Analysis of experimental mouse PRNP genetic polymorphisms and their susceptibility to prion diseases

Feng Guan\textsuperscript{1}, Zhao Wei CAI\textsuperscript{2}, Jun Tao AI\textsuperscript{3} and Jin Zhao*\textsuperscript{4}

College of Life Sciences, China Jiliang University, Hangzhou-310 018, China.
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ABSTRACT
Research studies showed that the polymorphisms in prion protein gene (PRNP) were associated with susceptibility to prion diseases in several animals, including humans and mouse. Several mouse strains carried natural PRNP mutations which had been identified and these could provide as animal models for human prion diseases. In this study, the genetic polymorphisms of PRNP in six common mouse strains were investigated. The experimental mice included KM mouse, ICR mouse, DBA mouse, C3H/He mouse, C57BL mouse and BALB mouse. The results showed only one new polymorphism was identified compared with the reference sequence. The identified new mutation site was C564T and it was homozygous, but this locus did not result in amino acid change. Sequence analyses suggested that these six mouse strains were susceptible to prion diseases and are suitable as susceptibility models of prion diseases.

Key words: Gene mutation, Genetic polymorphism, Mouse model, Prion protein gene.

INTRODUCTION
Prion diseases are a group of disorders caused by abnormally shaped proteins called prions, and occur in many disease forms in humans and several animals (Choudhary and Choudhary, 2013, Geschwind, 2015). The prion diseases include human Kuru and Jakob-Creutzfeldt disease (CJD), sheep and goat scrapie, bovine transmissible spongiform encephalopathies (BSE), chronic wasting disease (CWD) and others. It is believed that these diseases propagate by the misfolding and aggregation of the prion proteins (PrP) into scrapie affected prion proteins (PrP\textsuperscript{Sc}). Several research studies indicated that the sequence of host’s encoding gene for prion protein could affect the host’s susceptibility to prion diseases and is considered as the species barrier to transmission (Cortez and Sim, 2013). In other words, the genetic polymorphisms in prion protein encoding gene (PRNP) within species further affects the susceptibility or influence the incubation period, pathology and phenotype (Cortez and Sim, 2013, Geschwind, 2015). For example, a single nucleotide polymorphism of PRNP at codon 129 has been shown to be associated with the susceptibility and clinical symptom of human variant CJD (Jeong and Kim, 2014). Similar to the prion diseases, Alzheimer’s disease (AD), shows common type of dementia that result from the aggregation of norm protein (Viola and Klein, 2015). Studies on PRNP polymorphisms suggested a few mutation sites that affected human susceptibility to AD (Munoz-Nieto et al., 2013, Sassi et al., 2016, Smid et al., 2013). Research in the course of prion diseases revealed several PRNP polymorphisms in mice, which affected the incubation period and the susceptibility to prion diseases (Carlson et al., 1986, Kingsbury et al., 1983, Prusiner, 1982, Westaway et al., 1987), and a few SNPs showed a direct relationship with susceptibility (Lloyd et al., 2004, Moore et al., 1998, Westaway et al., 1987). Furthermore, these mutational mice had been partly used as animal models in the study of human prion diseases (Fernandez-Borges et al., 2015, Lee et al., 2013).

In view of the above progress, this study further investigated whether the commonly used mouse strains have the known genetic mutations and further assess the susceptibility. This study was performed using six mouse strains, including KM/615 mouse, ICR mouse, DBA/2J mouse, C3H/He mouse, C57BL/6 mouse and BALB/C mouse.
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MATERIALS AND METHODS

Experimental mice and samples collection: In this study, a total of 240 samples of six mouse strains, including KM/615 mouse, ICR mouse, DBA/2J mouse, C3H/He mouse, C57BL/6 mouse and BALB/C mouse were tested. KM and ICR mice were collected from Experimental Animal Center of Hangzhou Normal University in Hangzhou city. Other mice were collected from Shanghai SLAC laboratory animal center and Zhejiang Chinese Medical University Laboratory Animal Research Center. About 20 mg mouse tail tip was cut and stored in liquid nitrogen for use.

Genomic DNA was extracted from the tail tissue using animal tissue DNA extraction kit (Takara) in accordance with the manufacturer’s instructions. The purity and concentration of all the extracted samples were checked using a UV-Vis spectrophotometer (Nanodrop 2000, Thermo). DNA samples were diluted to a final concentration of 10ng/µl and stored at 4 °C for next use.

