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Global gene profiling of VCP-associated inclusion body myopathy

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Abstract

Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) is an autosomal dominant disorder caused by mutations in the Valosin Containing Protein (VCP) gene on chromosome 9p12-13. Patients demonstrate limb girdle muscle weakness, which eventually progresses to involve respiratory muscles, and death from respiratory and cardiac failure. This is the first investigation to analyze key molecular mediators and signaling cascades in skeletal muscle causing myopathy by global gene microarray in hopes of understanding the dysregulated genes and molecular mechanisms underlying IBMPFD and the hope of finding novel therapeutic targets. We determined expression profiles using Human Genome Array microarray technology in *Vastus lateralis* muscles from patients and their first degree relatives. We analyzed gene annotations by DAVID and identified differentially dysregulated genes with roles in several novel biological pathways, including regulation of actin cytoskeleton, ErbB signaling, cancer, in addition to regulation of autophagy, and lysosomal signaling, known disrupted pathways in VCP disease. In this report, we present data from the first global microarray analyzing IBMPFD patient muscles and elucidating dysregulated pathways to further understand the pathogenesis of the disease and discover potential therapeutics.

Keywords

Inclusion Body Myopathy with Paget Disease of Bone and Frontotemporal Dementia (IBMPFD); Valosin-containing protein (VCP); global microarray; signaling intermediates and cascades; actin cytoskeleton; autophagy; lysosome; growth factors; FoxO transcription factor

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Introduction

IBMPFD (Inclusion Body Myopathy associated with Paget's disease and Frontotemporal Dementia, OMIM 167320) is caused by mutations in the VCP gene[1], and is clinically characterized by adult onset of progressive proximal muscle weakness, Paget disease of bone, frontotemporal dementia[2] and clinical evidence of motor neuron disease in approximately 10% of individuals[3]. Clinical findings include muscle weakness, normal to mildly elevated serum creatinine kinase, electromyogram (EMG) findings of myopathic or mixed myopathic/neuropathic changes and typical skeletal muscle histology findings of rimmed vacuoles and inclusions stained with ubiquitin and TDP-43[4]. Large focal regions of non-specific myopathic changes are a common feature observed in some muscle biopsies, but rimmed vacuoles and inclusion bodies are more typical [2, 5-6]. TAR DNA-Binding Protein-43 (TDP-43), and ubiquitinated inclusions were most prominent in the myonuclei and the sarcoplasm[4].

Paget disease of bone (PDB) present in half the patients typically begins in the thirties and is caused by excessive osteoclastic activity and increased bone turnover and susceptibility to deformities and fractures[7]. The diagnosis of PDB is based on elevated serum alkaline phosphatase (ALP), and typical skeletal radiographic findings of coarse trabeculation, cortical thickening and spotty sclerosis typically in the skull, pelvis, spine, hip and scapulae[8] or hot spots on radionuclide scans[5].

Frontotemporal dementia (FTD) is seen at an average age of onset in the mid fifties. Clinical findings include altered judgment and planning, personality change with loss of social graces, and altered language and speech with relative preservation of memory[9]. Individuals die from progressive muscle weakness and cardiac and respiratory failure typically in their 50s to 60s, the clinical course being more rapid in the presence of central nervous system degeneration[2, 5, 11].

Valosin Containing Protein (VCP) is the gene responsible for IBMPFD, a progressive fatal genetic disorder [1]. Functional domains and disease mutations in VCP were found to cluster primarily in the ubiquitin binding domain, thus potentially defining this domain as having a critical role in bone cell activity, skeletal muscle, and brain function (reviewed in [3]). Inter- and intra-familial variations observed amongst patients make genotype-phenotype correlations quite difficult. VCP is a ubiquitously and highly expressed member of the type II AAA+ ATPase family and is involved in several cellular processes including post-mitotic nuclear envelope reformation and Golgi reassembly, cell cycle progression, endoplasmic reticulum-associated degradation of defective proteins and DNA damage repair[12].

Examination of patients' primary myoblasts demonstrate that defective cell fusion processes and terminal differentiation to myotubes from myoblasts in IBMPFD patients is abnormal [13], resulting in a paucity of normal differentiated replacement fibers. Vacuoles are identified as Light Chain 3 (LC3)-positive autophagosomes implicating autophagy in the pathogenesis of the disease [13]. Additionally, disease mutations were observed to interfere with the binding activities of interacting proteins [14]. At the tissue level, mutations in the VCP gene primarily affect muscle, brain and bone tissues, whereas many other tissues seem to be unaffected [13]. It is unknown why VCP function is indispensable for these specific tissues. Current theories suggest VCP disease mutations may cause disturbances in common signaling pathways [15], including autophagy [16], lysosomal pathway [17-18], ubiquitin-proteasomal system [12, 19], protein trafficking, ErbB signaling, activation and failure of inhibition of apoptotic pathways [20], and Erk1/Erk2 MAPK signaling [21].

DNA microarray technology is a powerful tool, thereby enabling the classification of dysregulated genes on a genome-wide scale by simultaneously monitoring the expression of

thousands of genes in human samples [22]. Gene expression comparisons in Duchenne Muscular Dystrophy (DMD)[23,24] and inflammatory myopathies including, Inclusion Body Myositis (IBM), polymyositis (PM), dermatomyositis (DM) and other degenerative muscle diseases[25-26] has yielded clinically and genetically significant information. We present data here from the first global microarray from muscles from patient with VCP mutations versus their first degree unaffected and non mutation carrying relatives. We have elucidated the dysregulated signaling pathways and their intermediates. Further analysis of the molecular mechanisms will provide unique insights into the pathogenesis of IBMPFD and ultimately the hope of discovering potential therapeutic targets.

