Phospholemman, a major regulator of skeletal muscle Na^+/K^+-ATPase, is not mutated in probands with hypokalemic periodic paralysis

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Abstract. The pathogenesis of hypokalemic periodic paralysis (HypoPP) remains unclear. Though some mutations in skeletal muscle ion channels were revealed previously, the exact mechanism remains to be fully elucidated. Increased Na^+/K^+-ATPase activity in skeletal muscle is postulated to contribute to attacks of HypoPP. Before the link between Na^+/K^+-ATPase dysfunction and these ion channel mutations is established, mutations in Na^+/K^+-ATPase and their regulators are the first to be excluded. Phospholemman, which is a protein encoded by the FXYD domain-containing ion transport regulator 1 (FXYD1) gene, is predominantly expressed in skeletal muscle and is the major regulator of Na^+/K^+-ATPase. Therefore, the aim of the present study was to determine the genetic involvement of phospholemman in HypoPP development. Genomic DNA was extracted from the peripheral blood of five HypoPP probands with typical manifestations. The coding exons of *FXYD1*, exons 2-7, were polymerase chain reaction (PCR)-amplified and sequenced. No mutations were detected in *FXYD1* in any of the subjects studied. To conclude, mutations in phospholemman encoding genes may not be involved with HypoPP and the relationship between phospholemman and Na^+/K^+-ATPase dysfunction in attacks of HypoPP requires further study.

Introduction

Hypokalemic periodic paralysis (HypoPP) is a potentially fatal disease characterized by recurrent episodes of hypokalemia and muscle weakness (1). It is now recognized that HypoPP is caused by aberrant potassium transport from the extracellular to the intracellular space (2). According to pedigree analysis, the inheritance pattern of the disease is autosomal dominant and several mutations in genes encoding ion channels have been identified (2-4). However, the known HypoPP-related genetic mutations do not account for all cases and electrophysiological studies (5-7) of functional changes in ion channels with such mutations have not satisfactorily explained the pathophysiological mechanism of HypoPP.

The total body potassium store consists of ~98% intracellular potassium, of which ~80% are present in skeletal muscles. The intracellular K^+ content in skeletal muscles is ~46 times that in the extracellular compartment (8). Furthermore, potassium movement in and out of skeletal muscles has a crucial role in maintaining extracellular potassium homeostasis (2). Specific membrane transporters, including sodium pumps (also known as Na^+/K^+-ATPase) and inward-rectifier K^+ channels, are vital for the exchange of potassium between the extracellular and intracellular spaces in skeletal muscle (9). Sodium pumps are responsible for potassium uptake; thus, increased sodium pump activity is postulated to be a direct mechanism of the transfer-related hypokalemia in HypoPP. In considering the genetic basis of the disease, sodium pump dysfunction during attacks of HypoPP may be caused by mutations in genes that encode for the sodium pumps.

The sodium pump consists of a 110-kDa catalytic α subunit (α1 to α4), a 31-kDa auxiliary β subunit (β1 to β3) and a regulatory FXYD subunit. Previous findings demonstrated that no mutations or polymorphisms in the coding regions of five sodium pump genes were exhibited in patients with thyrotoxic periodic paralysis (10), indicating that mutations involving sodium pumps in HypoPP seem less likely. Therefore, mutations in the regulators of sodium pumps should be considered. The maintenance of Na^+ and K^+ gradients requires distinct states of sodium pumps, which may be regulated by FXYDs. FXYD proteins constitute an evolutionarily conserved family of small (6.5 to 17 kDa) membrane proteins with homologous structures (11). In mammals, there are seven known FXYD isoforms, FXYD1 to 7 (12), which have been demonstrated to be associated with structures and functions of sodium pumps (13). To meet the requirements of physiological functions in different tissues, the expression of FXYDs is

