UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION AND CHARACTERIZATION OF TOLL-LIKE RECEPTOR (TLR) 2 AND 4 MUTATIONS IN COLORECTAL

HOMA DAVOODI

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IDENTIFICATION AND CHARACTERIZATION OF TOLL-LIKE RECEPTOR (TLR) 2 AND 4 MUTATIONS IN COLORECTAL

By

HOMA DAVOODI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2009
This thesis is dedicated to

My husband and my son, my late father, my beloved mother, sister, brothers and my parents in law with love and gratitude and also to the kids who have ability but don’t have facilities to obtain education.
IDENTIFICATION AND CHARACTERIZATION OF TOLL-LIKE RECEPTOR (TLR) 2 AND 4 MUTATIONS IN COLORECTAL

By
HOMA DAVOODI

Chairperson: Seow Heng Fong, PhD
Faculty: Medicine and Health Sciences

Toll-like receptors (TLRs) are the most important receptors in innate immunity that have been identified as a major class of pattern-recognition receptors. The TLR family comprises at least eleven members, these TLRs recognize a limited but highly conserved set of molecular structures, so called pathogen-associated molecular patterns (PAMPs). For example, TLR4 recognizes LPS, which is unique to gram-negative bacteria. Increasing evidence suggest that the ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms (SNPs) within TLR genes. The role of TLRs signaling and effect of SNPs mutations on cancer outcome and survival is not exactly determined yet. The objectives of this study were firstly to detect the two most common TLR2 (Arg677Trp, Arg753Gln) and TLR4 SNPs (Asp299Gly, Thr399Ile) in colorectal cancer (CRC), secondly to evaluate the TLR2 and 4 expressions in colorectal cancer cell lines and the effect of polymorphisms on the TLR4 expression. The cytokine profiles secreted by colorectal cancer cells with mutant and non- mutant TLR4 and the expression of some signal transduction molecules involved in TLR4 signalling were also determined.
Lastly, the impact of these SNPs on the cytotoxicity and apoptosis induction of the 5-Fluorouracil (5-FU) was also evaluated. PCR-RFLP was carried out on fifty normal blood samples and sixty human colorectal cancer paraffin-embedded blocks to determine the incidence of TLR2 and TLR4 mutations. The results showed two individuals were heterozygous for the Asp299Gly (D299G) and Thr399Ile (T399I) polymorphisms in the TLR4 gene. However, all samples in control group were the wild-type form. Since we could not find any TLR2 mutations in our samples, our study focused on the TLR4 gene. *In vitro* studies were performed on HCT116 cell line transfected with mutant and wild-type TLR4 genotype. A series of experiments were conducted to examine the effect of TLR4 variations on the expression of TLR4, LPS responsiveness and the response of the cells to the 5-FU as a chemotherapeutic agent. FACS analysis of TLR4 expression on transfected HCT116 cells showed that the expression of wild-type was higher than mutant TLR4. LPS induced TLR4 expression on transfected cells and the response of wild-type genotype to the LPS was more significant compared to the mutants. Western blot analysis and Dual Luciferase assay showed that the activity of pNF-kB was higher in cells transfected with plasmid for TLR4 D299G compared to the other cells. However, the activity of pAKT, pERK1 and pIRAK was higher in wild-type.
The results of cytokine measurements showed that IL-8 levels were increased in wild-type and basal VEGF was high in un-transfected cells. Secreted VEGF levels was decreased by LPS in wild-type cells but increased in un-transfected cells. IL-17 was secreted by transfected cells at a low level and was not significantly affected by LPS. The results of MTT assay showed that the cytotoxicity effect of 5-FU on transfected cells expressing D299G TLR4 mutant was lower compared to the other cells. 5-FU increased TLR4 expression on transfected cells and LPS has a synergistic effect with 5-FU. LPS increased the apoptosis induced by 5-FU and suggesting that it may be useful as an adjuvant in chemotherapy. HMGB1, an endogenous ligand for TLR4, was secreted by 5-FU- treated cells and also detected in cell lysate. TLR4 is functionally active on transfected HCT116 cell line. The increased activity of pNF-kB in cells transfected with plasmid expressing TLR4 D299G may lead to decreased-cytotoxicity effect of 5- FU in this variant. Therefore, it is possible that the CRC patients who harbor this polymorphism tend to be more resistant to drug compared to the wild-type. High level of pNF-kB can also explain the association of this polymorphism with Inflammatory Bowel Disease (IBD), susceptibility to infectious disease and even cancer.
PENGECAMAN DAN PENCIRIAN MUTASI PADA “TOLL-LIKE RECEPTOR” 2 DAN 4 (TLR) DALAM KANSER KOLOREKTAL

