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Expression Pattern of *WWP1* in Muscular Dystrophic and Normal Chickens

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The WW domain containing E3 ubiquitin protein ligase 1 (WWP1) is classified into one of ubiquitin ligases which play an important role in ubiquitin-proteasome pathway. Previously, we identified the *WWP1* gene as a candidate gene of chicken muscular dystrophy by linkage analysis and sequence comparison. However, the mechanism causing pathological changes and underlaying gene function remains elucidated. In the present study, we analyzed the *WWP1* gene expression in various muscles and tissues of normal chickens, and compared with those from muscular dystrophic chickens. Two mRNA isoforms were detected in all tissues examined and revealed almost equal expression level. The *WWP1* expression of dystrophic chickens was decreased in almost all skeletal muscles including unaffected muscles. These data indicate that there might not be a causal relationship between the alteration of *WWP1* expression level and the severity of muscular dystrophy.

Key words: chicken, expression analysis, fast twitch muscle fiber, muscular dystrophy, WWP1

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Introduction

The WW domain containing E3 ubiquitin protein ligase 1 (*WWP1*) is classified into an ubiquitin ligase (E3) which plays an important role in ubiquitin-proteasome pathway (UPP) to degrade unneeded or damaged proteins (Scheffner and Staub, 2007). E3 recognizes and catalyzes ubiquitin (Ub) conjugation to specific protein substrates (Liu, 2004). Comparative genome analysis reveals few genes encoding E1, tens of E2 encoding genes and hundreds of E3 encoding genes (Semple *et al.,* 2003).

The *WWP1* gene is classified into HECT (homologous to the E6-AP carboxyl terminus)-type E3 which possesses one C2 domain, multiple WW domains and one HECT domain (Pirozzi *et al.,* 1997; Flasza *et al.,* 2002). The C 2 domain binds to the cellular membranes in a Ca^{2+}-dependent manner (Plant *et al.,* 1997) and mediates interactions with other proteins (Plant *et al.,* 2000; von Poser *et al.,* 2000; Augustine, 2001). The WW domain has two conserved tryptophan residues and binds proline-rich region (Sudol *et al.,* 1985). HECT domain, similar to E2s structurally, has a cysteine residue as an active center that transfers the activated Ub from E2 onto first itself, and then onto its substrates (Jackson *et al.,* 2000).

The muscular dystrophies are the group of inherited diseases with progressive weakness and degeneration of skeletal muscle (Partridge, 1991). It is well known that abnormalities of muscle proteins linking sarcolemma and basal lamina lead to cause muscular dystrophies (Lisi and Cohn, 2007), but there are a number of muscular dystrophies and related diseases of which causes are still unknown. We identified *WWP1* gene as a candidate responsible for the chicken muscular dystrophy by the linkage analysis (Matsumoto *et al.,* 2007) and the sequence comparison between normal and dystrophic chickens (Matsumoto *et al.,* 2008). The R441Q missense mutation was found in *WWP1* gene to cause the phenotype of muscular dystrophy.

The WWP1s of human (Flasza *et al.,* 2002; Komuro *et al.,* 2004), mouse (Dallas *et al.,* 2006) and *C. elegans* (Huang *et al.,* 2000) were intensively studied and known

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that the *WWP1* gene is expressed ubiquitously, but strongly in liver, bone marrow, testis and skeletal muscles (Flasza *et al.,* 2002; Komuro *et al.,* 2004). In chicken, however, the *WWP1* expression has not been studied. The expression analysis of *WWP1* gene is important since it was reported that altered expression of known responsible gene could lead dystrophic phenotype (Smythe and Rando, 2006).

In this study, we analyzed the mRNA expression of *WWP1* in various skeletal muscles and other tissues of normal and dystrophic chickens by using Northern blotting and reverse transcription (RT)-PCR analysis to know the differences in the general expression pattern between them.

