

Clone and Bioinformatics Analysis of Chinese-Belgium Rabbit Metallothionein-1 MT_1 , MT_2 and MT_3 Genes CDS Region

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Abstract: Metallothioneins (MTs) play important roles in metal ion metabolism, detoxication of heavy metal, clearance of Reactive Oxygen Species (ROS). In the studies, Coding Sequences (CDS) of MT_1 , MT_2 and MT_3 genes from Chinese-Belgium rabbit were cloned and sequenced. The results showed: CDS length of MT_1 , MT_2 and MT_3 was 186, 186 and 201 bp, respectively encoded 61, 61 and 66 amino acids, respectively. Amino acid sequence of MTs includes 7 typical Cys-Xaa-Cys motifs and 20 cysteines which form 10 disulfide-bonds. Similarity analysis showed that the similarity of MT_1 and MT_2 reached to 87.6%; the similarity of MT_2 and MT_3 was 78.0%; the similarity of MT_3 and MT_1 was 76.3%. Alignment and cluster analysis of MTs genes from different species suggest MTs are highly conserved but different subtype of MTs genes have separated and formed in the early stages of the evolution of species and then they individually evolve.

Key words: Rabbit, Metallothionein (MT), clone, bioinformatics analysis, amino acids

INTRODUCTION

Metallothioneins (MTs) are ubiquitous low molecular weight proteins and polypeptides which can be specifically induced by trace metal ion (Dziegiel *et al.*, 2004). MTs play important roles in metal ion metabolism, detoxication of heavy metal, clearance of Reactive Oxygen Species (ROS) and then further influence body growth, development, reproduction, caducity, tumorigenesis, immunity, stress and the like physiological process (Carpene *et al.*, 2007). In 1957, Vajle and Margoshe firstly cloned MTs from a horse kidney, after those MTs were discovered in humans, other animals, plants and micro-organisms. The amino acids sequences of MTs protein have no aromatic amino acids and Histamine (His) but including rich Cysteine (Cys) (Romero-Isart and Vasak, 2002). Generally, isoelectric point (pI) of MTs is from 3.9-4.6. MTs usually take negatively charged under natural conditions. Due to the differences of different MTs pI, different types of MTs can stably bond with different metal ion (Vasak and Hasler, 2000).

MTs gene were located in mouse chromosome 8 in human chromosome 16. And the expression products from 17 closely related genes constitute different types of MTs in different combination ways (Tapiero and Tew, 2003). Different MT genes have similar structure including of

three exons, two introns and a typical polyadenylation sequence AATAAA in the 3'-Untranslated Region (UTR). The promoter region of mammalian MT genes contains several transcriptional regulatory elements such as Upstream Stimulating Factor-Antioxidant Response Element (USF/ARE), Metal Response element (MREs), Glucocorticoid Response Element (GREs), Major Late Transcription Factor-Antioxidant Response Element (MLTF/ARE), transcription factor Sp1 binding sites and Interleukin-6 (IL-6) responding element (Andrews, 2000).

Usually, mammalian MT proteins are single polypeptide chain containing 60-68 amino acid residues of which about 20 cysteine residues, the N-terminal acetylated methionine and C-terminal alanine. Tertiary structure of MT is an ellipsoid from two domains which were linked covalently between 31 lysine and 30 Lysine residues (Lys-Lys), other polypeptide sequences including seven Cys-Xaa-Cys tripeptide motif (Boulangier *et al.*, 1983).

Some studies on MTs genes from the human, rat, mouse, cow have been reported and the studies revealed MTs genes have different subtypes and pseudogenes. Up to now, the studies on sequence, structure and bioinformatics analysis of rabbit MT genes seldom been reported except for MT_{2a} , MT_3 , MT_4 from European hare, furthermore breeding rabbit's MT genes never been

cloned. Hence, the objective of study is to clone *MT* genes of Chinese-Belgium rabbit, a cultivated rabbit breed by introducing Belgium rabbit and then further analyzed coding sequence and structure of genes and evolutionary relationship with other species.

MATERIALS AND METHODS

Tissue samples collection: Five healthy and 70 days old Chinese-Belgium rabbits were from the farm of Sichuan Agricultural University. The fresh muscle, liver and brain tissues of rabbit were collected and stored in liquid nitrogen for total RNA extraction. The process of animal experiment obeyed the Act of Chinese Experimental Animal Management and Experimental Animal Management of Sichuan Agricultural University.

