〈Review〉

Identification of the Gene Responsible for Chicken Muscular Dystrophy

Hirokazu Matsumoto, Shinji Sasazaki and Hideyuki Mannen[†]

Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

ABSTRACT By a series of positional cloning, we successfully narrowed down the *AM* candidate region to approximately 1.2 Mbp on GGA2q including 7 functional genes. Subsequently, we identified *WWP1* gene as the most likely *AM* candidate by sequence comparison. The amino acid sequence around the candidate mutation was highly conserved among tetrapods, suggesting that *WWP1* is the causative gene of chicken muscular dystrophy. Transfection of mutated *WWP1* gene into C₂C₁₂ myoblasts disrupted muscle differentiation process. The abnormal muscle differentiation is a characteristic of chicken muscular dystrophy, so we could demonstrate a part of phenotype of the disease. Furthermore, western blotting revealed that accumulation of caveolin-3 protein is limited in damaged muscle of muscular dystrophic chicken, suggesting caveolin-3 may be associated with the pathological change of the disease. We could conclude that *WWP1* gene is the responsible one for chicken muscular dystrophy from these results, but the mechanism leading the onset should be clarified in the future. The information will contribute to the study of chicken muscular dystrophy and the corresponding human dystrophies.

(Key words : chicken, muscular dystrophy, WWP1)

BACKGROUND

Muscular dystrophy is defined as the group of inherited diseases with progressive weakness and degeneration of skeletal muscle, and there exist plural genetical backgrounds (Partridge, 1991). Main symptoms of the muscular dystrophy include progressive muscle wasting, limited range of movement, walking difficulty, respiratory difficulty and so on. Some types of muscular dystrophies can also affect the heart, causing cardiomyopathy or arrhythmias (Nonaka, 1987a). The prognosis for the patients varies widely. Some patients of muscular dystrophies die in infancy, while others are affected only moderately and live into adulthood. In addition, the affected muscles vary according to the type of the disease. Many types of muscular dystrophies tend to occur in early childhood, though some forms can affect adults.

The most famous and earliest identified protein responsible for muscular dystrophies is dystrophin. Dystrophin is one of muscle proteins to compose of the linkage between sarcolemma and basal lamina. Mutations in *dystrophin* gene lead loss or lack of dystrophin protein from sarcolemma, which results in two types of muscular dystrophies, Duchenne and Becker

muscular dystrophy (Nonaka, 1987a). Duchenne muscular dystrophy (dystrophin-deficient) is among the most severe forms of muscular dystrophies; hence the function of dystrophin and its related proteins has been intensively studied for these years. However, numbers of muscular dystrophies and related diseases need elucidating the responsible genes or the mechanisms (Lisi and Cohn, 2007).

Animal models are very useful tools to examine a pathophysiologic role of certain molecule, to elucidate a mechanism of a disease and to promote a therapeutic research. From this perspective, various animal models for muscular dystrophies have been established and utilized. Representative models in Japan are *mdx* mouse (dystrophin-deficient), *grmd* dog (dystrophin-deficient), *dy* mouse (merosin-deficient), muscular dystrophic hamster (δ -sarcoglycan-deficient) and muscular dystrophic chicken (Nonaka, 1987b). These models are ones with abnormalities of dystrophin or other related proteins, except for muscular dystrophic chicken. Thanks to these animal models, the studies about muscular dystrophies lacking dystrophin and/or its related proteins have been intensively progressed, while those have not about other types of muscular dystrophies.

[†] To whom correspondence should be addressed : mannen@kobe-u.ac.jp

Chicken muscular dystrophy with abnormal muscle (*AM*) has been known since 1956 (Asmundson and Julian, 1956), and several strains for this disease have been established. One of them is New Hampshire 413-strain (NH-413). This strain was introduced from University of California, Davis to Japan in 1976 (Kondo et al., 1982). The phenotype of *AM* dystrophic chickens is relatively mild and they are able to breed, which makes it easy to obtain fertilized eggs with *AM* gene and *AM/AM* homozygous chicks. Therefore, this animal model is regarded as the suitable for a diachronic study to examine when and how muscular dystrophies start to affect (Nonaka, 1987b). The disease in NH-413 is transmitted codominantly by a single gene, and the phenotype is modified by other backcross genes (Asmundson and Julian, 1956; Asmundson et al., 1966; Kikuchi et al., 1981; Wagner and Peterson, 1970). The responsible gene has not been identified yet, and dystrophin and/or other known causative proteins seem not to be responsible gene for this disease (Saito et al., 2005).