Materials and Methods

Chickens

A two-month-old dystrophic chicken (New Hampshire: NH-413) and an age-matched normal chicken (White Leghorn: WL-F) were used in this study. The New Hampshire (NH-413) strain is a homozygous dystrophic line introduced from University of California, Davis to Japan in 1976 (Kondo *et al.,* 1982). The disease in this strain is transmitted co-dominantly by a single gene, but the phenotype is modified by other background genes (Kikuchi *et al.,* 1981, 1987; Wilson *et al.,* 1979). The White Leghorn (WL-F) strain was established in 1970s, and maintained as closed colony in the Nippon Institute of Biological Science in Yamanashi, Japan. This study was carried out according to the guidelines of Animal Experimentation of Kobe University.

Expression analysis

For Northern blotting, mRNAs were isolated from *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 whole embryo (E) with PolyATtract mRNA Isolation kit (Promega, Madison, WI, USA). The 2μg of mRNAs, which were measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), were resolved by 1.2% agarose gel electrophoresis in the presence of formaldehyde and blotted on to Hybond-N+membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The mRNAs were visualized using digoxigenin (DIG) reagents, and kits for non-radioactive nucleic acid labeling and detection system (Roche Diagnostics, Basel, Switzerland) according to the procedure specified by the manufacturer excepting that the washing was done with 4×SCC 0.1% SDS at room temperature for 10 min, 4×SCC 0.1% SDS at 40℃ for 8 min and then 2×SCC 0.1% SDS at 40℃ for 8 min twice. The DIG-labeled DNA probes were prepared by PCR using DIG-dUTP using pectorals cDNA sample of a WL-F strain female as a template. The primers applied in this procedure were 5′-tccctcataaatgttgaaagcagaca-3′ (WWP1p-F), 5′-gtaataacccaaggtaatatgtaaac-3′ (WWP1 p-R) (NM_001012554), 5′-ccgtgtgccaacccccaatgt ctctg-3′ (GAPDHp-F) and 5′ -cagtttctatcagcctctcccacctc- 3′ (GAPDHp-R) (NM_204305). The PCR was done for 35 cycles at 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec (*WWP1*) and for 35 cycles at 94℃ for 30 sec, 63℃ for 30 sec, 72℃ for 30 sec (*GAPDH*) using TaKaRa Ex Taq® Hot Start Version (Takara Bio Inc., Tokyo, Japan). Quantitative analysis was performed with Scion Image (Scion Corporation, Frederick, MD, USA).

In order to analyze mRNA expression of *WWP1* gene in the PS, *M. anterior latissimus dorsi* (ALD) and H, RT-PCR method was applied. The concentration of cDNA derived from these muscles was calculated by NanoDrop ND-1000 (NanoDrop Technologies) and commeasurable cDNAs were used as template. The primers applied were 5′-attaggaagagccactgtagact-3′ (WWP1r-F) and 5′-tctgttgattgaggttctgctgt-3′ (WWP1r-R) (NM_001012554). The PCR was done for 35 and 40 cycles at 94℃ for 30 sec, 56℃ for 30 sec, 72℃ for 30 sec using TaKaRa Ex Taq® Hot Start Version (Takara Bio Inc.).

Histology

The PS, ALD and H were snap-frozen in liquid nitrogen-cooled isopentane and sectioned in a cryostat (Leica Microsystems Japan, Tokyo, Japan). The histopathology was made by hematoxylin-eosin staining (HE) method (Kikuchi *et al.,* 1981).

Results

The mRNA expression of *WWP1* gene was detected by Northern blotting in various muscles and other tissues of normal and muscular dystrophic chickens (Fig. 1). Two bands were detected in all tissues examined, and revealed almost equally expression level in any muscles and tissues observed.

In the PS, BF, TS, PL, B and K, *WWP1* gene was strongly expressed in normal than in dystrophic chickens (Fig. 1). *GAPDH* was used as an internal control of *WWP 1* expression analysis. In TFL, L and E, similar *WWP1* expression level was observed between two phenotypes (Fig. 1).