Methods

Human Subject Approval

This study was approved by Institutional Review Boards at University of Lexington, KY and at University of California, Irvine. Informed consent was obtained from all subjects.

Patients and Healthy Controls

Muscle from 10 individuals (7 affected, 3 unaffected first degree relatives) collected after informed consent for the muscle biopsy was obtained was utilized for these studies. The clinical characteristics of these individuals are provided in Table 1. The biopsy site was shaved and cleaned with an antiseptic (Betadine), and then anesthetized with 2-3 ml of Lidocaine under the skin and into the covering of the muscle. A 1-cm incision was made through the skin and muscle fascia using a sterilized disposable scalpel blade. Subjects were asked to relax their leg muscle while a Popper biopsy needle was inserted into the *Vastus lateralis* muscle and 2-3 small pieces of muscle (approximately 100 mg each) were excised. The muscle biopsy samples were frozen immediately in isopentane cooled in liquid-nitrogen and stored at −80°C until processing for these studies.

Microarray Expression Profiling

RNA samples from patient and controls were analyzed with GeneChip Human Genome U133 Plus 2.0 Array-technology (Affymetrix, Santa Clara, CA). An aliquot of 5 μg of total high-quality RNA from each sample was used to generate cDNA containing an initiation site for T7 RNA polymerase (Super Choice system, Invitrogen). Double-stranded cDNA was purified by Gene Chip Sample Cleanup Module (Affymetrix) and 1 μg of cDNA was subjected to an *in vitro* transcription reaction using biotinylated UTP and CTP (Enzo Bioarray, New York, NY). An aliquot of 20 μg of biotinylated cRNA was fragmented in 1x fragmentation buffer at 94°C for 35 min, of which 1 μl was analyzed on 1% agarose gel, after which 15 μg was hybridized to Affymetrix Gene-Chips HG U133 Plus2 (Affymetrix, Santa Clara, CA) for 16 hours. Hybridization, post-hybridization washes, staining and array scanning was performed in the Affymetrix Gene-Chip System following the manufacturer's instructions.

Gene Annotation

The results were analyzed using GeneSpring GX 10.0.2 Software (Agilent Technologies, Inc). All raw signal values were normalized using Probe Logarithmic Intensity Error algorithm (PLIER), and filtered on Expression (20.0 - 100.0th) Percentile. Only probe sets that had a present or marginal flag in at least 100% of values in any one out of the two conditions were selected for further analysis. Overall, 41,328 out of 54,675 probe sets represented on the array met these criteria. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [27] and are accessible through GEO Series accession number GSE30806

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30806. *T*-test unpaired unequal variance (Welch) [Affected] Vs [Controls] was first applied to each probe set and only probe sets with P-value<0.1, 0.01 and fold change>1.2 were included in the final results.

The final list of significantly changed probe sets was then additionally analyzed using the functional annotation tools provided by DAVID- Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov) [28-29] in order to classify the genes into pathways using the KEGG and BIOCARTA databases. Only pathways with EASE score ≤0.05 are presented in this analysis. EASE score is a modified Fisher Exact p-value in DAVID system used for gene-enrichment analysis. EASE score P-Value = 0 represents perfect enrichment. P-Value 0.05 is considered as gene-enrichment in a specific annotation category. (http://david.abcc.ncifcrf.gov/helps/functional_annotation.html#summary).

Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT qPCR)

Gene expression analyses were performed with the Real-Time system to simultaneously quantify both rare and abundant genes in the same sample. The genes of interest used for RT qPCR are listed in Table 4. A two-step approach was used in which the initial reverse transcription was followed by RT qPCR amplication. Standard TaqMan two-step thermocycling conditions with the LightCycler 480 were used: 1 cycle at 95°C for 10 min, 45 cycles of 15 seconds at 95°C, 1 minute at 60°C, with a PCR volume of 10 μl as per manufacturer's instructions (Roche Applied Science, Indianapolis, IN). All samples were run in duplicate with both test primer sets and the control genes glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) for mRNAs. These genes were used to control for differences in the amount of target material. The Relative Quantification ΔΔCt approach was used for the data analysis where the Threshold Cycle (Ct) reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. The Ct value assigned to a particular well, thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated, in that well, to be at a statistically significant point above the baseline. Fold changes were calculated by normalizing the test crossing thresholds Ct with the amplified controls Ct as a part of the Comparative CT method, which uses arithmetic formula for relative quantitation. The amount of target, normalized to an endogenous control is given by: $2^{-\Delta\Delta CT}$.

Results

Pathway analysis overview

In the present study, we performed global microarray platform analysis of muscle biopsies from seven individuals carrying VCP mutations from IBMPFD families and their three first degree non mutation carrying relatives to better understand the pathogenesis of the disease and elucidate the signaling mechanisms involved. Stringent analysis criteria were utilized to evaluate the data. Gene expression of up- and down-regulated gene lists was compared using an Affymetrix gene array representing more than 5,000 transcripts.