Elucidating the responsible gene and the mechanism leading the onset of chicken muscular dystrophy would be useful to understand muscular dystrophies and related diseases whose causes are unknown. We attempted to identify the responsible gene of the disease by genetical and biochemical technique. In this article, we will give an outline of our findings.

GENETICAL ANALYSIS FOR CHICKEN MUSCULAR DYSTROPHY

Previously, the *AM* locus was mapped to chicken chromosome 2q (GGA2q) using a linkage map constructed with the Kobe University resource family (Lee et al., 2002). Chicken consensus map by Schmid et al. (2000) revealed that this region was syntenic to the human chromosome 8q11-24.3, indicating the genes on this region were candidates for the disease, although approximately 600 functional genes exist on the human chromosome 8q11-24.3. Subsequently, the candidate region was narrowed down to 3.6 Mbp range on GGA2q by linkage analysis with 361 backcross chickens (Yoshizawa et al., 2004). This region included 34 functional genes, but there did not exist any known causative genes in this region. Therefore, further analyses were required to identify the responsible gene.

In order to narrow down the *AM* candidate region further

by positional cloning, we newly established F₂ resource family for chicken muscular dystrophy. One F₂ chicken, generated by F₁ parents, has the information equivalent to that of 2 backcross individuals theoretically, since the recombination events occurred both in a F₁ male and a female can be analyzed with a F₂ chicken. Highly polymorphic microsatellites were focused on as DNA markers. By haplotype analysis with 487 F₂ chickens and 22 DNA markers, *AM* candidate region was reduced to approximately 1.2 Mbp on GGA2q (Fig. 1). This region included seven functional genes, *ATP6V0D2*, *LOC420211*, *WWP1*, *LOC420213*, *LOC420214*, *LOC428367* and *MMP16*, indicating them as the most likely *AM* candidates (Matsumoto et al., 2007). None of the candidates have been determined to be responsible genes for other muscular dystrophies so far.

Subsequently, to identify the responsible gene and mutation, we carried out sequence comparison and expression analysis in normal and dystrophic chickens. The sequence comparison of seven candidate genes revealed three synonymous mutations and one missense mutation specific to the phenotype of muscular dystrophy. Two of the synonymous mutations were detected in *LOC420214* (C660T and C1009A), and the other in *LOC428367* (C954T). The *WWP1* missense mutation (G1321A) caused amino acid substitution from arginine to glutamine, leading to the molecular alteration from a basic side chain to an uncharged polar side chain (Fig. 2). We focused further studies on this missense mutation since it was predicted to influence the function of the *WWP1* protein.

The single nucleotide polymorphism (SNP) in *WWP1* gene was genotyped in 111 normal birds from 16 strains with genetically varied backgrounds. None of the normal birds exhibited this type of substitution. The region of *WWP1* including the SNP specific to chicken muscular dystrophy was highly conserved among normal birds. Additional homology research among tetrapods was conducted to estimate amino acid conservation in this region. The sequences of chicken, human, chimpanzee, monkey, mouse, rat, dog and cattle were available in the NCBI database. To obtain further information from other tetrapod species, we sequenced partial *WWP1* gene of pigeon, snake, alligator, lizard, turtle and frog. The amino acid sequence around the region was highly conserved among these tetrapods (Fig. 3), suggesting that the region was important for the function of *WWP1* protein. These data suggested that

Fig. 1. The abnormal muscle (*AM*) candidate region and locations of genetic markers used in this study. The region between *IMPA1* and *CBFA2T1* is the *AM* candidate region by Yoshizawa et al. (2004). The upper figure shows the region of 16 informative gene markers on first step. The lower figure shows reduced *AM* candidate region by 11 informative gene markers on the second step. The genes underlined are the *AM* candidate genes.