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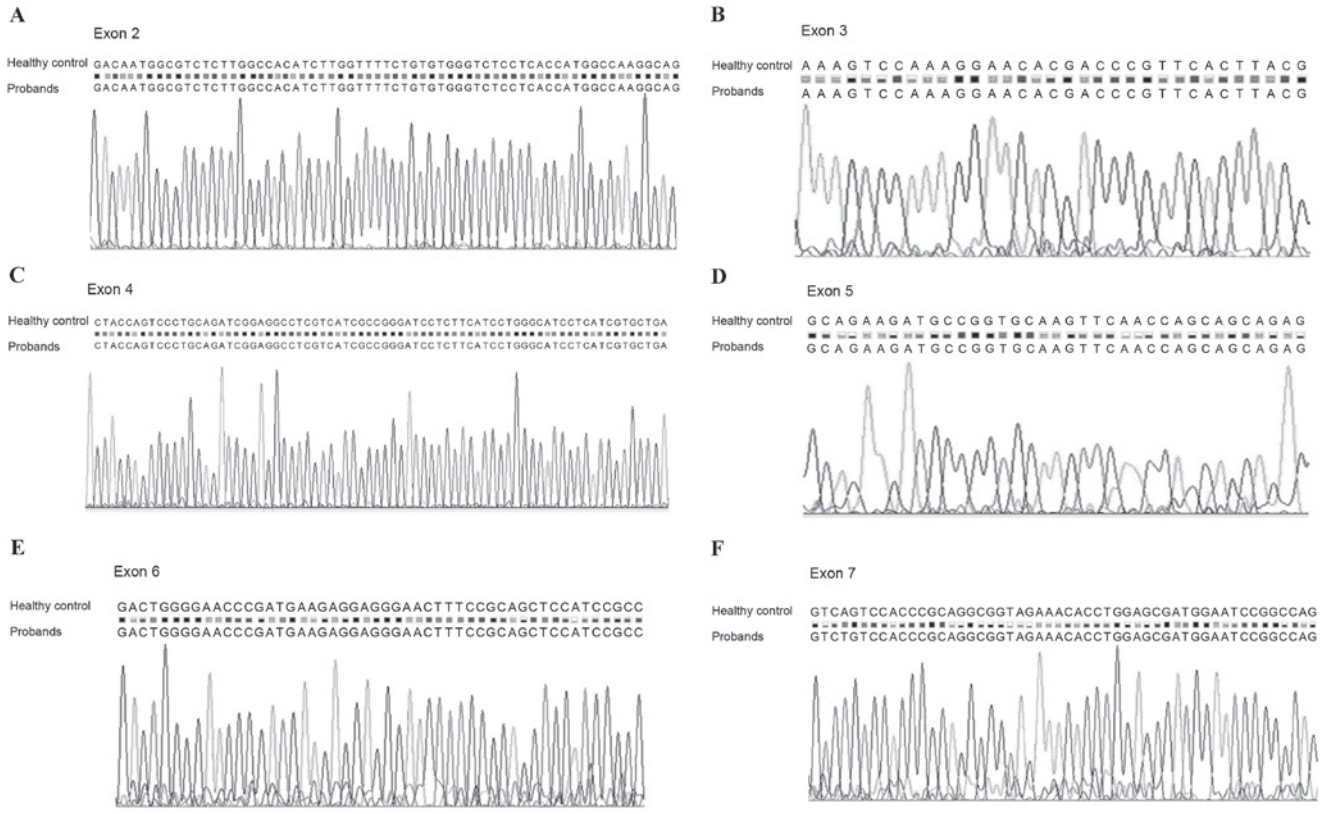

Figure 1. Sequence maps of *FXYD1* exons in the probands of HypoPP pedigrees. (A) Exon 2, (B) Exon 3, (C) Exon 4, (D) Exon 5, (E) Exon 6 and (F) Exon 7 of *FXYD1*. HypoPP, hypokalemic periodic paralysis; FXYD1, FXYD domain-containing ion transport regulator 1.

tissue-specific. Among the FXYD members, FXYD1 is predominantly expressed in the heart and skeletal muscle (14) and has been reported to exert an inhibitory effect on sodium pumps (15). Overexpression of FXYD1 in cardiac myocytes has been indicated to reduce sodium pump activity (16). Furthermore, a previous study indicated that sodium pump activity was significantly increased in isolated myocytes from *FXYD1* knockout mice (17). Alternative studies have demonstrated that the expression and phosphorylation of FXYD1 is essential for sodium pump regulation (18-21). Furthermore, phosphorylation of FXYD1 by protein kinase A and C has been revealed to increase sodium pump activity (22).

In the present study, the relationship between *FXYD1* and HypoPP susceptibility was investigated by screening for mutations in *FXYD1* in Chinese patients with HypoPP.

Materials and methods

Human subjects. Written informed consent was obtained from each patient. All experiments were approved by the Ethics Committee for Clinical Investigation of the PLA Navy General Hospital (Beijing, China) and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. A total of five probands (4 males and 1 female) from different HypoPP pedigrees (family trees not shown) were identified in the Department of Endocrinology, Chinese PLA Navy General Hospital and enrolled between December 2006 and December 2015. The age of the probands at the time of the study was 38±11 years old. The onset ages of these patients were from 15 to 45. All patients had suffered from typical and periodic attacks of limb paralysis with severe hypokalemia and were free from any other congenital disorder or inherited systemic disease. The diagnosis of HypoPP was made according to following aspects: Past histories of recurrent muscle weakness episodes; low muscle tone, and lowered or unapparent tendon reflexes during attacks of limb paralysis; hypokalemia during attacks of limb paralysis (concentration of serum potassium is often <3.0 mmol/l); and a possible characteristic electrocardiogram of hypokalemia during attacks of limb paralysis.