Total RNA extraction and preparation: About 50-100 mg tissue were homogenized in 1 mL Trizol (Takara, China) and processed for RNA isolation according to Trizol instructions.

The collected supernatant (500 µL) was precipitated with 500 µL of isopropanol, followed by washing with precooled 75% ethanol. The precipitated total RNA samples were dried and then dissolved in 50 or 100 µL of TE buffer (0.01 M Tris-HCl, pH 8.0, 1 mM EDTA). The concentration of total RNA samples were measured using a Gene Quant Pro RNA/DNA calculator (Amersham Pharmacia Biotech, UK) and then total RNA sample were treated with DNase I (Takara, China) in 10 µL reaction system (10 µg of total RNA, 1×DNase I buffer, 2 U of DNase I) by incubating at 37°C for 40 min on a Programmable Thermal Controller PTC-100 (MJ Co., USA) for removing genomic DNA.

cDNA synthesis and PCR amplification: The purified RNA samples were reverse-transcribed using Takara ExScript™ kit in 10 µL reaction system (5×ExScript™ Buffer: 2 µL; ExScript™ RTase (200 U µL): 0.25 µL; dNTP Mixture (10 mM): 0.5 µL; RNase Inhibitor (40 U µL): 0.25 µL; Oligo dT Primer (50 µM): 0.5 µL; Total RNA (500 ng): 1 µL; RNase Free of ddH₂O: 5.5 µL) on the PTC-100 (MJ Co.,

USA) by incubating 42°C for 15 min, followed 95°C for 2 min for inactivating reverse transcriptase. CDS of *MT*₁-*MT*₃ were respectively amplified using cDNA as template in a 25 µL PCR reaction system (2×Taq PCR Master Mix: 12.5 µL; cDNA (1 µg): 1 µL; upstream and downstream primers (10 µM) of: 1 µL; ddH₂O: 9.5 µL). All primers of *MT*₁-*MT*₃ were synthesized by Invitrogen Corporation. Primers of *MT*₁-*MT*₃ and PCR conditions were shown in Table 1. PCR products were detected with 1% agarose gel electrophoresis (Fig. 1).

PCR products cloning: PCR products were purified with TianGen DNA Recovering kit according to its manual instructions and then PCR products were respectively inserted pTA₂ vector (Invitrogen, USA) in 10 µL reaction system (1 L of 50 ng µL pTA₂ vector, 5 µL of 2×Ligation Buffer, 3 µL of PCR products, 1 µL of T4 DNA Ligase) by incubating at room temperature for 30 min, following 4°C for 12 h.

pTA₂-*MT*s vectors were transferred into competent *E. coli* DH5α cells (Invitrogen, USA) by heat shock (0°C for 30 min, 42°C for 60 sec for heat stressing finally 0°C for 3 min) and then *E. coli* DH5α cells were cultured in Luria-Bertani (LB) culture medium under 37°C for 1 h. Positive *E. coli* DH5α cells were screened by Amp/IPTG/X-Gal LB agarose culture plate, processed for positive *E. coli* DH5α cells screening according to the manual of pTA₂ vector.

The positive *E. coli* DH5α cells were cultured in LB medium including Amp at 37°C, overnight CDS of *MT*₁-*MT*₃ were amplified using the positive *E. coli* DH5α as template and then PCR products were electrophoresed on 1% agarose gel (Fig. 2).

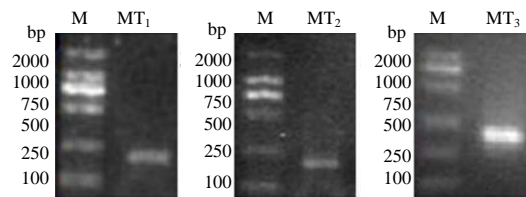


Fig. 1: PCR product of rabbit *MT*₁-*MT*₃ gene (M: Marker)