Fig. 2. The domain structure of chicken *WWP1* and the site of missense mutation. Chicken *WWP1* protein is composed of 922 amino acids. Here are shown *WWP1* functional domains: C2 domain, three WW domains and HECT domain. C in HECT domain indicates an active cysteine residue. The arrow indicates the site of missense mutation detected in this study.

WWP1 is the causative gene of chicken muscular dystrophy (Matsumoto et al., 2008).

The expression analysis of seven candidate genes were performed with the pectoral muscle derived from both normal and dystrophic chicken. Pectoral muscle is the one selectively affected in chicken muscular dystrophy (Barnard et al., 1982). Therefore, we expected that the expression of the *AM* responsible gene might differ between normal and dystrophic chicken. The expression of *WWP1*, *LOC420213* and *LOC420214* could be detected by Northern blotting. To *ATP6V0D2*, *MMP16*

and *LOC420211*, RT-PCR analysis was applied because the expression of these genes was not detected by Northern blotting. The expression of *LOC428367* was not confirmed in either genotype, which meant that this gene was not expressed or expressed at extremely low level in the pectoral muscle. The expression of human *LOC428367* (*CNGB3*) is limited only in retina (Kohl et al., 2000), suggesting this gene is not involved in muscular dystrophic phenotype.

There was no difference in size of mRNA between affected and normal birds in any of these genes. *LOC420213* was highly expressed in affected individuals. Two bands were detected in *WWP1* and *LOC420214*. *WWP1* was expressed slightly higher in normal than in affected chickens, while a slightly higher level of expression was exhibited for *LOC420214* from affected chickens. Higher expression of *ATP6V0D2* and *MMP16* in dystrophic chickens was observed than in normal birds. The pectoral muscles from both genotypes expressed *LOC420211* to identical level. Since no extreme alteration of expression level and abnormal splicing were observed, the onset of chicken muscular dystrophy might not be attributed to aberrant expression of these genes (Matsumoto et al., 2008).

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Chicken N ⁽¹⁾	RNQLQGAMQQFNQRYLYSASMLSAENDPLGPLPPGWERRVDSNDRVYFVNHNKTQWED									
Chicken A	Q.....									
Human ⁽¹⁾	A.....Y.....K.....T.....									
Chimpanzee ⁽¹⁾	A.....Y.....K.....T.....									
Monkey ⁽¹⁾	A.....Y.....K.....T.....									
Mouse ⁽¹⁾	A.....Y.....K.....T.....									
Rat ⁽¹⁾	A.....Y.....K.....T.....									
Dog ⁽¹⁾	A.....Y.....K.....T.....									
Cattle ⁽¹⁾	AD.....A.....Y.....K.....T.....									
Pigeon										
Snake										
Alligator	T.....									
Lizard										
Turtle										
Frog	T.....T.....									

Fig. 3. Homology study of *WWP1* among tetrapods. Chicken N and A show amino acid sequences of normal and dystrophic chickens, respectively. R441Q *WWP1* mutation is specific to chicken muscular dystrophy, and the amino acid sequence on this region is highly conserved among tetrapods. Dots indicate the same amino acids with above sequences. Asterisk indicates the residue that the substitution was detected in dystrophic chicken. (1) Amino acid sequence published in NCBI.