Oleh
HOMA DAVOODI
Disember 2009

Pengerusi: Seow Heng Fong, PhD
Fakulti: Perubatan Dan Sains Kesihatan

Toll like receptors (TLRs) merupakan reseptor penting dalam imuniti semulajadi, yang telah dikenalpasti sebagai komponen utama ‘pattern recognition receptor’. TLR merangkumi sekurang-kurangnya sebelas komponen dan TLR boleh mengenalpasti struktur molekul secara terperinci yang dikenali sebagai pathogen-associated molecular patterns (PAMPS). Sebagai contoh, TLR4 boleh mengenalpasti LPS, yang merupakan komponen bakteria gram negatif. Kajian telah menunjukkan bahawa kebolehan seseorang individu untuk bertindak balas terhadap ligan TLR mungkin terganggu oleh polimorfisme mononukleotida (SNPs) pada gen TLR. Peranan utama isyarat TLRs dan kesan mutasi SNPs terhadap kanser belum dikenalpasti lagi. Objektif utama kajian adalah untuk mengenalpasti dua TLR yang utama iaitu TLR2 (Arg 677 Trp, Arg 753 Gln) dan TLR4 SNPs (Asp 299 Gly, Thr 399 Ile) dalam kolorektal kanser (CRC). Kedua, menilai ekspresi TLR2 dan TLR4 dalam titisan sel kanser kolorektal dan juga kesan polimorfisme terhadap ekspresi TLR4. Mengenalpasti profil sitokin yang dirembes oleh sel-sel kanser kolorektal antara TLR4 mutan dan bukan mutan.
mutan D299G TLR4 adalah rendah berbanding sel-sel lain. 5-FU telah meningkatkan ekspresi TLR4 terhadap sel yang ditransfeksikan dan LPS mempunyai kesan sinergistik. LPS meningkatkan kadar apoptosis yang diaruhkan oleh 5-FU dan boleh digunakan sebagai adjuvan dalam kemoterapi 5-FU. HMGB1 yang merupakan ligan endogenus untuk TLR4 telah dirembbes oleh sel yang dirawat dengan 5-FU dan dikenalpasti dalam media dan lisat sel. TLR4 adalah aktif terhadap titisan sel HCT116 tertransfeksii Aktiviti yang tinggi oleh p-NFkB dalam D299G boleh mengurangkan kesan kesitotoksikan 5-FU dalam varian ini. Oleh itu, ada kemungkinan bahawa peasakit CRC yang mempunyai polimorfisme ini, adalah lebih rintang terhadap dadah berbanding jenis liar. Kadar pNFkB juga boleh menjelaskan hubungan antara polimorfisme dan ‘Inflammatatory Bowel Disease’ (IBD), kecenderungan mendapat penyakit berjangkit dan juga kanser.
ACKNOWLEDGEMENTS

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I certify that a Thesis Examination Committee has met on 7 December 2009 to conduct the final examination of Homa Davoodi on her thesis entitled "Identification and characterization of Toll-like receptor (TLR) 2 and 4 mutations in colorectal cancer" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor of Philosophy (PhD).

Members of the Thesis Examination Committee were as follows:

**Sabariah Abdul Rahman, PhD**  
Associate professor  
Institute of Bioscience  
University Putra Malaysia  
(Chairman)

**Chong Pei Pei, PhD**  
Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Daud Ahmad Israf Ali, PhD**  
Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Kennet Scott, PhD**  
Senior lecturer  
Faculty of Science  
University of Auckland  
New Zealand  
(External Examiner)

*****************************************************************************

BUJANG BIN KIM HUAT, PhD  
Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:
This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

**Seow Heng Fong, PhD**  
Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairperson)

**Maha Bt Abdullah, PhD**  
Senior Lecturer  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

**Sharmili Vidyadaran, PhD**  
Senior Lecturer  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

---

**HASANAH MOHD GHAZALI, PhD**  
Professor and Dean  
School of Graduate studies  
Universiti Putra Malaysia  

Date: 11 February 2010
DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously and it is not concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

HOMA DAVOODI
Date: 11 February 2010
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<table>
<thead>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
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<tr>
<td>Abs</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigens</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Human colon carcinoma epithelial cell line</td>
</tr>
<tr>
<td>CBA</td>
<td>Cytometric bead array</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's Disease</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster of differentiation 8</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
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<tr>
<td>HMGB1</td>
<td>High-mobility group box 1</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Median Inhibition Concentration (the concentration of substance that provides 50% inhibition to certain reaction)</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IEC</td>
<td>intestinal epithelial cells</td>
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<td>IL</td>
<td>Interleukins</td>
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<td>IRAK1</td>
<td>IL-1 receptor associated kinase</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>Myeloid differentiation protein 88</td>
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<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
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<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>pH</td>
<td>Hydrogen ion concentration</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>The transforming growth factor beta</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet ray</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
### Units
- **°C**: Degrees centigrade
- **g**: Unit for measuring centrifugation force
- **g**: Gram
- **μl**: Microlitre
- **h**: Hour
- **l**: Liter
- **min**: Minute
- **mL**: Milliliter
- **ng**: Nanogram
- **nm**: Nanometer
- **w/v**: Weight by volume

