Journal of Animal and Veterinary Advances 11 (21): 4034-4039, 2012
ISSN: 1680-5593

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 mental ion metabolism, detoxication of heavy mental, 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 high 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, China

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 mental ion metabolism, detoxication of heavy mental, clearance of Reactive Oxygen Species (ROS) and then further influence body growth, development, reproduction, caducity, tumorgensis, 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 mental 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 Responseelement (MREs), Glucocorticoid Response Element (GREs), Major Late Transcription Factor-Antioxidant Response Element (MLTF/ARE), transcription factor Sp1 binding sites and Interleukin-6 (IL-6) responsing 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 (Boulanger *et al*., 1983).

MATERIALS AND METHODS

Tissue samples collection: Five healthy and 70 days old Chinese-Begium rabbits were from the farm of Sichuan Agricultural University. The fresh muscle, liver and brain tissue 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.

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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 Oharmacia Biotec, 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^{-1}): 0.25 μL; dNTP Mixture (10 mM): 0.5 μL; RNase Inhibitor (40 U μL^{-1}): 0.25 μL; Oligo dT Primer (50 μM): 0.5 μL; Total RNA (500 ng): 1 μL; RNase Free of ddH_2O: 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_2 and MT_3 were respectively amplified using cDNA as template in 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; ddH_2O: 9.5 μL). All primers of MT_1, MT_2, MT_3 were synthesized by Invitrogen Corporation. Primers of MT_1, MT_2, 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 pTA_2 vector (Invitrogen, USA) in 10 μL reaction system (1 μL of 50 ng μL^{-1} pTA_2 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. pTA_2-MTs 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 or 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 pTA_2 vector. The positive *E. coli* DH5α cells were cultured in LB medium including Amp at 37°C, overnight CDS of MT_1, MT_2 and MT_3 were amplified using the positive *E. coli* DH5α as template and then PCR products were electrophoresed on 1% agarose gel (Fig. 2).

Sequencing: Coding sequence of MT_1, MT_2 and MT_3 from Postive *E. coli* DH5α were sequenced with ABI 3100 genetic analyzer (ABI Biosystem, USA) by Invitrogen Inc.

Bioinformatics analysis: Coding Sequence (CDS) of MT_1, MT_2 and MT_3 were edited with BioEdit Software and proteins similarity was analyzed with MegAlin Software. Due to sequences of *MT_1* gene being the most abundant from different species, researchers obtained sequence of

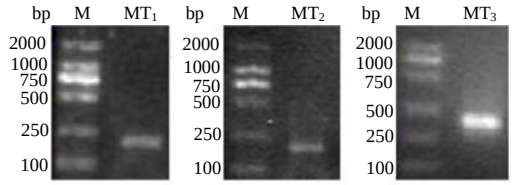

Fig. 1: PCR product of rabbit *MT_1*, *MT_2* and *MT_3* gene (M: Marker)

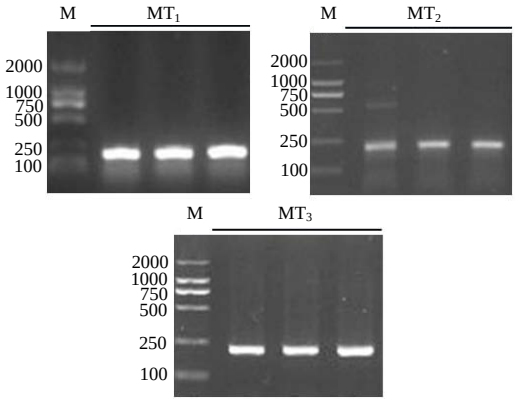

Fig. 2: Identification of MT_1, MT_2 and MT_3 recombinant plasmid (M: Marker)

Table 1: Rabbit *MT_1*, *MT_2* and *MT_3* gene PCR amplification primer information and PCR reaction conditions

Gene	Primer sequences (5'→3')	Product size (bp)	PCR reaction conditions
MT_1	F: TCTGACCCCAACTGCTCCTGCGCCACAGGCA R: CAGTCAGGCGCAGCAGCTGCACTTGTCCGATGC	186	94°C/5 min-32 cycles*(94°C/50 sec-58°C/50 sec-72°C/1 min)-72°C/10 min
MT_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
MT_3	F: ATGGACCCTGAGRCCTGCCCCTG 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

MT_1 from some species in GenBank such as human, monkey, chimpanzee, *Sumatran orangutan*, giant panda, dog, bowhead whale, cattle, yak, rhesus monkey, marmoset, pigs, *Chinchilla lanigera*, rat and mouse and then the phylogenic tree was computed using MEGA-4 Software according to Neighbor-Joining Method based on CDS of rabbit MT_1 and other known MT_1 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 *MT_1*, *MT_2* and *MT_3* gene: Electrophoresis bands of PCR products of MT_1, MT_2 and MT_3 (Fig. 1) and PCR amplification of postive *E. coli* DH5α suggested that coden sequence of rabbit MT_1, MT_2 and MT_3 inserted pTA_2 vector. Coden sequences of Rabbit *MT_1*, *MT_2* and *MT_3* 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 *MT_1* 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 *MT_2* 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 *MT_3* 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 MT_1, MT_2 and MT_3 contain 20 cysteine residues of which form 7 Cys-Xaa-Cys tripeptide motif (Fig. 4). Isoelectric point (pI) of MT_1, MT_2 and MT_3 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 MT_1, MT_2 and MT_3 protein have 20 cysteine residues which form 10 disulfide bonds (Table 2).

