in the nucleus changes is shown with the timing of treatment (hours). The average of the density in two experiments of HK-2 *JADE1* sh2 cells is shown in the *JADE1* sh2 curve.
C. Cytoplasmic Fraction

Relative abundance of MSH2

Time (hours)

D. Nuclear Fraction of MSH2

Relative abundance of MSH2

Time (hours)

E. Nuclear Fraction of Jade-1

Relative abundance of Jade-1

Time (hours)
Figure 18: pVHL may change the timing of Jade-1 and MSH2 translocation.
786-O Flag-control and 786-O Flag-VHL renal cancer cells were pretreated with 4 µg/ml O6-BG for 1 h, then treated with 5 µM MNNG for 0.5, 1 and 4 h; DMSO is vehicle control (veh). Experiments were repeated twice, (A) and (B). After treatment, cells were collected, and nuclear fractions were extracted and assessed by immunoblot with Jade-1 antiserum and MSH2, fibrillarin and α-tubulin antibodies, while cytoplasmic fractions were probed with pVHL and α-tubulin antibodies. The relative densitometry of Jade-1 and MSH2 is shown in (B) and graphed in (C)-(E). The relative abundance of cytoplasmic MSH2 of (A) and (B) with time of treatment is shown as a curve in (C). The relative abundance of MSH2 and Jade-1 in nuclear fractions of (B) changes with time points shown in curves (D) and (E), respectively.
Figure 19: Colony forming assay with HK-2 stable cell lines.

Cells were trypsinized and counted, then 100, 200 and 300 cells were seeded in each well for DMSO group, while 500, 800 and 1000 cells were seeded in each well for MNNG group. After 18-24 hours, cells were treated with DMSO or 50 μM MNNG. After 45 min treatment with MNNG, medium was removed and replaced with fresh medium. Cells were grown for about two weeks, then stained with 0.5 % crystal violet, and colonies were counted manually.

DMSO treated: (A) HK-2 parental; (B) nonsi; (C) JADE1 sh1; (D) JADE1 sh2.

MNNG treated: (a) HK-2 parental; (b) nonsi; (c) JADE1 sh1; (d) JADE1 sh2.
Table 2: Colony forming efficiency in HK-2 cell lines treated with MNNG.

A. Number of cells seeded and colonies formed of HK-2 cell lines. In DMSO control group, 600 cells in each cell lines were seeded in three wells; while in MNNG group, 2300 cells in total were seeded, and the total number of colonies were counted.

B. Colony forming efficiency (CFE) of HK-2 JADE1 sh and HK-2 nonsi cells with MNNG treatment. In order to compare the role of Jade-1 in colony formation, the CFE of HK-2 parental cells and nonsi cells were processed together as nonsilencing, and JADE1 sh1 and JADE1 sh2 were regarded as JADE1 sh.

A

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<tr>
<th>Treatment</th>
<th>Parental</th>
<th>Nonsi</th>
<th>JADE1 sh1</th>
<th>JADE1 sh2</th>
<th>Cells seeded</th>
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<td>DMSO</td>
<td>9</td>
<td>29</td>
<td>91</td>
<td>40</td>
<td>600</td>
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<tr>
<td>MNNG</td>
<td>2</td>
<td>16</td>
<td>74</td>
<td>123</td>
<td>2300</td>
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B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DMSO</th>
<th>MNNG</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonsilencing (nonsi)</td>
<td>6.3</td>
<td>0.7</td>
</tr>
<tr>
<td>JADE1 sh1</td>
<td>15.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Ratio (JADE1 sh1 / nonsi)</td>
<td>2.4</td>
<td>4.6</td>
</tr>
<tr>
<td>JADE1 sh2</td>
<td>6.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Ratio (JADE1 sh2 / nonsi)</td>
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<td>7.6</td>
</tr>
<tr>
<td>JADE1 sh</td>
<td>21.9</td>
<td>8.5</td>
</tr>
</tbody>
</table>
Ratio ($JADE1$ sh / nonsi) | 3.5 | 12.1

*CFE: Colony Forming Efficiency (%) = number of colonies formed / number of cells seeded × 100%

Figure 20: The resistance to MNNG in $JADE1$-silenced cells.