The WW domain containing E3 ubiquitin protein ligase 1 (*WWP1*) is classified into a ubiquitin ligase (E3) which plays an important role in ubiquitin-proteasome pathway (UPP) to degrade unneeded or damaged proteins (Scheffner and Staub, 2007). In chicken, the *WWP1* expression has not been studied so far. Therefore, the mRNA expression of *WWP1* in various skeletal muscles and other tissues of normal and dystrophic chickens was analyzed to know the differences in the general expression pattern between these chickens. The mRNA expression of *WWP1* gene was detected by Northern blotting in various muscles and other tissues of normal and muscular dystrophic chickens (Fig. 4). Two bands were detected in all tissues examined, and revealed almost equally expression level in any muscles and tissues observed. Flasz et al. (2002) reported that there are plural splice variants of human *WWP1* gene, and our results indicated that chicken *WWP1* gene also has plural splice variants. The downward alteration of *WWP1* expression was observed commonly in almost all dystrophic muscles examined, but even in unaffected muscles the expression was lowered. Not only affected muscles but unaffected ones exhibited the same pattern (Fig. 5). Moreover, the expression was altered in liver and kidney, unaffected tissues. The result suggested that *WWP1* expression level lowered in dystrophic phenotype is not directly related to the cause of disease in chicken muscular dystrophy, whereas mutated *WWP1* does

Fig. 4. Expression of chicken *WWP1* in various tissues. A *WWP1* cDNA probe was used to detect *WWP1* mRNA transcripts by Northern blotting using blots containing 2 μ g of mRNAs from chicken muscles or various other tissues. *M. pectoralis superficialis* (PS), *M. tensor fascia lata* (TFL), *M. biceps femoris* (BF), *M. triceps surae* (TS), *M. peroneus longus* (PL), heart (H), brain (B), liver (L), kidney (K) and embryo (E) were analyzed. A doublet band is detected at variable levels in all tissues. “A” indicates mRNAs from dystrophic chickens. “N” indicates mRNAs from normal chickens. The numbers below the *GAPDH* bands represent the relative ratios of *WWP1*/*GAPDH*.

not function properly to cause the onset of chicken muscular dystrophy (Matsumoto et al., 2009).

FUNCTIONAL ANALYSIS TOWARD THE MOST LIKELY CANDIDATE GENE OF CHICKEN MUSCULAR DYSTROPHY

Though oncogenic character of *WWP1* is intensively studied (Chen and Matesic, 2007), the role of *WWP1* in muscle

Fig. 5. RT-PCR detection of *WWP1* gene and histological analysis for three representative muscle types. *M. pectoralis superficialis* (PS), *M. anterior latissimus dorsi* (ALD) and heart (H) expressed *WWP1* less in muscular dystrophic chicken, but only dystrophic PS was severely harmed. A) Expression of *WWP1* in PS, ALD and H was analyzed by RT-PCR method. PCR was performed for 35 or 40 cycles. B) The PS, ALD and H of affected and normal chickens were analyzed with HE staining. Vacuoles (arrows) and fatty infiltration (asterisk) are observed in PS of dystrophic chickens. It is also remarkable that, in dystrophic PS, many muscle fibers have many nuclei in cytoplasm and vary widely in size. These pathological features are not observed in ALD and H of dystrophic chicken. Scale bar = 120 μ m.

remains elucidated. The switching of adult phenotype in fast muscle is inhibited in chicken muscular dystrophy, resulting in the continued expression of slow twitch myosin heavy chain (MyHC) isoform in adult fast muscles (Bandman, 1985; Bandman and Bennett, 1988; Kaprielian et al., 1991; Tidymann et al., 1997). This inhibition is proposed to contribute to the onset of chicken muscular dystrophy. To investigate the effects of the overexpression and the expression of mutated *WWP1* gene on the *MyHC* genes expressions, we transfected the wild and mutated types of *WWP1* gene into C₂C₁₂ cells which are myoblasts derived from mice skeletal muscle. The expression of muscle-differentiation markers, *Myogenin* (*Myog*), *myogenic differentiation 1* (*MyoD*) and *MyHCs* (*MyHC Ia* and *MyHC Iib*), was analyzed by real-time PCR. *Myog* and *MyoD* were analyzed as the markers for early stages (Langlands et al., 1997) and *MyHCs* for later stages of muscle differentiation (Silberstein et al., 1986). *MyHCs* are divided into two classes, type I composed of fast twitch fibers and type II of slow twitch fibers (Larsson and Salviati, 1989).