Table 1: Rabbit *MT*₁-*MT*₃ gene PCR amplification primer information and PCR reaction conditions

| Genes | Primer sequences (5-3) | Product size (bp) | PCR reaction conditions |
|------------------------|---|-------------------|---|
| <i>MT</i> ₁ | F: TCTGACCCCAACTGCTCCTGCGCCACAGGCA R: CAGTCAGGCGCAGCAGCTGCACTGTCCGATGC | 186 | 94°C/5 min-32 cycles*(94°C/50 sec-58°C/50 sec-72°C/1 min)-72°C/10 min |
| <i>MT</i> ₂ | F: ATGGAYCCCAACTGCTCCTG R: TCAGGCRCAGCAGCTGCACT | 186 | 94°C/5 min-32 cycles*(94°C/50 sec-59°C/50 sec-72°C/1 min)-72°C/10 min |
| <i>MT</i> ₃ | F: ATGGACCCTGAGRCCTGCCCTG R: TCACTGGCAGCAGCYGCACTTCT | 201 | 94°C/5 min-35 cycles*(94°C/30 sec-58°C/60 sec-72°C/1.5 min)-72°C/10 min |

Sequencing: Coding sequence of MT₁-MT₃ from positive *E. coli* DH5α were sequenced with ABI 3100 genetic analyzer (ABI Biosystem, USA) by Invitrogen Inc.

Bioinformatics analysis: Coding sequence of MT₁-MT₃ were edited with BioEdit software and proteins similarity was analyzed with MegAlin software. Due to sequences of MT₁ gene being the most abundant from different species researchers obtained sequence of MT₁ from some species in GenBank such as human, monkey, chimpanzee, orange utan, giant panda, dog, wolf, balaena mysticetus, cattle, yak, rhesus monkey, marmoset, pigs, chinchilla lanigera, rat and mouse and then the phylogenic tree was computed using MEGA4 software according to Neighbor-Joining method based on CDS of rabbit MT₁

and other known MT₁ sequence. Disulfide bonds and secondary structure of MT protein were predicted using a online tools (<http://expasy.org/tools>) (Cheng *et al.*, 2005).

RESULTS AND DISCUSSION

Coding sequences of rabbit MT₁-MT₃ gene: electrophoresis bands of PCR products of MT₁-MT₃ (Fig. 1) and PCR amplification of positive *E. coli* DH5α suggested that coden sequence of rabbit MT₁-MT₃ inserted pTA₂ vector. Coden sequences of rabbit MT₁-MT₃ genes were shown in Fig. 3, their sequences length was 186, 186 and 201 bp, respectively and coded 61, 61 and 66 amino acids, respectively. Base composition of CDS region of MT₁ gene is 22.04% of A base, 26.34% of G, 17.20% of T and 34.41% of C; base composition of CDS of MT₂ gene is 14.52% of A, 30.65% of G, 20.97% of T and 33.87% of C and base composition of CDS of MT₃ gene is 21.39% of A, 32.84% of G, 18.91% of T and 26.87% of C.

Characteristics of rabbit MTs mature protein: Mature protein of rabbit MT₁-MT₃ contain 20 Cysteine residues, of which form 7 Cys-Xaa-Cys tripeptide motif (Fig. 4). Isoelectric point (pI) of MT₁-MT₃, respectively is 5.12, 5.34 and 5.32. Secondary structure prediction shows, rabbit MTs mature protein mainly contain random coil and α helix (Fig. 4). MTs are cysteine-rich protein, rabbit MT₁-MT₃ protein have 20 Cysteine residues which form 10 disulfide bonds (Table 2).

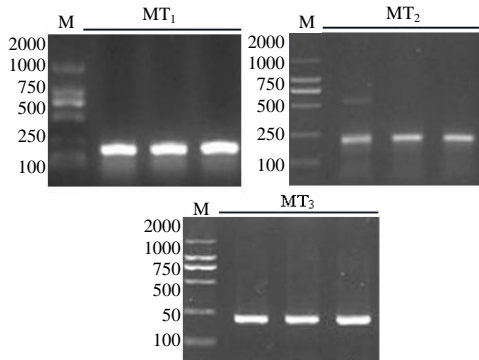


Fig. 2: Identification of MT₁-MT₃ recombinant plasmid (M: Marker)

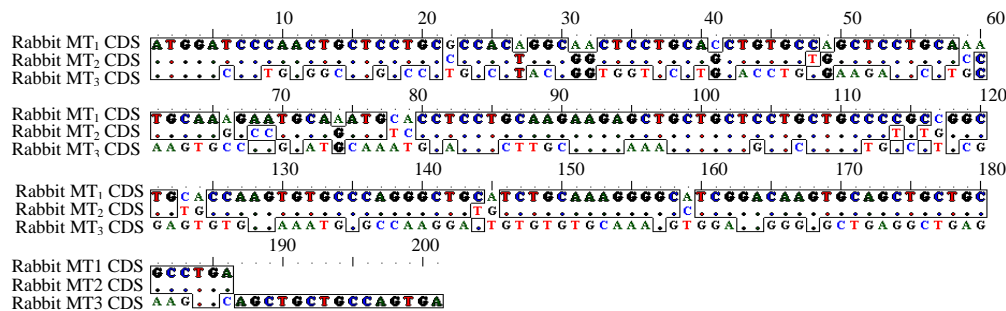


Fig. 3: The sequences of MT₁-MT₃ genes CDS region (means the same base as above)