RT-PCR analysis indicated that *WWP1* gene was expressed in slow tonic ALD, not only in PS and H of both phenotypes (Fig. 2A). Figure 2B shows histopathological changes in PS, ALD and H of normal and dystrophic chickens. The pathological findings in dystrophic PS were characterized by the degenerating fibers with many vacuoles in cytoplasm, the fatty infiltration into connective tissue, and the proliferation of nuclei within muscle fibers with large variation in sizes. However, no such lesions were observed in ALD and H from age-matched dystrophic chickens (Fig. 2B).

Discussion

Northern blotting with *WWP1* specific probe detected two bands in all tissues and muscles examined (Fig. 1). Northern blot analysis of *WWP1* expression in human tissues also exhibited two bands (Mosser *et al.,* 1998), and RT-PCR analysis showed that human *WWP1* gene had at

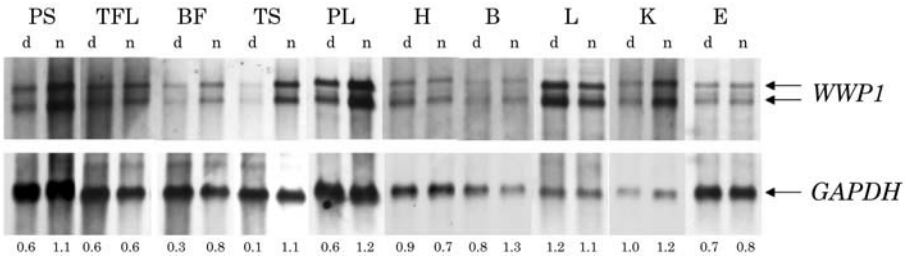

Fig. 1. **Expression of chicken *WWP1* in various tissues.**
A *WWP1* cDNA probe was used to detect *WWP1* mRNA transcripts by Northern blotting using blots containing 2μg of mRNAs from chicken muscles or various other tissues. *M. pectoralis superficialis* (PS), *M. tensor fascia lata* (TFL), *M. biceps 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. "d" indicates mRNAs from dystrophic chickens. "n" indicates mRNAs from normal chickens. The numbers below the *GAPDH* bands represent the relative ratios of *WWP1/GAPDH*.

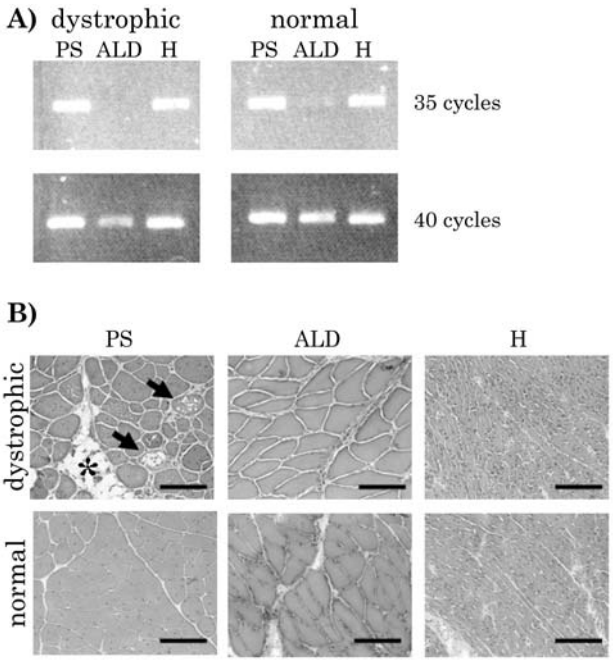

Fig. 2. **RT-PCR detection of *WWP1* gene and histological analysis for three representative muscle types.**
M. pectoralis superficialis (PS), *M. anterior latissimus dorsi* (ALD) and heart (H) expressed *WWP1* less in muscular dystrophic chicken, but only dystrophic PS was severely harmed. A) Expression 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 dystrophic (NH-413) and normal (WL-F) 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=120μm.

least six mRNA isoforms synthesized through the alternative splicing, two of which were strongly expressed and commonly observed in various tissues (Flasza *et al.,* 2002). The mRNA doublet bands of chicken *WWP1* by Northern blot analysis might be equivalent to two bands of human tissues, while a single band was observed by RT-PCR analysis in chicken (Fig. 2A), suggesting that the amplified region does not include alternative spliced site. Flasza *et al.* (2002) also mentioned that the relative ratio of these isoforms from human *WWP1* varied in a tissue-specific manner, but the doublet bands of chicken *WWP1* were expressed almost equally in all tissues examined.