Transfecting mouse *WWP1* gene into C₂C₁₂ mouse myoblasts resulted in the disturbed expressions of *MyHC* genes, though clear difference was not observed in the myotube morphology and the proliferation rate of myoblasts. The expression level of *MyHC Ia* gene was higher in wild type *WWP1*-introduced cells, but *MyHC Iib* gene expression was lower (Fig. 6), indicating that *WWP1* promotes to transform C₂C₁₂ cells into fast twitch characteristics. However, R436Q-transfected cells

persisted in the high expression of both fast *MyHC Ia* and slow *MyHC Iib* isoforms, suggesting retainment in slow and fast twitch isoforms characteristic (Matsumoto et al., 2010a). Furthermore, the *WWP1* suppression by RNAi technique reduced the proliferation rate of C₂C₁₂ myoblasts, while clear difference was not observed in the proliferation rate by the transfection of the mutated *WWP1* gene into the cells (Fig. 7). These data indicated that the mutation responsible for chicken muscular dystrophy does not eliminate the enzymatic activity and provides some new function for the gene (Matsumoto et al., 2010b). Taken together, the R436Q mutation in *WWP1* gene seems to inhibit the normal fiber type differentiation. The results suggest that *WWP1* plays an important role in myoblasts' differentiating process.

The *WWP1* is classified into a ubiquitin ligase to degrade unneeded or damaged proteins (Scheffner and Staub, 2007), suggesting that muscular dystrophic phenotype might be triggered by aberrant regulation of some *WWP1*'s substrates. From this perspective, we focused on caveolin-3 as a candidate protein interacting with *WWP1*, since an electron microscopic analysis revealed increase of caveolae in dystrophic chickens (Costello and Shafiq, 1979), and caveolin-3 is a main component of caveolae in muscle cells (Song et al., 1996). Gene-encoding caveolin-3 is responsible for limb-girdle muscular dystrophy type 1C (LGMD-1C) and other muscle diseases (Dowling et al., 2008). Caveolin-3 deficiency induces a muscular dystrophic phenotype (Galbiati et al., 2000a; Hagiwara

Fig. 6. Muscle-differentiation markers in *WWP1*-transfected (WT and R436Q) and empty vector-transfected (control) C_2C_{12} cells. After transfection of wild-type (WT) and mutated (R436Q) *WWP1* into C_2C_{12} cells, expression levels of muscle-differentiation markers (*Myog*, *MyoD*, *MyHC Ia* and *MyHC IIb*) in differentiating day 6 cells were analyzed by real-time PCR. The expression of *Myog* in R436Q group was significantly lower than other groups, while no significant difference was seen in the expression level of *MyoD* gene. However, the expression of fast *MyHC Ia* in *WWP1*-transfected cells was significantly higher than in control cells, while that of slow *MyHC IIb* was significantly lowered in *WWP1*-transfected cells compared to control cells. The R436Q-transfected cells retained the high expression of both fast *MyHC Ia* and slow *MyHC IIb* isoforms compared to control cells. Y-axis indicates relative expression level of each gene to the *GAPDH* gene expression. Bars indicate standard deviations. Different letters indicate significant differences ($p < 0.05$) among column graphs.

et al., 2000), while its overexpression is also harmful to muscle cells (Galbiati et al., 2000b).

Western blotting analysis revealed the higher expression of caveolin-3 protein in only affected muscle in dystrophic chickens (Fig. 8(A)), suggesting a close correlation between higher caveolin-3 expression and dystrophic phenotype. On the other hand, semi-quantitative RT-PCR analysis revealed that affected muscle did not present such higher caveolin-3 expression at the mRNA level (Fig. 8(B)). The amount of caveolin-3 protein might be controlled in a posttranslational fashion such as ubiquitination. Furthermore, the expression of other caveolae-

related proteins showed similar tendency as that of caveolin-3. These results suggest that the accumulation of caveolin-3 protein may be associated with the causative process of chicken muscular dystrophy (Matsumoto et al., 2010c). However, more detailed analysis is required in order to elucidate the relationship between *WWP1* and caveolin-3.