### Common abbreviations
- **e. g.**: for example
- **et al.**: and others
- **i.e.**: for example

### Statistical terms
- **P**: Probability
- **SD**: Standard deviation
- **SE**: Standard error
Chemical elements and compounds

- CO₂: Carbon dioxide
- DW: Distilled water
- EtOH: Ethanol
- HCl: Hydrochloric acid
- HOAc: Acetic acid
- K: Potassium
- KH₂PO₄: Potassium di-hydrogen phosphate
- K₂SO₄: Potassium sulfate
- PBS: Phosphate buffered saline
- MeOH: Methanol
- NaCl: Sodium chloride
- NaOH: Sodium hydroxide
CHAPTER I

INTRODUCTION

Toll-like receptors (TLRs) are the most important receptors in innate immunity that have been identified as a major class of pattern-recognition receptors. The TLR family comprises at least eleven members, these TLRs recognize a limited but highly conserved set of molecular structures, so called pathogen-associated molecular patterns (PAMPs). For example, TLR4 recognizes LPS, which is unique to gram-negative bacteria, and TLR2 recognizes peptidoglycan found in gram-positive bacteria.

Recognition of pathogen-associated molecular patterns (PAMPs) by TLRs activates numerous signal-transduction pathways that target several transcription factors, a major signalling target of the TLRs is activation of the transcription factor NF-κB, a key regulator of immune and inflammatory responses, which control the expression of genes encoding cytokines, chemokines and enzymes that regulate innate and adaptive immune responses (Kaleigh, 2002). Recently researchers have shown that chronic or recurrent inflammation has been implicated in the initiation and development of several human cancers including those of the stomach, liver, colon, and urinary bladder. The fact that colorectal cancer is more often in patients suffering from inflammatory bowel disease, such as ulcerative colitis (UC) and Crohn’s disease (CD), raises the question of the relationship between chronic inflammation and malignant transformation (Boraska et al., 2006).
It has been reported that TLRs are not only expressed in immune cells, but also on endothelial and epithelial cells. In particular, TLR4 was found in epithelial cell layers that are in contact with the outer environment, such as intestine, lung, gingival, corneal and renal epithelial cells. Bacterial infection could affect the function of these cells through TLR4 which leads to local infections or inflammatory processes. It was reported that proinflammatory cytokines induced by inflammatory stimuli can counteract immune surveillance and facilitate tumor outgrowth (He et al., 2007). Toll-like receptors are increasingly implicated in disease pathogenesis. Population studies of polymorphisms in genes encoding TLRs or their downstream signalling molecules have directly linked these pathways to multiple human disease processes.

The great majority of polymorphisms studied are single nucleotide polymorphisms (SNPs) that occur with a frequency of >1% in the normal population (in contrast to ‘mutations’ that occur with a frequency of <1%) (El-Omar et al., 2008). Hundreds of SNPs have been identified in TLRs but the functional consequences of these have not been revealed completely. Many associations have been reported between TLR polymorphisms and infectious disease or cancers and it is the interaction between infection and chronic inflammation that most likely mediates the increased risk of cancer development. SNPs in TLR2 have been reported to alter the susceptibility to various inflammatory and infectious diseases. The two most common TLR2 SNPs are Arg677Trp and Arg753Gln. The Arg677Trp mutation has been suggested to cause impaired function of the intracellular domain of the TLR2 protein. This
polymorphism has been reported to be associated with lepromatous leprosy in a Korean population (Kang and Chae, 2001) and recurrent bacterial infections in Turkish children (Kutukculer et al., 2007). The other well-described TLR2 SNP is also located in the coding region, which is at amino acid position 753 substituting an arginine residue to a glutamine residue. This polymorphism has been reported to increase the risk of gram-negative sepsis in a Caucasian population. In addition to these two SNPs, Boraska et al. (2006) reported that the allele frequency of a TLR2 guanine thymine (GT) microsatellite repeat polymorphism in the second intron has an impact on the susceptibility to sporadic colorectal cancer. Individuals who developed sporadic colorectal cancer tended to have decreased frequency of alleles 20 and 21 GT repeats and increased frequency of alleles with 31 GT repeats (Boraska et al., 2006). The TLR-4 gene consists of three exons mapped to chromosome 9q32–33 two nonsynonymous single nucleotide polymorphisms (SNPs), A896G (rs4986790) and C1196T (rs4986791) in exon 3 lead to Asp299Gly and Thr399Ile amino acid substitutions, respectively. SNP Asp299Gly was later shown to co-segregate with SNP Thr399Ile—both in the third exon of TLR4. These SNPs are present in 10% of Caucasian populations and are reported to have a positive correlation with susceptibility to several infectious diseases (including gram-negative sepsis), atherosclerosis, asthma, malaria and also H. pylori-induced gastric cancer (El-Omar et al., 2008). The SNPs disrupt the normal structure of the extracellular region of the TLR4 and are therefore hypothesized to decrease responsiveness to lipopolysaccharide (LPS) through alterations in binding.