We characterized the genes that were differentially expressed in the patients' muscle using gene pathways. 1868 ($p<0.01$) and 261 ($p<0.001$) probe sets were differentially expressed in the patients' muscle. These genes have been analyzed and categorized into specific pathways (Tables 3 and supplemental Table 5). We found 5 KEGG pathways and 3 BIOCARTA pathways that were enriched with genes that expressed differently in the patients' muscle (Ease score ≤0.05; Table 2). Many of the pathways identified were involved in proliferation, differentiation, and protein degradation processes, including: Regulation of Actin Cytoskeleton, ErbB signaling pathway, Cancer, Regulation of Autophagy, and Lysosomal Pathway (Table 3). Additional analysis using BIOCARTA

revealed disrupted mechanisms of Protein Import into the Nucleus, Agrin in Postsynaptic Differentiation, and Erk1/Erk2 MAPK signaling pathway (*provided as* supplemental Table 5). Table 3 lists the differentially expressed genes identified in IBMPFD by microarray analyses ($p<0.01$), gene symbols, AFFY ID, and fold change values.

To identify potential pathophysiological and genetic relations between IBMPFD and others muscular dystrophies, we also compared IBMPFD gene expression profiles with 63 existing profiles of 6 groups of known and unknown muscular dystrophies and Amyotrophic Lateral Sclerosis (ALS) samples from Dr. Eric Hoffman's laboratory (Washington, DC).

Novel Dysregulated pathways identified by KEGG analysis

Regulation of actin cytoskeleton

A significant number of human diseases are attributable to mutations in genes encoding proteins expressed in skeletal muscles. In this study, microarray analysis results showed a dysregulation of the actin cytoskeleton pathway including both downregulation and upregulation of proteins involved in this axis (Table 3). The cytoskeleton spans the cytoplasm, forms a structural link between molecules involved in cell communication and gene expression and also provides the framework for nearly all cellular processes. The cytoskeleton consists of hundreds of proteins involved in multiple structural and dynamic processes including maintenance of cell shape, cell replication, cell movement, cell signaling, cell differentiation and apoptosis [30-31]. Since the cytoskeleton is involved in various cellular processes, aberrations in these proteins lead to many pathological phenotypes.

Downregulated genes from our microarray analyses included genes modulating the activity of Rho GTP-binding proteins in cytoskeletal intracellular signaling networks which stimulate the exchange of GDP for GTP including Rho guanine nucleotide exchange factor (GEF) 12, T-cell lymphoma invasion and metastasis 1 and 2 (TIAM1 and 2), modulates activity of Rho-like proteins and connects signals to cytoskeletal events; the alpha actinin 1 (ACTN1), a cytoskeletal protein involved in multiple roles in cells.

Upregulated genes in the actin cytoskeleton pathway included PTK2 protein tyrosine kinase 2 (PTK2), involved in cell motility, cell proliferation, and apoptosis; actin related protein 2/3 complex (ARPC5L), plays a role in the regulation of actin polymerization and mediates formation of actin networks; cofilin 1 (CFL1), controls actin polymerization; cytoplasmic FMR1 interacting protein 1 (CYFIP1), regulates formation of membrane ruffles and is important in axonal growth; Guanine nucleotide binding protein (G protein) gamma 12 (GNG12), a modulator in transmembrane signaling systems; integrin beta 1 (ITGB1), involved in cell adhesion, hemostasis, and tissue repair; mitogen-activated protein kinase kinase 1 (MAP2K1), activates ERK1 and ERK2 MAP kinases; moesin (MSN); p21 protein-activated kinase 2 (PAK2), a critical effector that links Rho GTPases to the cytoskeletal reorganization and nuclear signaling, and is involved in stimulating cell survival and growth; protein phosphatase 1, regulatory (inhibitor) subunit 12A (PPP1R10), plays a role in the control of chromatin structure and cell cycle progression; and slingshot homolog 2 (SSH2), regulates actin filament dynamics (Table 3).

Many of the dysregulated genes included growth factors, mainly: Fibroblast growth factor 12 (FGF12) and Fibroblast Growth Factor 2 (basic) (FGF2) are present in basement membranes where they play a role in wound healing of normal tissues; fibroblast growth factor 4 (FGF4), involved in embryonic development, cell growth, and tissue repair; fibroblast growth factor receptor 2 (FGFR2), a high-affinity receptor for acidic/basic keratinocyte growth factor; integrin alpha 11 (ITGA11); p21 protein-activated kinase 1

(PAK1), involved in the regulation of microtubule biogenesis and inhibited in cells undergoing apoptosis; and tubulin tyrosine ligase-like family, member 3, monoglycyclase (TTLL3), which modifies alpha and beta tubulin. Other dysregulated growth factors included the downregulation of Epidermal Growth Factor Receptor (EGFR), TGFA and the upregulation of ITGB1, growth factor receptor-bound protein 2 (GRB2), and IGF2R. This is not surprising since growth factors play essential functions in skeletal development and adult homeostasis, and if not tightly regulated may result in disease.