Genomic DNA preparation. Blood samples were collected from patients with confirmed HypoPP using EDTA-containing blood collection tubes and were stored at -80˚C. Genomic DNA was isolated from white blood cells using a WizardTM Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA).

Mutation analysis. Sequences of *FXYD1* were obtained online (www.ncbi.nlm.nih.gov/) and synthesized by polymerase chain reaction (PCR) using genomic DNA extracted from patients with confirmed HypoPP as the template. High fidelity Pyrobest DNA polymerase was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Exon 2 and exon 3 were PCR amplified with primers forward 5'-CACTTCTGT GATTCAGTCCC-3' and reverse 5'-GGGAAGGAGAATGAC AGAGA-3'; exon 4 and exon 5 with primers forward 5'-CTC TTGCCTGCGTCTGTCTC-3' and reverse 5'-GTTTTTTCC TCGCTTGGTCC-3'; and exon 6 and exon 7 with primers forward 5'-TCCCCCACCAACTTCACATC-3' and reverse 5'-CCTTTCCTTCCTCCCTCCTT-3'. PCR thermal cycling

conditions consisted of one cycle of denaturation at 95˚C for 3 min, followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 54˚C for 30 sec, and extension at 72˚C for 30 sec, followed by a final extension at 72˚C for 7 min. The amplified products were sequenced using ABI 377 DNA Sequencer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sequencing results were searched using BLAST (https://blast.ncbi.nlm.nih.gov/) against GeneBank data.

Results

The probands, four males and one female, were from five HypoPP pedigrees. The HypoPP pedigrees indicated an autosomal dominant mode of inheritance. No mutations were identified in any of the six *FXYD1* exons in these probands. Sequence maps of *FXYD1* exons from the probands are shown in Fig. 1.

Discussion

HypoPP has severe effects on the life quality of patients and may be potentially lethal. Although the exact mechanism remains unclear, attacks are attributable to defects in extracellular potassium homeostasis (2). It is well known that the majority of potassium in the body is intracellular and, as the largest pool of K^+ in the body, skeletal muscle tissue is important for buffering and balancing the concentration of serum potassium. The ionic concentration gradient of Na^+ and K^+ across the plasma membrane is established and maintained by sodium pumps. A rapid increase in serum potassium may be quickly corrected by sodium pumps that are present in skeletal muscles. Accordingly, it may be speculated that transfer-related hypokalemia in HypoPP is accompanied by an unusual increase in sodium pump activity. Family heritage results suggest a vital role of genetic factors in the pathogenesis of HypoPP (23). However, to date, no mutations in the genes encoding for sodium pumps have been identified. Numerous mutations in voltage-gated calcium channel, sodium channel and potassium channel genes have been discovered in HypoPP pedigrees and mouse models exhibiting these mutations have been established (24-26). However, studies investigating the electrophysiological changes in these ion channels have not revealed the underlying mechanism for the attacks of the disease (5-7).

Sodium pump dysfunction may occur at different levels, including changes in gene expression, pump movement to or from the plasma membrane or tissue-specific (27) changes in intrinsic properties via FXYDs (12,21). Among these possibilities, mutations in the sodium pumps and their regulatory FXYD subunit are the first that need to be excluded. Since no association was indicated between sodium pumps encoding genes and HypoPP in a previous study (10), investigating possible mutations in the sodium pump regulator was the predominant focus in the present study.

FXYD1 is highly expressed in skeletal muscle and has been demonstrated to have an important influence on sodium pump function (28-30). Studying the relationship between FXYD1 and HypoPP may yield an improved understanding of the mechanism of the disease. In the present study, mutation screening of HypoPP probands failed to detect any mutations in *FXYD1*. Our results, together with those of Kung *et al* (10), who detected no HypoPP mutations in the α or β subunit of the sodium pump, suggests that a constitutive abnormality in the sodium pump is not a primary cause of HypoPP. This raises the possibility that the level of expression or the phosphorylation of FXYD1 or of α or β sodium pump subunits may contribute to sodium pump dysfunction. Furthermore, the intracellular cascade of signaling pathways may have a role in the entire process.

In conclusion, the present study explored whether mutations in the gene encoding the sodium pump regulator, phospholemman, are present in HypoPP. The present findings demonstrated an absence of *FXYD1* mutations in the HypoPP probands. A major implication of the present study is that the dysfunction of sodium pumps cannot be attributable to gene mutations in *FXYD1*. Consequently, changes in signaling pathways related to sodium pumps or their regulators may be involved in HypoPP. Establishment of a link between altered pump function and defective FXYD1 regulation in HypoPP attacks requires further investigation.

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