There are some endogenous proteins which bind and stimulate TLR4, including HSP60, HSP70, oxidized low-density lipoprotein, surfactant protein A,
hyaluronan breakdown products, fibronectin, b-defensin, and the alarmin high-mobility group box 1 protein (HMGB1). The term ‘alarmin,’ denotes an array of structurally diverse host proteins rapidly released during infection or tissue damage that have mobilizing and activating effects for host defence and tissue repair. HMGB1 is a highly mobile nuclear protein (non-histone chromatin binding protein) that influences transcription and other nuclear transactions.

Apetoh *et al.* (2007) reported that dying tumor cells promoted by cancer therapies trigger an immune response in a TLR4-dependent way and that TLR4 triggering requires HMGB1 released by dying tumor cells. In this study they also showed that in the breast cancer patients the metastasis-free survival was significantly longer in the cohort carrying the normal allele of TLR4 compare to the patients who carrying the Asp299Gly polymorphism (50% of relapse in mutated versus 37.4% in nonmutated patients at 10 years). Similar results were obtained by analyzing the second missense mutation (Thr399Ile) that co-segregates with the Asp299Gly substitution. Importantly, mutant TLR4 also reduced the interaction of endogenous TLR4 with HMGB1, suggesting that it acts as a dominant-negative form of TLR4 with respect to the binding capacity to HMGB1 (Apeteh *et al.*, 2007). Experimental evidence suggests that the neoplastic process may interfere with TLR signalling pathways to advance cancer progression. Current *in vivo* genetic studies highlight a more fundamental and direct role for epithelial cell TLRs and NF-kB in tumour development. Multiple chronic inflammatory diseases have been associated with cancer development including inflammatory bowel disease, hepatitis and chronic Helicobacter pylori
infection predisposing to colorectal, hepatocellular and gastric cancer respectively. *TLR4* is induced by proinflammatory cytokines and is highly expressed in IECs, resident macrophages, and dendritic cells in inflamed mucosa of IBD patients. Dendritic cell maturation and the development of adaptive immunity seem to require correct *TLR4* signalling.

Disruption of *TLR4* signalling could create an inappropriate innate and adaptive immune response necessary to eradicate pathogens which would result in a more severe inflammation. Studying SNPs in molecules involved in bacterial recognition will be essential to understand individual responses to bacterial components and define genetic backgrounds at risk of IBD (Franchimont et al., 2004). Now *TLRs* are hot targets for drug development. The industry has spent hundreds of millions of dollars developing new substances that they hope will kill cancer by *TLR* activation (Schmidt, 2006). The most important *TLRs* against bacterial cell walls components are *TLR2* & 4 and mutation in these genes decrease the response to bacterial components, impact gut homeostasis and results in impairment of *TLRs* activation which may affect on cancer development and progression.
Hypotheses

➢ TLR4 mutant alleles are more frequent in colorectal cancer (CRC) patients compared to the control group.

➢ The expression of mutant TLR4 on colorectal cancer cells is lower compared to the wild-type.

➢ Mutant TLR4 allele affect on cytokine profiles and some molecules involved in TLR4 signaling to induce inflammatory response.

➢ CRC patients with mutant TLR4 genotype are more resistant to 5-Fu chemotherapy compared to the wild-type.

Objectives

The objectives of this study were to:

1. Determine the TLR2 and TLR4 polymorphisms in colorectal cancer.

2. Evaluate the TLR2 and 4 expressions in colorectal cancer cell lines and the effect of polymorphisms on the TLR4 expression.

3. Determine the cytokine profiles secreted by colorectal cancer cells with mutant and non-mutant TLR4.

4. Determine the effect of polymorphisms on the expression of some molecules which are involved in TLR4 signalling.

5. Determine the impact of these SNPs on the cytotoxicity of the 5-Fluorouracil.

6. Determine the impact of these SNPs on the apoptosis induced by 5-Fluorouracil.
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