Similarity analysis of rabbit MTs: The similarity analysis of rabbit *MT_1*, *MT_2* and *MT_3* genes were computed by MegAlin Software and the results revealed that the similarity between MT_1 and MT_2 was 87.6%; the similarity

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	MT_1		MT_2		MT_3	
index	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

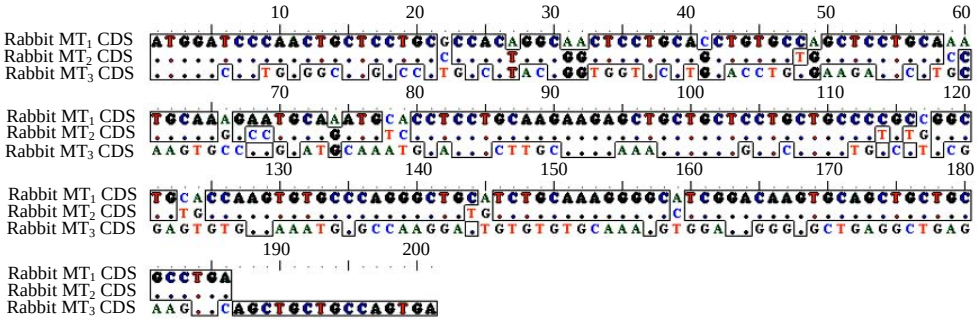

Fig. 3: The sequences of *MT_1*, *MT_2* and *MT_3* genes CDS region (“.” means the same base as earlier)

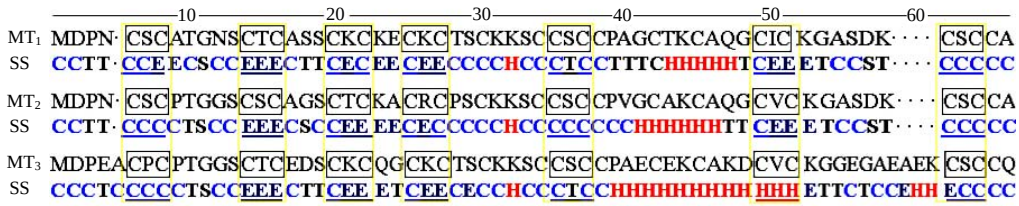

Fig. 4: Amino acid sequences and secondary structure of rabbit MT mature protein (“.” means gap; SS: Secondary Structure; C: The rest; T: Turn; E: Extended strand; S: Bend; H: Alpha-helix. The amino acids in the box are typical Cys-Xaa-Cys structure in MTs)

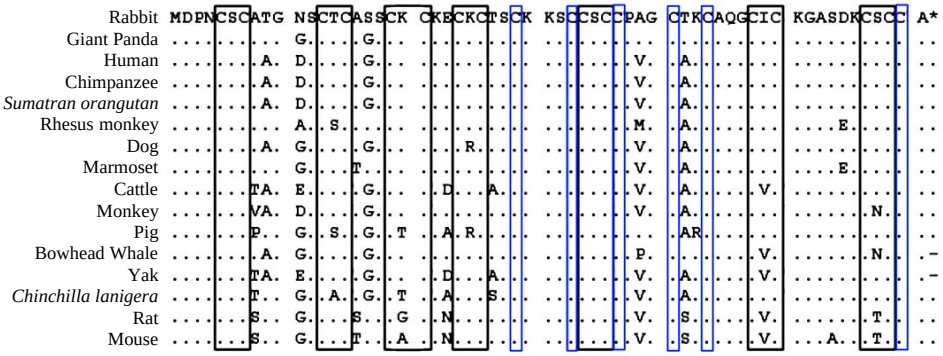

Fig. 5: Alignment analysis of MT mature protein from different species (“.” is the same amino acid; “-” is the gap; “*” is terminator. The animal names and the GenBank accession number of MTs are as follows: Chimpanzee: XM_526603; Monkey: V01532; *Sumatran orangutan*: NM_001133593; Human: M26637; Giant 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)

between MT_2 and MT_3 was 78.0% and the similarity between MT_3 and MT_1 was 76.3%. It suggests they have a high similarity.