ErbB signaling pathway

The ErbB signaling transduction axis is significantly important in regulating diverse biological processes including cell motility, cell survival, proliferation and differentiation [32-33]. Several genes were dysregulated in the ErbB pathway in IBMPFD disease. Downregulated genes included epidermal growth factor receptor oncogene homolog (EGFR), p21 protein-activated kinase 1 (PAK1), protein kinase C, beta (PRKCB), and transforming growth factor, alpha (TGFα). Upregulated genes included calcium/calmodulin-dependent protein kinase II Delta, glycogen synthase kinase 3 beta (GSK3B), a protein involved in glycogen metabolism and neuronal cell development; growth factor receptor-bound protein 2 (GRB2), an adaptor protein which links cell surface growth factor receptors and the Ras signaling pathway; protein tyrosine kinase 2 (PTK2), implicated in signal transduction pathways in cell motility, proliferation, and apoptosis; mitogen-activated protein kinase kinase 1 (MAP2K1), ERBB2, a protein tyrosine kinase which plays a role in several cell surface receptor complexes; and MYCLK1, a possible new functional member of the myc family of proto-oncogenes (Table 3).

Regulation of Cancer

Expression analysis revealed dysregulated genes in cancer in affected IBMPFD patients versus control subjects. Downregulated genes included adenomatosis polyposis coli 2 (APC2) and epidermal growth factor receptor (EGFR). Upregulated genes included glycogen synthase kinase 3 beta (GSK3B), growth factor receptor-bound protein 2 (GRB2), mitogen-activated protein kinase kinase 1 (MAP2K1), mutL homolog 1 (MLH1), v-myc myelocytomatosis viral oncogene homolog (MYCLK1), and adenomatous polyposis coli (APC) (Table 3).

Regulation of autophagy

VCP is a key regulatory protein and plays a critical role in regulating this proteolytic pathway involving non-dividing differentiated cells. Constitutive autophagy plays an important role in protein quality control [34]. Studies have demonstrated mutations in VCP disrupt its normal role in protein homeostasis, causing a defect in autophagy, the main route for sequestration of cytoplasm to the lytic compartment [16]. The autophagy pathway is one of the three proteolytic systems involved in mediating the breakdown of short-lived or long-lived proteins, and plays a central role in differentiation and development [35] and is activated under environmental stress conditions in pathological situations.

Our microarray analyses depicted an upregulation in ATG4 autophagy related 4 homolog A (ATG4), a cysteine protease required for autophagy. Presently, at least four ATG4 mammalian homologs have been identified. ATG4s may act as both conjugating and deconjugating enzymes and thus their activities are tightly regulated. In the process of autophagy, following Atg8 cleavage, ATG4 must become inactive to ensure conjugation of Atg8 to the autophagosomal membrane. Expression of this regulatory mutant prevents autophagosomal formation in cells.

Downregulated genes included Forkhead Box O3 (FoxO3) and mechanistic target of rapamycin (mTOR), FoxO3 has been shown to be sufficient to induce autophagosome formation in the adult mouse skeletal muscle, subsequently promoting lysosomal proteolysis showed a downregulation in IBMPFD patient muscle [36]. Studies on FoxO3 have demonstrated transcriptional induction of a number of autophagy genes including LC3B, Atg12, Atg4B, ulk2, and Beclin1. Studies have also demonstrated the FoxO transcription factors to activate protein degradation and promote autophagy in cardiomyocytes [37-38]. Another gene which demonstrated downregulation was mechanistic target of rapamycin (mTOR), a serine/threonine kinase known for its multiple roles in cell growth, proliferation, motility, survival and protein synthesis. Studies have demonstrated that the mTOR pathway is dysregulated in a variety of human diseases. A partial list of the autophagy genes is listed in Table 3.

Lysosomal pathway

VCP is a key regulatory protein and plays a critical role in regulating this proteolytic pathway involving non-dividing differentiated cells. There are two pathways for lysosomal degradation, mainly the golgi-endosome pathway and autophagy pathway. Studies have demonstrated mutations in VCP cause a defect in protein degradation. In this study, microarray analysis results showed an upregulation of the lysosomal pathway, mainly in the adaptor-related protein complex 1 (gamma 1, mu 1, sigma 2 subunits) and 3 (AP1G1, AP3S2), Iduronate 2-sulfatase (IDS), lysosomal multi-spanning membrane protein 5, prosaposin (PSAP), ATPase H+ transporting lysosomal V0 subunit d1, adaptor-related protein complex 1/3, cathepsin B (CTSB), cystinosis (neuropathic) (CTNS), and ectonucleoside triphosphate diphosphohydrolase (ENTPD1). The dysregulated lysosomal pathway showed downregulation of glucosidase beta, golgi associated, gamma adaptin ear containing ARF binding protein 2 (GGA1), and solute carrier family 11 member 1 (SLC11A2) (Table 3). Future mechanistic studies will highly focus on this intricate pathway in VCP disease.

Three dysregulated pathways by BIOCARTA: Mechanism of protein import into the nucleus, Agrin in postsynaptic differentiation, and Erk1/Erk2 MAPK signaling pathways

Signaling transduction cascades regulate numerous cellular processes including differentiation, apoptosis, and proliferation. Improper functioning of these pathways leads to the progression of diseases and developmental abnormalities. Signals from these cascades need to enter the nucleus for modulation of transcription factors and chromatin remodeling enzymes. Nuclear transport signaling proteins play an important role in the regulation of gene expression and may have consequences when these processes become disrupted. This process includes 2 steps: mainly, an energy-independent docking of the protein to the nuclear envelope and an energy-dependent step of translocation via the nuclear pore complex. The specific mechanisms involved in the localization of nuclear transport factors are currently being investigated.