The colony forming experiment was shown in Figure 19. The fold shows colony forming efficiency of the HK-2 $JADE1$ sh1, $JADE1$ sh2 and $JADE1$ sh (all $JADE1$-silenced cell lines) cell lines with MNNG treatment compared to DMSO.
Figure 21: *Jade1* E148 mouse genotyping and absence of loss of wild-type *Jade1* in spontaneous mouse tumors.

Genomic DNA was extracted from mouse tissues, and genotyping was performed by PCR. *Jade1* wild-type gene was detected with a wild-type reverse primer and the *Jade1* knock-out gene was detected with a mutant reverse primer. These two PCR reactions share the same forward primer. The arrows point to expected PCR products. *Jade1*\(^{+/+}\) DNA was used for negative control for the mutant band, while *Jade1*\(^{+/-}\) DNA was used for positive control for the mutant band. This experiment showed the same results as genotyping by another method, X-gal staining.
DISCUSSION

4.1 DNA mismatch repair in renal cancer

DNA repair is critical for normal cellular function, and its dysfunction is associated with the malignant state. Cells incapable of repairing DNA errors insidiously accumulate an escalating number of mutations throughout their genome. Those mutations can apply to oncogenes, tumor suppressor genes, and other genes related to oncogenesis (Chung and Rustgi 1995). Germline mutation in any of 5 MMR genes, *MSH2, MSH6, MLH1, PMS1* or *PMS2* can cause Lynch syndrome (Rhyu 1996). MS2 mutation was found in 60% of Lynch syndrome families (Fishel, Lescoe et al. 1994). Biallelic loss of any of these MMR genes causes genome-wide instability, a mutator phenotype and microsatellite instability (MSI) (Paige 2003). MSI is one of the features of the early onset of Lynch syndrome.

The phenotypic features of Lynch syndrome include, early onset colorectal carcinomas (CRC) and a variety of other cancers, particularly carcinoma of the endometrium, ovary, stomach, small bowel, ureter, and renal pelvis (Lynch, Shaw et al. 1966). A study of 446 unaffected carriers of an MMR gene mutation (*MSH2, n=222*) and 1029 their unaffected relatives showed that mutation carriers had a 11-fold greater risk of renal cancer (95% CI, P<0.001) (Win, Young et al. 2012). Moreover, in another study with 129 ccRCC patients, the MSH2 negative group showed trends for decreased rates of recurrence-free survival, progression-free survival and overall survival, although it did not reach statistical significance (Yoo, Won et al. 2014). Based on this evidence, DNA mismatch repair defects may contribute to renal cancer, such as ccRCC. More work is
needed to confirm this conclusion.

4.2 Jade-1 may be involved in the initiation process of DNA mismatch repair

4.2.1 Jade-1 binds to MSH2

The well-known MSH2 binding proteins are MSH3, MSH6 and the MutL complex. MSH2 also recognizes and binds to G-T mismatched base pairs (Figure 9). In our study, we first identified Jade-1 as a novel MSH2 binding partner. These observations suggest a new role for Jade-1 in MMR.

The protein interaction between endogenous Jade-1 and endogenous MSH2 in HK-2 cells is stronger than in HEK 293T cells (Figure 13). HEK 293T cells have a defect in MMR: these cells lack hMLH1/hPMS2 but express normal levels of MSH2 (Stojic, Mojas et al. 2004). The 293T cells were highly resistant to killing by MNNG, but MMR signaling was initiated (Stojic, Mojas et al. 2004). We detected nuclear translocation of MSH2 and Jade-1 in 293T cells (data not shown) with MNNG treatment. These observations support a role for Jade-1 in the initiation of MMR. Due to the lack of MMR, 293T cells should have other supplementary repair mechanisms to compensate for the deficiency. This may also help explain the reason why the MSH2 and Jade-1 interaction is weaker in 293T than in MMR proficient cells.