PCR primers design and amplification: The sequences of mouse PRNP (accession number NM011170) was retrieved from NCBI database and used as a reference template for primer design. A pair of mouse specific primers were designed using Primer Premier 5.0 software, and the primer set could generate 541 bp length fragment from PRNP, and this amplified region contained all the known mutations. The primer sequences are as follows: Forward primer: (5’-GCTTA TTTA TCTTTCACGGG-3’) and reverse primer (5’-TGTGCA TAAGCTCCTTGAAG-3’).

PCR reactions were carried out in a total volume of 20 µl mixture, and the 20 µl PCR mixture was prepared as follows: 2 µl 10X PCR buffer, 1.2 µl MgCl$_2$ (25 mM), 0.2 µl dNTP mixture (2.5 mM), 1 U Taq polymerase, 2 µl of each forward and reverse primer (10 µM), 3.0 µl DNA template (30 ng), added ddH$_2$O to 20 µl. Amplification was carried out with the following program: initial denaturation at 94°C for 8 min and 30 cycles at 94°C for 30 sec, 60.5°C for 30 sec, and 72°C for 35 sec followed by 72°C for 5 min. 5.0 µl PCR product was analyzed using 2% agarose gel in 1X TAE buffer stained with 4S Red as a visualizing agent and run for 40 min at 80V.

Genetic polymorphism analysis: Recovery of the PCR products were performed using Gel Recovery Kit after separating on 2% agarose gel, and then were bidirectionally sequenced by Hangzhou Qingke Biotechnology Co., Ltd. Part of PCR products from different mouse strains were cloned into pMD18-T vector for sequencing after transformation of DH5α cells. Sequence alignments and comparisons were carried out using Mutation Surveyor v3.97 and DNAsstar7.0 software package.

RESULTS AND DISCUSSION

PRNP amplification and specificity: The PCR products were analyzed on 2% agarose gel after amplification, and the results showed clear electrophoresis strips and demonstrated the same length with expected size, 541bp. Part of the results were shown in Figure 1, and no other non-specific PCR product was observed, judging from the figure. All the samples were successfully amplified.

PRNP polymorphisms in six mouse strains: In this study, a total of 240 PCR products from mouse samples were bidirectionally sequenced or cloned sequenced. The forward and reverse sequencing results of all the samples were in complete agreement, and showed exactly the same sequences at the reported mutational sites compared with the reference sequences. The reported mutational amino acid sites included 103, 104, 105 and others, the data were shown in Table 1. In addition, one homozygous mutation (C-T) was identified at amino acid site 188 of prion protein, which had not been reported previously.

Mouse PRNP mutational site analysis: The C188T mutation locus was detected in ICR mouse, compared with the reference sequence, and the mutation located at 564 position in CDS region of PRNP. This mutation was homozygous at this point, and forward sequencing (Figure 2) was in complete agreement with reverse sequencing.
Table 1: Data of mouse PRNP mutation sites and comparison of reported data