In this report, microarray analysis demonstrated a dysregulation of the protein import factors including RAN a member RAS oncogene family a, small GTP binding protein belonging to the RAS super family involved in nucleo-cytoplasmic transport; Karyopherin (importin) beta 1 (KPNB1) which functions in nuclear protein import; Karyopherin alpha 2 (RAG cohort 1, importin alpha1) (KPNA2) which functions as an adaptor protein for nuclear receptor KPNB1; Nuclear transport factor 2 (NUTF2), facilitates protein transport into the nucleus; and Nucleoporin 210KDa (NUP210), essential for nuclear pore assembly and fusion, and nuclear pore spacing. All these nuclear transport molecules demonstrated an upregulation in VCP affected patients when compared with their control counterparts (*provided as* supplemental Table 5).

Agrin, a proteoglycan released by motoneurons has been shown to induce/maintain the acetycholine receptor clustering and postsynaptic differentiation. In our analysis, we detected two genes to be upregulated in this pathway, mainly protein tyrosine kinase 2 (PTK2) and Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12) (ITGB1), involved in cell adhesion and recognition in multiple processes such as hemostasis, immune responses and tissue repair. Downregulated genes in this pathway included Cortactin (CTTN), which may contribute to the organization of cellular structure; Laminin, alpha 3 (LAMA3), is a basement membrane component which mediates attachment, migration, and organization of cells into tissues; p21 protein (cdc42/Rac)-activated kinase 1 (PAK1); and Utrophin (UTRN), which shares structural and functional similarities with the dystrophin gene. Recently, mouse studies have suggested that this gene may serve as a substitute for the dystrophin gene thus serving as a potential therapeutic for muscular dystrophies (*provided as* supplemental Table 5).

The extracellular signal-regulated kinase 1 and 2 (Erk1/Erk2) pathway is one of four different mammalian Mitogen Activated Protein Kinase (MAPK) cascades which have been identified and participate in the regulation of all stimulated processes within cells. Within this cascade, the signal is propagated by phosphorylations and activations of sequential kinases, eventually leading to the phosphorylation of regulatory proteins by MAPK and its components [39-40]. The Erk1/2 pathway activates a large number of substrates and induces a variety of cellular processes including proliferation and differentiation, transcriptional suppression, chromatin remodeling, regulation of nuclear import, and more recently has been identified in cancers [41-42]. In our microarray analysis with the VCP-associated IBMPFD patients, we found two downregulated genes including GNAS complex locus (GNAS) and Epidermal growth factor receptor (EGFR) and four upregulated genes including Cyclin-dependent kinase-like 3 (CDKL3); Growth factor receptor-bound protein 2 (GRB2); Integrin beta 1 (ITGB1); and Mitogen Activated Protein Kinase 1 (MAP2K1). We are currently investigating the molecular signaling intermediates in the Erk1/2 pathway to better understand the regulatory role of this transduction pathway in IBMPFD disease (*provided as* supplemental Table 5).

Verification of Dysregulated Genes

Findings of the gene expression study were validated by using quantitative reverse transcriptase polymerase chain reaction analyses to test genes involved in various signaling cascades. Several genes were chosen from our global microarray results, represent both increased and decreased expression and were selected according to the P values and the FC values for the differentially expressed sequences obtained in the comparisons. Several of the genes had the same directional change (for example PTK2, ITGB1, PAK1, and FoxO3) however FoxO3 was the most statistically significant ($p<0.05$) (Figure 1, Table 4). The forkhead box O3 (FoxO3) transcription factor has been shown to induce autophagosome formation in the adult mouse skeletal muscle subsequently promoting lysosomal proteolysis [36]. Studies on FoxO3 have demonstrated transcriptional induction of a number of autophagy genes including LC3B, Atg12, Atg4B, ulk2, and Beclin1 and activation of protein degradation [37-38]. Targeting FoxO3 a component of the autophagy pathway, which is important in the pathogenesis of VCP-associated disease, offers the potential of a novel therapeutic strategy for these patients.

Discussion

Microarrays reveal gene expression profiles of human tissues and provide valuable insight into molecular signaling pathways involved in pathogenesis or abnormally regulated in disease. IBMPFD is a clinically heterogeneous, dominantly-inherited disorder with a variable phenotype and is caused by mutations in VCP, an important gene which plays an

integral role in several diverse cellular processes, functions, and signaling cascades. Patients may express only one or two phenotypic components and need not demonstrate all three phenotypic features. We were hoping that these studies would provide an explanation for the variable phenotype seen.

Our exploratory study is the first to compare gene expression profiling in IBMPFD patients and control subjects, thereby providing insights to the intricate signaling transduction pathways involved in VCP disease. Post-natal muscle growth involves satellite cells, which are incorporated into the developing fibers. In the adult muscle, there is increased. Keeping the delicate balance between the protein synthesis and degradation pathways is influenced by physical activity, mechanical stress, and growth factors. Elucidating the signaling mechanisms which regulate muscle atrophy and hypertrophy are thus keys for the prevention and treatment of neuromuscular diseases. The global microarray analyses conducted revealed dysregulation mainly of the genes involved in the actin cytoskeleton cascade, involving several critical growth factor receptors, such as FGFR2 and EGFR; and disruption of the autophagy pathway genes, namely ATG4A, MTOR, and FoxO3. The signaling intermediates involved in these pathways are important for the crosstalk between protein breakdown and synthesis in IBMPFD disease.