Alignment analysis of rabbit MT_1 mature protein and other species: Alignment analysis of MT_1 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 earlier, researchers BLAST and obtained *MT_1* or *MT_2* 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 (*MT_1* gene of bowhead whale and chinchillas hadn’t been submit to the GenBank, so researchers constructed the phylogenic tree based on amino acid sequences of their MT_2 mature protein and for the rest of animals researchers computed the phylogenic tree based on amino acid sequences of MT_1 mature protein). The phylogenic tree was computed using MEGA4 Software according to Neighbor-Joining Method based on amino acid sequence of MT_1 and MT_2 mature protein (Fig. 6a). In GenBank, researchers got MT_3 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 MT_3 mature protein (Fig. 6b). The overall topology of the neighbor-joining tree based on MT_1-MT_2 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 MT_3 showed that dogs, pigs and cattle, human and chaimpanzee, rhesus monkey, rabbit and hare, mouse and rat were individually clustered into separate branches.

Coding sequences of rabbit *MT_1*, *MT_2* and *MT_3* gene: In this study, Coding Sequences (CDS) of Chinese-Belgium rabbit *MT_1*, *MT_2* and *MT_3* gene were cloned and sequenced. The length of MT_1 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 MT_2 CDS was 186 bp, encoded a single peptide 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 MT_3 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%). Galtier (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 (Galtier, 2003). Nicolas’s conclusion inferred coding regions of rabbit MT_1, MT_2 and MT_3-maintain a lower nucleotide substitution rate because content of their C and G is

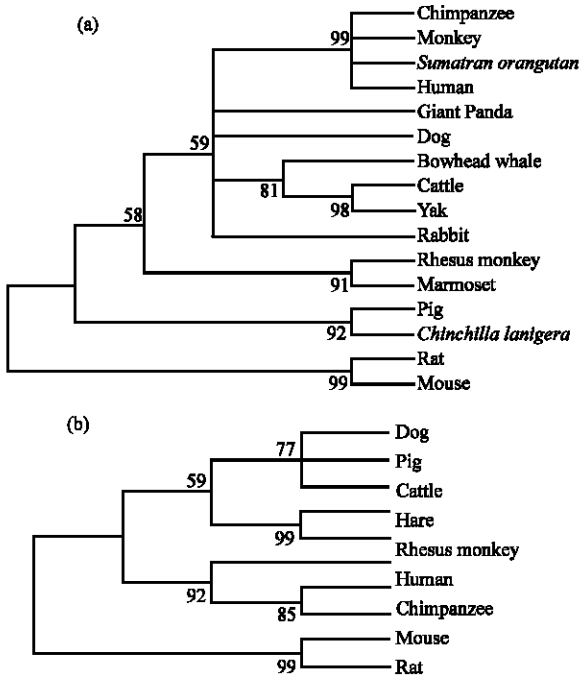

Fig. 6: The Neighbor-Joining phylogenic tree based on amino acid sequence of MTs mature protein; a) Clustering analysis based on amino acid sequences of MT_1 or MT_2 mature protein; b) Clustering analysis based on amino acid sequences of MT_3 mature protein. The number at each branch indicate the percents of times a node was supported in 1000 bootstrap pseudoreplication by neighbor-jioning. 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

greater than that of A and T which MT_1, MT_2 and MT_3 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. MT_1 and MT_2 was high similar but they belong to different subtype. If clustering analysis is based on MT_1 and MT_2, great divergence of MT_1 and MT_2 will impact the phylogenetic tree. Maybe it is possible reason that clustering analysis of MT_1 and MT_2 lead to divergence increasing. On the contrary, the evolution relationship which was revealed by the phylogenetic tree which was constructed based on CDS of MT_3 conformed with the phylogenies of these species (Nielsen, 1998). The results suggested that MT_1, MT_2 and MT_3 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 MT_1, MT_2 and MT_3 mature protein: Carpene *et al.* (2007) revealed isoelectric point (pI) of MTs protein generally ranged from 3.9-4.6 (Carpene *et al.*, 2007). But in the studies, the pI of rabbit MT_1, MT_2 and MT_3 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 mental ion. Secondary structure prediction showed that MT_3 was more one α-helix than MT_1 and MT_2 (Fig. 4). From the point of view of alignment analysis of amino acid sequence, MT_3 is big different with MT_1 and MT_2. In human, rat and mouse, MT_1 and MT_2 have abundant variants and pseudogenes (Toriumi *et al.*, 2005; Scheede-Bergdahl *et al.*, 2005; Huang *et al.*, 2009) but MT_3 has almost no. On the one hand, it was possible reason that phylogenetic time of MT_3 was later than that of MT_1 and MT_2 and on the other hand MT_3 may have unique biological functions. MT_3 can inhibit *in vitro* growth of nerve cell and have higher ability to combine with copper than MT_1, MT_2 (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.

CONCLUSION

Some studies on *MTs* genes form 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 this 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.

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