<table>
<thead>
<tr>
<th>Locus</th>
<th>Location in CDS</th>
<th>Base</th>
<th>Amino acid</th>
<th>Mutation</th>
<th>Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>103</td>
<td>307-309</td>
<td>AAA</td>
<td>K</td>
<td>-</td>
<td>104P105T</td>
</tr>
<tr>
<td>104</td>
<td>310-312</td>
<td>CCA</td>
<td>P</td>
<td>K105R</td>
<td>12.1</td>
</tr>
<tr>
<td>105</td>
<td>313-315</td>
<td>AAA</td>
<td>K</td>
<td>P104T</td>
<td>11.1</td>
</tr>
<tr>
<td>108</td>
<td>322-324</td>
<td>CTC</td>
<td>L</td>
<td>-</td>
<td>12.3</td>
</tr>
<tr>
<td>109</td>
<td>325-327</td>
<td>AAG</td>
<td>K</td>
<td>V121I</td>
<td>14.8</td>
</tr>
<tr>
<td>111</td>
<td>331-333</td>
<td>GTG</td>
<td>V</td>
<td>A117V</td>
<td>14.8</td>
</tr>
<tr>
<td>115</td>
<td>343-345</td>
<td>GCG</td>
<td>A</td>
<td>A115S</td>
<td>15.5</td>
</tr>
<tr>
<td>117</td>
<td>349-351</td>
<td>GCT</td>
<td>A</td>
<td>A115V</td>
<td>14.8</td>
</tr>
<tr>
<td>119</td>
<td>355-357</td>
<td>GCA</td>
<td>A</td>
<td>-</td>
<td>12.3</td>
</tr>
<tr>
<td>121</td>
<td>361-363</td>
<td>GTG</td>
<td>V</td>
<td>V121I</td>
<td>23.3</td>
</tr>
<tr>
<td>127</td>
<td>379-381</td>
<td>TAC</td>
<td>Y</td>
<td>Y127I</td>
<td>13.4</td>
</tr>
<tr>
<td>131</td>
<td>391-393</td>
<td>AGC</td>
<td>S</td>
<td>-</td>
<td>12.3</td>
</tr>
<tr>
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<td>400-402</td>
<td>AGC</td>
<td>S</td>
<td>-</td>
<td>12.3</td>
</tr>
<tr>
<td>137</td>
<td>409-411</td>
<td>ATG</td>
<td>M</td>
<td>-</td>
<td>12.3</td>
</tr>
<tr>
<td>176</td>
<td>526-528</td>
<td>CAC</td>
<td>H</td>
<td>H176R</td>
<td>15.6</td>
</tr>
<tr>
<td>189</td>
<td>565-567</td>
<td>ACC</td>
<td>T</td>
<td>-</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Note: - represents no mutation was found.

(Figure 3). But this mutation site does not result in change of amino acid, which is considered as a silent mutation.

The outbreak of bovine spongiform encephalopathy (BSE) from 1985 to 1995 in UK had a high risk on human health and food safety. BSE belongs to prion diseases, there are several diseases which belong to this, such as scrapie in sheep and chronic wasting disease in deer, and Kuru, Creutzfeld-Jakob disease (CJD) in human, etc. Prion diseases belong to a group of fatal infectious diseases with no effective therapies available. A part of prion diseases in humans (Cortez and Sim, 2013), sheep (Choudhary et al., 2013, Probsthensch et al., 2013), and cattle (Cortez and Sim, 2013), can be inherited as a result of mutations in PRNP. Furthermore, molecular research showed that prion diseases were caused by the recruitment of normal cellular form of the protein (PrP<sup>c</sup>) which self-propagates into polymeric forms were often referred to as PrP<sup>sc</sup> (Risse et al., 2015). Similarly, neurodegenerative diseases such as Parkinsons and AD involve the fundamental molecular processes of protein
misfolding and aggregation. Recently, PrP<sup>C</sup> was identified as a receptor for amyloid β (Aβ) oligomers, which are considered as key pathogenic factors in AD (Kessels et al., 2010, Lauren et al., 2009, Um and Strittmatter, 2013). In 2013, it was reported that about 5.2 million Americans had AD and 200,000 people younger than 65 years had AD, and 5 million comprise the older onset AD population, in the United States (Thies and Bleiler, 2013). AD begins with memory loss for short-term events and finally robs the patients of their sense of self. AD is involved in 50-70% of dementia cases, and nearly half of people older than 85 years suffer from it. The disease poses a great threat to older populations and their families, becoming a serious social problem with increasing longevity (Amemori et al., 2015). Currently, there are no curative treatments for AD and other prion diseases, and also the underlying mechanism had not been fully elucidated (Karamanos et al., 2015, Visanji et al., 2013, Winans and Drummond, 2016). On the other hand, there were no appropriate animal models or limited animal models for the drug development research (Fernandez-Borges et al., 2013, Ghate et al., 2014, Shirai et al., 2014). In the last 20 years, several mouse models had been established and were used in a number of research fields of prion diseases, including pathology (Corda et al., 2015, Ghate et al., 2014) and genetics (Asante et al., 2015, Friedman-Levi et al., 2011). Through the last 35 years, less than 50 different drugs have been tested in different experimental animal models without any hope for the results. An important limitation was the existence of appropriate models of the disease (Fernandez-Borges et al., 2013). In order to study the molecular mechanism of the prion diseases and to test the drugs, many mouse models had been established and they are required (Asante et al., 2015, Bajsarowicz et al., 2012, Bruce et al., 2002, Fernandez-Borges et al., 2015, Friedman-Levi et al., 2011, Lee et al., 2013, Morales et al., 2015, Shirai et al., 2014). Among these mouse models, there included a number of transgenic models which are expensive, with short life, clinical symptoms and genetic instability, and other associated problems. So it was difficult to fully meet the application of these mouse models for the research of prion diseases.