The actin cytoskeleton pathway was implicated by downregulation of Rho guanine nucleotide exchange factor 12, Alpha Actinin 1, Actin related protein 2/3 complex, integrin alpha 11, myosin light chain 10, p21 protein activated kinase 1, and tubulin tyrosine ligase-like family, member 3. These proteins are expressed in muscle and may be involved in attaching muscle tissue to the extracellular matrices. Thus, a downregulation of this pathway may lead to aberrations in the cytoskeleton, thereby leading to increased weakness and pathology of the muscle. Signaling by growth factors play an important role in muscle physiology, thus disruption in these cascades may ultimately lead to pathogenesis of disease. Furthermore, FGFR2 is linked directly to cytoskeletal integrity. Hypothetically, decreased cellular response to general growth signals due to decreased overall numbers of these three receptors could cause involutional effects on muscle fiber protein, including proteins associated with the cytoskeleton. Secondly, disruption of normal protein disposal pathways, particularly autophagy, could trigger cell death pathways and loss of myocytes or myoblasts needed to regenerate muscle. Finally, there is preliminary evidence of upregulation in several nuclear envelope-related genes associated with importation of transcriptional regulatory proteins. Although other interpretations are possible, this observation may reflect a compensatory response to disruption in the normal availability of these signals from the nuclear periphery. The net effect of the alterations we observe is thus increased apoptosis, impaired autophagic degradation and defective myotube formation, consequently leading to significant muscle weakness in IBMPFD patients. EGFR is linked to the ErbB, agrin, cancer and Erk1/Erk2 pathways and its downregulation would be expected to have multiple downstream effects, including the cytoskeletal pathway. The Erk1/Erk2 pathway and is critical to muscle replenishment. How these signaling pathways crosstalk and interact with one another for muscle homeostasis will require further investigation.

Autophagy is an important process which provides the degradation of damaged organelles through the autophagosomal-lysosomal fusion process. The homeostasis of skeletal muscles is dependent upon the proper regulation of the autophagic flux. Excessive autophagy or a defect therein is detrimental for healthy muscles and plays a pathogenic role in several muscle diseases. Three autophagy genes ATG4A, MTOR, and FOXO3 were dysregulated in patient muscle samples. Studies suggest that the autophagic process is associated with cell cycle regulation, adaptation to starvation, and cancer development. The importance of autophagy was demonstrated in previous *in vitro* studies of patient myoblasts whereby expression of IBMPFD mutations led to accumulated ubiquitinated proteins [43-46].

Additionally, VCP interacts with aggregated proteins [47-48] and defects in these protein aggregate (such as expanded polyglutamine-containing proteins) degradation impairs autophagosome maturation, thereby explaining the observed IBMPFD pathology [49]. Previous studies have shown an accumulation of LC3II, and LAMP1/2 in immature autophagosomes [16]. IBMPFD patients as well as transgenic mouse models have demonstrated rimmed vacuoles lined with p62 and LC3 [16]. These data suggest the significance of VCP in the autophagosome-lysosome fusion, and its disruption may lead to pathogenesis.

Recent studies have shown increasing evidence suggesting that p97 or some of its adaptors may play a role in cancer. Patients with VCP-associated inclusion bodies have shown some unusual tumors. We are currently investigating the role of VCP-associated IBMPFD in the involvement of various cancers. Further analyses will help us understand the key disease mechanisms/pathways associated with causing muscle weakness in IBMPFD disease and will prove to be useful in studying effects of potential treatments. We systematically assessed the dysregulated signaling cascades and their intermediates in IBMPFD patients and their first degree relatives. We have used powerful analytical methodology and stringent criteria in analyzing the microarray data. The results we have obtained are extremely encouraging and statistically significant. However, there are still many questions remaining in the treatment of the disease, which may be understood by further dissecting the patho-physiological signaling transduction pathways and examining crosstalk between these cascades. The study limitations in this investigation included: a small cohort of subjects, and limited amount of tissue/RNA for protein analyses. Microarrays are primarily a screening tool. A further weakness includes decreased sensitivity of the arrays to the detection of genes with low expression levels.

Thus, future studies will be aimed at collecting larger cohorts of patient samples and elucidating these intricate pathways by performing mechanistic characterizations of these signaling transduction cascades. Although by RT-PCR, the four selected genes had the same directional change, only FOXO3 was statistically significant. Future studies will be aimed at elucidating these intricate pathways by performing mechanistic characterizations of these signaling transduction cascades and their intermediates.