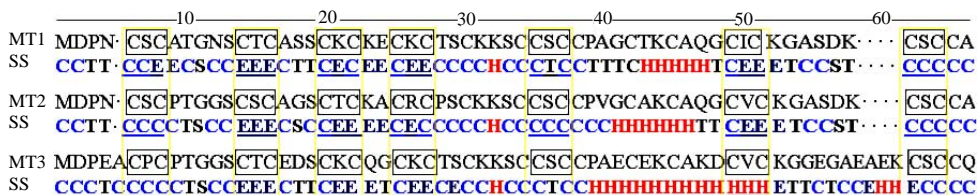


Fig. 4: Amino acid sequences and secondary structure of rabbit MT mature protein (Means gap; SS: Secondary structure; C: The rest; T: Turn; E: Extended strand; S: Bend; H: Alpha-helix. The amino acids in the box are typical Cys-Xaa-Cys structure in MTs)

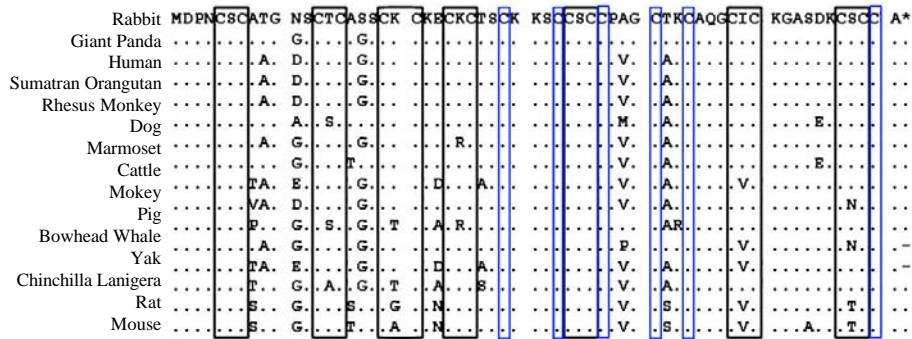


Fig. 5: Alignment analysis of MT mature protein from different species; . is the same amino acid, - is the gap; * is terminator. The animal names and the GenBank accession number of MTs are as follows: Chimpanzee, XM_526603; Monkey, V01532; Sumatran orangutan, NM_001133593; Human, M26637; Giant panda, XM_002912252; Dog, NM_001003149; Bowhead whale, AF022117; Cattle, NM_001075140; Yak, AY513745; Rhesus monkey, XM_002760985; Marmoset, XM_002760985; Pig, NM_001001266; Chinchilla lanigera, AY533220; Rat, NM_138826; Mouse, NM_013602)

Table 2: The prediction of disulfide bonds of cysteine pairs (the number indicate the cysteine position in amino acid sequences of MT mature protein)

| Bond index | MT ₁ | | MT ₂ | | MT ₃ | |
|------------|-----------------|-------|-----------------|-------|-----------------|-------|
| | Cys 1 | Cys 2 | Cys 1 | Cys 2 | Cys 1 | Cys 2 |
| 1 | 50 | 60 | 50 | 60 | 51 | 65 |
| 2 | 7 | 19 | 7 | 19 | 45 | 62 |
| 3 | 37 | 48 | 44 | 57 | 49 | 64 |
| 4 | 44 | 57 | 37 | 48 | 8 | 20 |
| 5 | 5 | 13 | 5 | 15 | 22 | 37 |
| 6 | 15 | 29 | 21 | 34 | 16 | 30 |
| 7 | 41 | 59 | 29 | 41 | 27 | 35 |
| 8 | 26 | 34 | 24 | 33 | 6 | 14 |
| 9 | 21 | 36 | 13 | 26 | 25 | 38 |
| 10 | 24 | 33 | 36 | 59 | 34 | 42 |

Similarity analysis of rabbit MTs: The similarity analysis of rabbit *MT₁-MT₃* genes were computed by MegAlin software and the results revealed that the similarity between *MT₁* and *MT₂* was 87.6%; the similarity between *MT₂* and *MT₃* was 78.0% and the similarity between *MT₃* and *MT₁* was 76.3%. It suggests they have a high similarity.