During the study of mouse models in prion diseases, the researchers found that the mice had different incubation periods, which showed a high degree of association with PRNP polymorphisms (Carlson et al., 1986, Kingsbury et al., 1983, Prusiner, 1982, Westaway et al., 1987). The mouse PRNP polymorphisms directly affected the susceptibility and incubation period, and DNA sequencing confirmed that the polymorphisms were at L108-T189 and F108-V189 sites, respectively (Carlson et al., 1986, Lloyd et al., 2004, Moore et al., 1998, Westaway et al., 1987). The incubation period showed a significant difference between the two genotypes of PRNP L108-T189 and F108-V189, they were 113±2.6 d and 200–385 d, respectively (Carlson et al., 1986, Westaway and Prusiner, 1986). Later, the genotyped mouse of PRNP at F108-T189 was also found (Lloyd et al., 2004). The discovery of PRNP mutational mouse filled the gap of existing AD mouse models in this research area, which showed a potential application in the study of pathogenesis, new drug design and testing aspects. There has been five mouse strains with resistant genotypes identified till now, which included I/LnJ mouse, Rbb11/S/J mouse, P/J mouse, MA mouse and MyJ mouse (Westaway et al., 1987). On the contrary, 21 mouse strains with susceptible genotypes were identified, such as NZW/LacJ mouse, BALB/cJ mouse, BUB/BnJ mouse, CBA/J mouse, CE/J mouse, C3H/HeSn mouse, C57BL/6J mouse, C57BL/10Sn mouse and DBA/2J mouse (Westaway et al., 1987). Shirai et al. (2014) had performed a systematic study on all the known point mutations using cell models and evaluated the risk of prion disease susceptibility. As a result, the residual sites in which the point mutations tend to increase the susceptibility were mainly localized at K103–A119 and S131–M137 in the PrP. The ten most effective sites (with the highest average fold-increase of PrP<sup>C</sup> conversion over all amino acids) included were V121I (23.3-fold increase), P104T (12.1), K105R (17.1), A115S (16.5), A115W (15.5), A115N (12.7), A115Y (10.6), A117V (14.8), Y127I (13.4), and H176R (15.6) (Shirai et al., 2014). So, the susceptibility of mutation mouse models demonstrated a judgment to a certain extent for progression of this study.

In China, many mouse strains such as KM mouse, ICR mouse, BALB mouse, C3H mouse, C57BL mouse and DBA/2J mouse, were widely used in research fields, of course, including the study of the prion disease models. In order to analyze the PRNP polymorphism in these mice and exploit the utilization in disease research, six common mouse strains are selected and analyzed. As a result, all obtained sequences in these samples were consistent with the relative reference sequences except a new single nucleotide homozygous polymorphism in ICR mouse. But, we propose that all the tested mice have susceptible PRNP genotypes based on the other reported genotypes, such as K103, P104, K105, K109, V111, A115, A117, V121, Y127, S131, S134 and M137. Furthermore, all the tested mouse samples were detected for L108-T189 genotype, which was a susceptible genotype (Westaway et al., 1987). So, we propose that KM mouse, ICR mouse, BALB mouse, C3H mouse, C57BL mouse and DBA/2J mouse strains are susceptible to prion diseases, and they fit as mouse models for research in prion diseases. But on the other hand, these mouse strains lack genetic diversity because they belong to the inbred lines.

In a word, genetic mutational mouse model is a new animal model for study on prion diseases, which can be an important supplement to the existing models. Several mutational loci had been identified in mouse PRNPs and showed susceptibility to prion diseases. To study PRNP
genetic polymorphism and search for susceptible mouse, six common mouse strains were tested and identified one new mutational locus. Comprehensive consideration of gene sequences and mutations suggested that the six mouse strains were susceptible to prion diseases. Furthermore, the new mutational locus was a homozygous silent site.

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