Abnormality in *WWP1* protein might lead to aberrant regulation of its substrates. Therefore, identification of *WWP1*'s substrates in muscle will greatly contribute to elucidation of the mechanism leading to the onset of chicken muscular dystrophy. Recently, some *WWP1*'s substrates have been iden-

Fig. 7. The effects of four WWP1-shRNA sequences on C₂C₁₂ cells. Four WWP1-shRNA sequences (shWWP1-O1-4) were prepared and their effects on the proliferation rate of C₂C₁₂ cells were observed. All cells with shWWP1-O1-4 vectors showed significant downward tendencies twenty-four hours after transfection (24 H) (shWWP1-O1: 89 ± 35 %, shWWP1-O2: 101 ± 22 %, shWWP1-O3: 83 ± 16 % and shWWP1-O4: 110 ± 22 %). Y-axis indicates relative proliferation rate based on the cell numbers of immediately after transfection (0 H). Proliferation rates were calculated as relative levels toward the cell numbers of immediately after transfection. Three individual samples were prepared for each calculation.

tified, but any of them are oncogenic proteins and might not be involved in the causative event of chicken muscular dystrophy (Chen and Matesic, 2007). To identify novel substrates, study with WWP1-specific antibody is inevitable. Unfortunately, any commercially available WWP1-specific antibody for chicken muscle sample must be developed for further study to understand the mechanism of chicken muscular dystrophy.

RESENT FINDINGS OF THE MOST LIKELY CANDIDATE GENE OF CHICKEN MUSCULAR DYSTROPHY

Aberrant glycosylation of α -dystroglycan in muscular dystrophic chicken was reported (Saito et al., 2005). α -dystroglycan is one of dystrophin-related proteins and shortage of its glycosylation causes muscular dystrophic phenotype (Dalkilic and Kunkel, 2003). In addition, certain mutations in glycosyltrans-

ferases result in muscular dystrophic phenotypes including Fukuyama type congenital muscular dystrophy, Walker-Warburg syndrome and Muscle-Eye-Brain disease (Nonaka, 1987a). Therefore, abnormality of α -dystroglycan's glycosylation was also expected to trigger the onset of chicken muscular dystrophy. However, there was not glycosyltransferases and their related proteins in the *AM* candidate region narrowed down by haplotype analysis and the most likely *AM* candidate gene identified by sequence comparison, *WWP1*, has not been reported to associate with glycosyltransferases so far. Some F-box type ubiquitin ligases, such as Fbx2 and Fbx2, are known as the ones that recognize sugar chain (Yoshida et al., 2002, 2003), while WWP1 is classified into HECT type ubiquitin ligase (Scheffner and Staub, 2007). HECT type ubiquitin ligases responsible for sugar chain regulation have not been reported so far. The possibility that WWP1 might control glycosylation of α -dystroglycan still remains, but short sugar chain of α -dystroglycan in muscular dystrophic chicken may be the secondary result of the pathological change and new

Fig. 8. Expression of caveolin-3 at protein and mRNA level. (A) Expression of caveolin-3 in *M. pectoralis superficialis* (PS), *M. anterior latissimus dorsi* (ALD) and heart (H) was analyzed by Western blotting. PS expressed higher amount of caveolin-3 protein in muscular dystrophic chickens (A), while the expression in ALD and H was undetectable as in normal chickens (N). (B) The caveolin-3 expression in PS was assessed at mRNA level. No drastic difference was observed between D and N.

explanation for this disease may be required.

Many HECT type ubiquitin ligases ubiquitinate membrane proteins (Chen and Matesic, 2007). Therefore, WWP1 may also catalyze some membrane protein in muscle. In fact, WWP1 can bind a membrane protein, β -dystroglycan which is one of dystrophin-related proteins, *in vitro* (Pirozzi et al., 1997). β -dystroglycan is normally expressed in chicken muscular dystrophy (Saito et al., 2005); hence, this interaction between WWP1 and β -dystroglycan is unlikely to relate with the pathological change of chicken muscular dystrophy. Identification of WWP1's new substrates, which may accumulate in muscle and harm muscle physiology, will be needed in order to elucidate the mechanism for mutated WWP1 to cause the muscular dystrophic phenotype.

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