Alignment analysis of rabbit *MT₁* mature protein and other species: Alignment analysis of *MT₁* mature protein of rabbit and other species were shown in Fig. 5. The results showed that MTs mature protein from 17 species of animals such as human, monkey, chimpanzee, orange-utan, giant panda, dog, bowhead whale, cattle, yak, rabbit, rhesus monkey, marmoset, pigs, chinchilla lanigera, rat and mouse, all contain 20 cysteines of which from 7 typical Cys-Xaa-Cys motif.

Phylogenic analysis based on MTs: Just as the above, researchers BLAST and obtained *MT₁* or *MT₂* gene of human, monkey, gorilla, orangutan, giant panda, dog,

wolf, bowhead whale, cattle, yak, rabbit, rhesus monkey, apes, pig, chinchillas, rats and mouse in GenBank (*MT₁* gene of bowhead whale and chinchillas had not been submit to the GenBank so researchers constructed the phylogenic tree based on amino acid sequences of their *MT₂* mature protein and for the rest of animals researchers computed the phylogenic tree based on amino acid sequences of *MT₁* mature protein). The phylogenic tree was computed using MEGA4 software according to Neighbor-Joining method based on amino acid sequence of *MT₁* and *MT₂* mature protein (Fig. 6a). In GenBank, researchers got *MT₃* of human, chimpanzee, dog, pig, cattle, rabbit, hare, rhesus monkey, rat and mouse and the phylogenic tree was solely constructed based on amino acid sequences of *MT₃* mature protein (Fig. 6b). The overall topology of the neighbor-joining tree based on *MT₁-MT₂* showed that people, monkeys, gorillas and orangutans were clustered into one branch and then cattle and yak, rhesus monkey and marmoset, pigs and chinchillas lanigera, rat and mouse, giant panda, bowhead whales, rabbits, dogs were individually clustered into separate branches. The overall topology of the Neighbor-Joining tree based on *MT₃* showed that dogs, pigs and cattle, human and chimpanzee, rhesus monkey, rabbit and hare, mouse and rat were individually clustered into separate branches.

Coding sequences of rabbit *MT₁-MT₃* gene: In this study, Coding Sequences (CDS) of Chinese-Belgium rabbit *MT₁-MT₃* gene were cloned and sequenced. The length of *MT₁* CDS was 186 bp, encoded a single peptide of 61 amino acids, its content of G and C base (60.76%) was higher than the content of A and T base (39.24%). The length of *MT₂* CDS was 186 bp, encoded a single peptide