Conclusion

In conclusion, we have examined some of the key molecular mediators and cascades which are dysregulated in skeletal muscle in IBMPFD by global gene microarray. We will continue to explore these dysregulated genes as molecular targets for development of novel therapeutic strategies for IBMPFD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IBMPFD	Inclusion Body Myopathy with Paget's Disease of Bone and Frontotemporal Dementia
ALS	Amyotrophic Lateral Sclerosis
VCP	Valosin Containing Protein
CK	Creatinine kinase
ERAD	Endoplasmic Reticulum Associated Degradation
DAVID	Database for Annotation, Visualization and Integration Discovery

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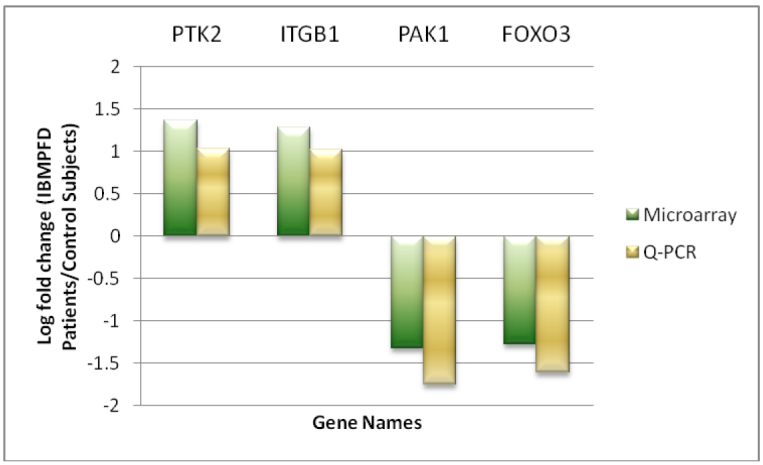

Figure 1.
Validation of a sample of microarray genes by RT qPCR.

Table 1

Summary of clinical features and characteristics of IBMPFD patients and their unaffected relatives.

Patient Sample ID[1]	**Age**[2]	**Sex**[3]	**Clinical features**[4]	**Mutation**[5]	**Myopathy Onset**[6]	**Paget Disease of Bone Onset**[7]	**Dementia Onset**[8]
01-001	59	F	Myopathy, Paget's disease	R155H	33	—	—
01-003	43	M	Myopathy, Paget's disease	R155H	40	—	—
01-004	56	F	Paget's disease	R155H	—	40	—
01-005	41	F	Paget's disease	R155H	—	31	—
03-001	42	M	Myopathy, ALS	R155H	—	—	—
16-001	49	M	Myopathy, Paget's disease	R155H	45	32	47
30-002	41	F	Myopathy	L198W	34	—	—
16-002	44	F	Unaffected	Normal	—	—	—
16-004	41	M	Unaffected	Normal	—	—	—
16-005	48	F	Unaffected	Normal	—	—	—

[1] Patient Sample ID

[2] Age

[3] Sex

[4] Clinical symptoms

[5] Mutation

[6] Myopathy Onset

[7] Paget Disease of Bone Onset

[8] Dementia Onset

Table 2

Gene pathways as classified by KEGG and BIOCARTA: Pathways enriched with genes in VCP patients.

[1]Pathways <0.05	[2]Pathway	[3]Number of genes	[4]EASE Score
KEGG_PATHWAY	Regulation of actin cytoskeleton	29	2.00E-03
KEGG_PATHWAY	ErbB signaling pathway	12	2.50E-02
KEGG_PATHWAY	Cancer	8	3.50E-02
KEGG_PATHWAY	Regulation of autophagy	3	4.00E-02
KEGG_PATHWAY	Lysosome	15	4.80E-02
BIOCARTA PATHWAY	Mechanism of Protein Import into the Nucleus	5	7.90E-03
BIOCARTA PATHWAY	Agrin in Postsynaptic Differentiation	5	1.00E-02
BIOCARTA PATHWAY	Erk1/Erk2 Mapk Signaling pathway	4	5.10E-02

Complete gene lists resulting from microarray analysis were separately analyzed with DAVID and the resulting KEGG and Biocarta pathways and associated p values are represented.

[1]Pathways <0.05

[2]Pathway

[3]Number of genes

[4]EASE Score

Table 3

List of dysregulated genes in IBMPFD and control subjects by KEGG analysis.

AFFY ID	Gene Symbol	Gene Name	FC (↑↓)
SIGNALING TRANSDUCTION PATHWAYS			
Regulation of Actin Cytoskeleton Genes			
1559833_at	ARHGEF12	Rho guanine nucleotide exchange factor (GEF)	12 1.20↓
231536_at	TIAM1	T-cell lymphoma invasion and metastasis 1	1.35↓
232022_at	TIAM2	T-cell lymphoma invasion and metastasis 2	1.31↓
237401_at	ACTN1	Actinin, alpha 1	1.20↓
217174_s_at	APC2	Adenomatosis polyposis coli 2	1.58↓
243327_at	EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian	1.30↓
240067_at	FGF12	Fibroblast growth factor 12	1.32↓
204421_s_at	FGF2	Fibroblast growth factor 2 (basic)	1.42↓
1552982_a_at	FGF4	Fibroblast growth factor 4	1.21↓
208225_at	FGFR2	Fibroblast growth factor receptor 2	1.88↓
222899_at	ITGA11	Integrin, alpha 11	1.65↓
221659_s_at	MYL10	Myosin, light chain 10, regulatory	1.29↓
1565772_at	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	1.32↓
243911_at	TTLL3	Tubulin tyrosine ligase-like family, member 3; actin related protein 2/3 complex, subunit 4, 20kDa	1.23↓
241387_at	PTK2	PTK2 protein tyrosine kinase 2	1.37↑
239397_at	ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	1.21↑
220966_x_at	ARPC5L	Actin related protein 2/3 complex, subunit 5-like	1.30↑
216933_x_at	APC	Adenomatous polyposis coli	1.72↑
1555730_a_at	CFL1	Cofilin 1 (non-muscle)	1.25↑
212133_at	CYFIP1	Cytoplasmic FMR1 interacting protein 1	1.33↑
224681_at	GNAI1	Guanine nucleotide binding protein (G protein) alpha 12	1.22↑
222834_s_at	GNG12	Guanine nucleotide binding protein (G protein) gamma 12	1.44↑
205782_at	FGF7	Hypothetical LOC100132771; fibroblast growth factor 7 pseudogene 2	1.39↑
1561042_at	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12	1.29↑
202670_at	MAP2K1	Mitogen-activated protein kinase kinase 1	1.37↑
240960_at	MSN	Moesin	1.28↑
1559052_s_at	PAK2	p21 protein (Cdc42/Rac)- activated kinase 2	1.29↑
201602_s_at	PPP1R10	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	1.66↑
1555425_x_at	SSH2	Slingshot homolog 2 (Drosophila)	1.20↑
ErbB Signaling Pathway Genes			
2287817_at	PRKCB	Protein kinase C, beta	1.28↓
211258_s_at	TGFA	Transforming growth factor, alpha	1.21↓
1565772_at	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	1.32↓