4.2.2 Jade-1 regulates kinetics of MSH2 nuclear translocation

In Msh6 deficient yeast cells, Msh2 translocates to the nucleus, but with decreased abundance. Moreover, Msh6 localization depended on the Msh2 nuclear localization
signal (NLS) (Hayes, Sevi et al. 2009), suggesting a binding and stabilization pattern of MSH2 and MSH6 heterodimer to prevent free monomer accumulation (Hayes, Sevi et al. 2009). Based on our data, in cells with low levels of Jade-1, the kinetics of MSH2 translocation changed, although the differences in nuclear MSH2 levels is not conclusive (Figure 17). Apart from MSH6, Jade-1, another binding partner of MSH2, may also contribute to the balance of abundance of MSH2 monomer and MSH2-MSH6 heterodimer. Moreover, several of my experiments have shown the timing of Jade-1 and MSH2 translocation is similar and may predict a positive role of Jade-1 for MSH2 nuclear translocation. We need further experiments to illustrate whether Jade-1 and MSH2 form a heterodimer to translocate or Jade-1 regulates the amount of nuclear MSH2.

4.2.3 Resistance to MNNG in JADE-1 silenced level cells

Conventional chemotherapy works to slow or stop cancer cell growth and is commonly used to treat solid tumors. Most cancer cells are sensitive to DNA damage, so alkylating agents are common cancer chemotherapy drugs. Alkylating agents induce DNA lesions that cancer cells are unable to repair, causing apoptosis. However, renal cancer is highly resistant to chemotherapy, and no agent is considered standard for its treatment. Part of the reason for this is that P-glycoprotein 1, also known as multidrug resistance protein 1 (MDR1), is constitutively expressed in the proximal tubule of the kidney, liver cells, intestine, capillaries of brain, testes and ovaries to pump out xenobiotics, such as drugs and toxins (Chen, Chin et al. 1986).

Kidney proximal tubule cells with lower levels of Jade-1 show higher survival and
growth ability following MNNG treatment. Similar to MSH2-silenced cells, JADE-1-silenced HK-2 cells showed resistance to MNNG, which suggests similar roles for MSH2 and Jade-1 in MMR. Jade-1 expression in ccRCC cells is low (Zhou, Wang et al. 2002), which may disturb the normal function of MMR, and ccRCC cells show resistance to alkylating agents. To overcome chemotherapy resistance, elevation of endogenous Jade-1 levels may be a possible approach. ccRCC cells treated with proteasome inhibitor showed dramatically increased Jade-1 abundance (Latif, Tory et al. 1993). Our laboratory is trying to identify small molecules to stabilize or induce endogenous Jade-1 that may be useful therapeutics in ccRCC.

4.3 Jade-1 might mediate MMR by pVHL

The kinetics of MSH2 translocation was altered in HK-2 JADE1-silenced cells. Endogenous Jade-1 protein was low or undetectable in VHL-deficient renal cancer cell lines (Latif, Tory et al. 1993). Moreover, Jade-1 is stabilized by pVHL: Jade-1 abundance is 3-~10-fold higher in VHL-stably transfected cell lines (Zhou, Wang et al. 2002). We hypothesized that reintroduction of pVHL into VHL-deficient ccRCC cell lines may induce more Jade-1 and MSH2 translocation, because nuclear Jade-1 and MSH2 increased 12.5-fold and 15.4-fold, respectively, in response to MNNG in HK-2 parental cells, which have wild-type pVHL. However, the experiments we have done showed opposite: more Jade-1 and MSH2 translocated in VHL-null 786-O control cells (Figure 18). We need to do more repeat experiments to confirm this point.
4.4 Jade-1 in colon cancer

The most commonly mutated gene in colorectal cancer (CRC) is the *adenomatous polyposis coli (APC)* gene, which encodes the tumor suppressor APC. APC and other proteins form a complex to degrade oncoprotein β-catenin. With mutated APC, β-catenin level remains high and translocates to the nucleus and activates oncogene transcription. Another subset of CRC is HNPCC, which arise from mutations in MMR genes, including MSH2. MMR deficiency contributes to microsatellite instability.

Assessment of tumors from 557 cases of CRC showed that microsatellite instability was inversely associated with β-catenin overexpression (Wangefjord, Brandstedt et al. 2013). Moreover, Jade-1 is involved in MMR and regulates the translocation of MSH2. Jade-1 is also an E3 ligase for β-catenin and regulates the degradation of β-catenin. It might possible that the MMR and wnt pathway crosstalk via Jade-1.