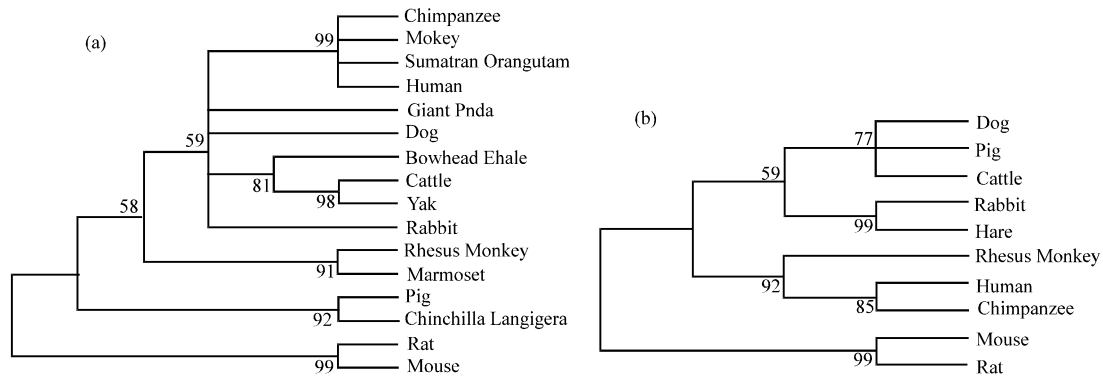


Fig. 6: The Neighbor-Joining phylogenetic tree based on amino acid sequence of MTs mature protein; a): Clustering analysis based on amino acid sequences of MT₁ or MT₂ mature protein; b): Clustering analysis based on amino acid sequences of MT₃ mature protein. The number at each branch indicate the percents of times a node was supported in 1000 bootstrap pseudoreplication by neighbor-joining. The animal names and the GenBank accession number of MTs are as follows: Chimpanzee, XM_526603; Monkey, V01532; Sumatran orangutan, NM_001133593; Human, M26637; Giant panda, XM_002912252; Dog, NM_001003149; Bowhead whale, AF022117; Cattle, NM_001075140; Yak, AY513745; Rhesus monkey, XM_002760985; Marmoset, XM_002760985; Pig, NM_001001266; Chinchilla lanigera, AY533220; Rat, NM_138826; Mouse, NM_013602; Hare, NM_001082220)

of 61 amino acids, its content of G and C base (64.51%) was higher than the content of A and T base (35.49%). The length of MT₃ CDS was 201 bp, encoded a single peptide of 66 amino acids, its content of G and C base (59.71%) was higher than the content of A and T base (40.29%). Nicolas (2003) thought the greater was the content of A and T base in gene, the higher was the frequency of nucleotide substitution, contrarily the greater was the content of G and C in gene, the lower was the frequency of nucleotide substitution. Therefore the gene in which the content of C and G was higher than that of A and T is more conserved in evolution. Nicolas's conclusion inferred coding regions of rabbit MT₁-MT₃ maintain a lower nucleotide substitution rate because content of their C and G is greater than that of A and T which MT₁-MT₃ was conserved in evolution. The results of nucleotide sequence and amino acid sequence alignment showed that *MT* gene and mature protein from rabbit, human, monkeys, gorillas, orangutans, giant pandas, dogs, bowhead whales, cattle, yak, rabbit, rhesus monkey, ape, pigs, chinchillas lanigera, rats and mice have high homology and all of their mature protein includes 20 cysteines residues and seven typical Cys-Xaa-Cys motif. These evidences suggest MT is much conserved.

Phylogenetic analysis of Mts: CDS of MTs from people, monkeys, gorillas and orangutans were similar and clustered into one branch and then cattle and yak, rhesus monkey and marmoset, pigs and chinchillas lanigera, rat and mouse, giant panda, bowhead whales, rabbits, dogs

were individually clustered into eight separate branches. There was a big difference between the evolution relationship which was revealed by phylogenetic tree and the phylogenies of these species. MT₁ and MT₂ was high similar but they belong to different subtype. If clustering analysis is based on MT₁ and MT₂, great divergence of MT₁ and MT₂ will impact the phylogenetic tree. Maybe it is possible reason that clustering analysis of MT₁ and MT₂ lead to divergence increasing. On the contrary, the evolution relationship which was revealed by the phylogenetic tree which was constructed based on CDS of MT₃ conformed with the phylogenies of these species (Nielsen, 1998).

The results suggested that *MT₁-MT₃* gene separated and formed different subtypes in the early stages of the evolution of species and then they individually evolve.

Structure and function of rabbit MT₁-MT₃ mature protein: Carpene *et al.* (2007) revealed isoelectric point (pI) of MTs protein generally ranged from 3.9-4.6. But in the studies, the pI of rabbit MT₁-MT₃ mature protein which were predicted with Isotopident Software (http://education.expasy.org/student_projects/isotopident/htdocs/) were 5.12, 5.34 and 5.32, respectively. Predicted pI of Mts protein were greater than the measured pI. Maybe pI of MTs protein have slightly changed after post-translational modification or binding metal ion. Secondary structure prediction showed that MT₃ was more one α -helix than MT₁ and MT₂. From the

point of view of alignment analysis of amino acid sequence, MT₃ is big different with MT₁ and MT₂. In human, rat and mouse, MT₁ and MT₂ have abundant variants and pseudogenes (Toriumi *et al.*, 2005; Scheede-Bergdahl *et al.*, 2005; Huang *et al.*, 2009) but MT₃ has almost no. On the one hand, it was possible reason that phylogenetic time of MT₃ was later than that of MT₁ and MT₂ and on the other hand MT₃ may have unique biological functions. MT₃ can inhibit *in vitro* growth of nerve cell and have higher ability to combine with copper than MT₁, MT₂ (Izzo *et al.*, 2010) which also show the uniqueness of its biological function. Bioinformatics prediction showed MTs contains 10 disulfide bonds. These disulfide bonds were functional domain and it is import for uptake and detoxification of metal ion.

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