AFFY ID	Gene Symbol	Gene Name	FC (↑↓)
SIGNALING TRANSDUCTION PATHWAYS			
Regulation of Actin Cytoskeleton Genes			
243327_at	EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.30↓
209945_s_at	GSK3B	Glycogen synthase kinase 3 beta	1.39↑
215075_s_at	GRB2	Growth factor receptor-bound protein 2	1.33↑
241387_at	PTK2	PTK2 protein tyrosine kinase 2	1.37↑
231042_s_at	CAMK2G	Calcium/calmodulin-dependent protein kinase II delta	1.28↑
202670_at	MAP2K1	Mitogen-activated protein kinase kinase 1	1.37↑
1559052_s_at	PAK2	p21 protein (Cdc42/Rac)-activated kinase 2	1.29↑
226213_at	ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3(avian)	2.83↑
202431_s_at	MYCLK1	v-myc myelocytomatosis viral oncogene homolog (avian)	1.98↑
Cancer Genes			
2171714_s_at	APC2	Adenomatosis polyposis coli 2	1.58↓
243327_at	EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	1.30↓
209945_s_at	GSK3B	Glycogen synthase kinase 3 beta	1.39↑
223049_at	GRB2	Growth factor receptor-bound protein 2	1.39↑
202670_at	MAP2K1	Mitogen-activated protein kinase kinase 1	1.37↑
202520_s_at	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E.coli)	1.31↑
202431_s_at	MYCLK1	v-myc myelocytomatosis viral oncogene homolog (avian)	1.98↑
216933_x_at	APC	Adenomatous polyposis coli	1.72↑
Regulation of Autophagy Genes			
217399_s_t	FOXO3	Forkhead box O3; forkhead box O3B pseudogene	1.27↓
215381_at	MTOR	Mechanistic target of rapamycin (serine/threonine kinase)	1.46↓
213115_at	ATG4A	ATG4 autophagy related 4 homolog A (S. cerevisiae)	1.25↑
Lysosomal Pathway Genes			
213448_at	LOC149533	Glucosidase, beta; acid (includes glucosylceramidase)	1.28↓
214233_at	GGA1	Golgi associated, gamma adaptin ear containing, ARF binding protein 2	1.21↓
217507_ at	SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1.21↓
212041_at	Atp6v0d1	ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	1.30↑
241294_at	AP1G1	Adaptor-related protein complex 1, gamma 1 subunit	1.23↑
223025_s_at	AP1M1	Adaptor-related protein complex 1, mu 1 subunit	1.25↑
243745_at	AP1S2	Adaptor-related protein complex 1, sigma 2 subunit pseudogene; adaptor-related protein complex 1, sigma 2 subunit	1.40↑
202398_at	AP3S2	Adaptor-related protein complex 3, sigma 2 subunit	1.28↑
200838_at	CTSB	Cathepsin B	1.46↑
204925_at	CTNS	Cystinosis, nephropathic	1.39↑
204076_at	ENTPD1	Ectonucleoside triphosphate diphosphohydrolase	1.29↑
202439_s_at	IDS	Iduronate 2-sulfatase	1.30↑

AFFY ID	Gene Symbol	Gene Name	FC (↑↓)
SIGNALING TRANSDUCTION PATHWAYS			
Regulation of Actin Cytoskeleton Genes			
201392_s_at	IGF2R	Insulin-like growth factor 2 receptor	1.40↑
201721_s_at	LAPTM5	Lysosomal multispanning membrane protein 5	1.70↑
200866_s_at	PSAP	Prosaposin	1.27↑

Table 4

Validation of dysregulated genes by RT qPCR.

Gene Symbol	Gene Name	Signaling Transduction Pathway	Microarray	RT qPCR
PTK2	PTK2 protein tyrosine kinase 2	Regulation of Actin Cytoskeleton	+1.37	↑1.04
ITGB1	Integrin, beta 1 (fibronectin receptor)	Regulation of Actin Cytoskeleton	+1.29	↑1.02
PAK1	P21 protein (cdc42/Rac)-activated kinase 1	Regulation of Actin Cytoskeleton	−1.327	↓−1.75
FOXO3	Forkhead box O3	Autophagy	−1.27	↓1.61