The *WWP1* gene expression in *M. pectoralis superficialis* (PS) of dystrophic chicken was less than that of normal chicken (Fig. 1). The PS of chicken is a fast twitch muscle composed of two types of fast twitch fibers (αW and βW). TFL, BF, TS and PL muscles from wing and leg are mixed muscles co-existing fast twitch (αW and βW) with slow twitch fibers (βR) in a mosaic pattern (Ashmore and Doerr, 1971a), except that the ALD and *M. adductor magnus* are composed of slow tonic fibers (ST) innervated multiply (Ashmore *et al.,* 1978; Kikuchi *et al.,* 1986). In chicken muscular dystrophy, fast twitch fibers are initially and most severely affected, while slow twitch and slow tonic muscles persist relatively harmless throughout the life span (Ashmore and Doerr, 1971b; Barnard *et al.,* 1982). The *WWP1* expression in dystrophic BF, TS and PL showed a similar downward trend as observed in dystrophic PS (Fig. 1). These data indicate that there might not be a causal relationship between the alteration of *WWP1* expression level and the severity of muscular dystrophy, since not only affected muscles but unaffected ones exhibited the same pattern. Moreover, the alteration of *WWP1* expression level was observed in other unaffected tissues, such as B and K, which reinforces our hypothesis that the alteration of *WWP1* expression levels

does not link directly to the dystrophic phenotype (Fig. 1).

To assess the genetic influence of mutant *WWP1* upon chicken muscular dystrophy, we examined *WWP1* gene expression and histological changes in three distinct muscle types, PS as a fast twitch type, ALD as a slow tonic type, and H as a different type of muscle. RT-PCR was applied to this study since ALD was not enough quantity of mRNA for Northern blotting. The *WWP1* mRNA expression was confirmed in all muscles examined (Fig. 2 A).

Figure 2B shows HE stained sections of PS, ALD and H from normal and dystrophic chicken. The dystrophic PS was severely affected, while ALD and heart of dystrophic chicken remained relatively intact (Fig. 2B) as described in a previous study (Kikuchi *et al.,* 1981). The *WWP1* was expressed even in unaffected muscles and the downward alteration of *WWP1* expression was observed commonly in almost all dystrophic muscles examined (Figs. 1, 2). The observation suggests that the alteration of *WWP1* might not be the cause of the pathological change in chicken muscular dystrophy. Hence, the mutation identified previously (Matsumoto *et al.,* 2008) might play a crucial role in leading the onset of chicken muscular dystrophy. The detected mutation lay between WW domains, highly conserved region among tetrapods (Matsumoto *et al.,* 2008), which has been predicted as substrate binding region (Pirozzi *et al.,* 1997; Flasza *et al.,* 2002). This suggests that mutated *WWP1* could not recognize its substrates.

Many HECT-type E3s with WW domains including *WWP1* regulate membrane proteins (Chen and Matesic, 2007). Therefore, aberrant regulation of membrane protein may lead the onset of chicken muscular dystrophy. For example, *WWP1* could bind to β-dystroglycan, which is one of important muscle proteins consisting of membrane (Pirozzi *et al.,* 1997). Abnormal glycosylation of α-dystroglycan in chicken muscular dystrophy has been reported (Saito *et al.,* 2005). Furthermore, the fact that some E3s can recognize sugar chain (Yoshida *et al.,* 2002, 2003; Lederkremer and Gliskman, 2005) leads to the hypothesis that mutated *WWP1* might not be able to recognize the sugar chain of α-dystroglycan to regulate the glycosylated molecules, and that insufficiently glycosylated α-dystroglycan accumulates and causes the disease.

In the present study, we analyzed the mRNA expression of *WWP1* in various skeletal muscles and other tissues of normal and dystrophic chickens. The results suggest 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 not function normally to cause the onset of chicken muscular dystrophy.

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