Interestingly, in *Jade1* E148 mice, 5 of 10 *Jade1* heterozygous mice have metastatic tumors, 3 of which appear to arise from bowel. Reduced levels of Jade-1 increase the probability of bowel tumors in the mice. We need immunohistochemistry (IHC) to further characterize the MSH2, Jade-1 and β-catenin level in those mice bowel tumors. Furthermore, we can analyze the data in the Catalogue of somatic mutations in cancer (COSMIC) to check Jade-1 mutations and expression in CRC, which will provide insight into the role of Jade-1 beyond renal cancer.
BIBLIOGRAPHY


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Thesis: The role of Jade-1 in DNA mismatch repair in renal cancer

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Advisor: Jin-Tang Dong, Ph.D.
Thesis: The interactions between Kruppel-Like Factor 5 and Histone Deacetylases

RESEARCH EXPERIENCES
Master’s Research Project, Boston University, Boston, MA 2014–current
Thesis advisor: Herbert T Cohen, M.D.
Lab interests: the biological role of tumor suppressor Jade-1 in renal cancer
  • The role of tumor suppressor Jade-1 in DNA mismatch damage in renal cancer
  • The relationship between Jade-1 and MutS Homolog 2 (MSH2) in DNA mismatch repair
  • Transgenic mice genotyping
Jade-1 and MSH2 interaction: co-immunoprecipitation and immunofluorescence; DNA mismatch was induced by MNNG: colony forming assay, protein fractionation, and western blotting were performed to evaluate Jade-1 contribution to mismatch repair; genotyping: β-gal staining and PCR

Senior Thesis, Nankai University, Tianjin, China 2014
Thesis advisor: Jin-Tang Dong, Ph.D.
Lab interests: transcription factors KLF5 and ATBF1 in normal epithelial development, and their tumor suppressor roles in breast cancer and prostate cancer pathogenesis
  • Study the interactions between Kruppel-Like Factor 5 and Histone Deacetylases:
    co-immunoprecipitation, followed by western blot

Undergraduate Training Project, Nankai University, Tianjin, China 2013
Advisor: Yanqiang Liu, Ph.D.
  • The function of mammillary body and its surrounding areas in arterial blood pressure regulation:
    Used electricity and rabbit as stimulator for mammillary body and animal model, respectively.

Undergraduate Summer Research, Peking University Health Science Center, Beijing, China 2013
Advisor: Yuxin Yin, M.D., Ph.D.  
Lab interests: tumor suppressors p53 and PTEN in cell cycle regulation, apoptosis and genomic stability  
- Study the translation mechanism and function of PTEN-α:  
  created of point mutation of PTEN-α expression vector, cultured cells transfection  

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2011~2013  
Advisor: Zhangyong Hong, Ph.D.  
Lab interests: drug delivery and immune-induced targeted therapy for cancer  
- IR-780-loaded PLGA nanoparticles as infrared fluorescent probes for tumor detection:  
  generation of IR-780-loaded PLGA nanoparticles and biological characterization in vitro, in cultured mammalian cells and mice  

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Advisor: Jin-Tang Dong, Ph.D.  
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- The regulation of signaling pathways in carcinogenesis  
- Post-translational modification of tumor suppressors and oncoproteins  
- Nanomedicine in tumor detection and treatment  

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- Excellence Award, Molecular Biology Experiment Contest, Nankai 

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- Third Prize, Cell Biology Experiment Contest, Nankai University 2012
- Second-class Scholarship, Nankai University 2012
- First Prize, Biochemistry Experiment Contest, Nankai University 2011
- Second Prize, Plant Biology Experiment Contest, Nankai University 2011
- Third-class Scholarship, Nankai University 2011
- Second Prize, the 11th Beijing High School Mathematics Competition, Beijing, China 2008
- Third Prize, the 17th National Junior High School Applied Physics Competition, China 2007

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- **Secretary** of Student Organization, College of Life Science, Nankai University, Tianjin, China 2010~2012
  Poster preparation for sporting events and dancing social
- **Minister** of Nankai University Student International Communication Association, Tianjin, China 2010~2012
  Organized a new member orientation and a seminar for exchange students
- **Charity Volunteer** for disabled students, Tianjin, China 2011
  Gave fine art and algebra lectures to students with disability