DYSREGULATION OF AMP-ACTIVATED PROTEIN KINASE SIGNALING IN ALZHEIMER'S DISEASE

BY

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LIST OF ABBREVIATION

Abbreviation	Definition
4EBP1	eukaryotic translation initiation factor 4e-binding protein 1
AD	Alzheimer's disease
ABC	Avidin-biotin complex
ACC1	Acetyl CoA carboxylase 1
ACN	Acetonitrile
ADP	Adenosine diphosphate
ADSCT	AD signature cortical thicknesses
AICAR	5-aminoimidazole-4-carboxamide ribonucleoside
AK1	Adenylate kinase-1
AKT	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
APOE	Apolipoprotein E
APP	Amyloid precusor protein
ATP	Adenosine triphosphate
AUC	Area under the curve
Aβ	Amyloid β
BACE1	β-amyloid converting enzyme 1
BCA	Bicinchoninic acid assay
BMI	Body mass index

CaMkkβ	Calcium/calmodulin-dependent protein kinase kinase-β
CPT1	Carnitine palmitoyl transferase 1
CSF	Cerebral spinal fluid
ECL	Enhanced chemiluminescence
eEF2	Eukaryotic elongation factor 2
EF1A	Elongation factor 1A
eIF4E	eukaryotic initiation factor 4E
ELISA	Enzyme-linked immunosorbent assay
FASN	Fatty acids synthase
Fgf21	Fibroblastic growth factor 21
FOXO	Class O of forkhead box transcription factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBD	Glycogen-biding domain
GLUT	Glucose transporters
GSK3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HDAC2	histone deacetylase 2
HMGCR	Hydroxymethylglutaryl CoA reductase
IACUC	Institutional Animal Care and Use Committee
JNK3	c-Jun N-terminal kinase 3
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LKB1	Liver kinase B1

LMII	Logical Memory test II
MCI	Minor cognitive impairment
MMSE	Mini-Mental State Examination
MR	Magnetic resonance
mTORC1	mechanistic target of rapamycin complex 1
NeuN	Neuronal nuclei
NF-κB	Nuclear factor κB
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PHF	Paired helical filaments
PI3K	Phosphatidylinositol -3-kinase
PiB	Pittsburgh compound B
PP	Phosphatase protein
RAGE	Receptor for advanced glycation end products
Rheb	Ras homologue enriched in brain
RIPA	Radioimmunoprecipitation buffer
ROC	Receiver operating characteristic
S6K	Ribosomal S6 kinase
SDS	Sodium dodecyl sulfate
SIRT1	silent information regulator type 1
SREBP1	sterol regulatory element-binding protein 1
TBS-T	Tris-buffered saline and Tween 20

TSC1/2	Tuberous sclerosis complex 1/2
ULK1	unc-51-like autophagy-activating kinase 1
WWS	Working wash solution

ABSTRACT

The etiology of Alzheimer's disease (AD), one of the most grievous neurodegenerative disease, is still unclear despite decades of study and early diagnosis of this disease remains a challenge. Energy metabolic dysfunction has been found to be a distinct characteristic in Alzheimer's disease, and may be the underlying pathogenic mechanism of Alzheimer's disease. AMP-activated protein kinase (AMPK) is a master energy sensor which can sense the ratio of AMP/ATP and regulates catabolic and anabolic pathways to keep the energy metabolism in balance, and has been found to play an important role in the pathogenesis of Alzheimer's disease. AMPK has 3 subunits, the α, β and γ. The α subunit is the catalytic subunit and has 2 isoforms, α1 and α2. Phosphorylation of α subunit determines the activity of AMPK. In this study, I found AMPKα2 was dysregulated in a vervet monkey model of Alzheimer's disease, which exhibited AD-like pathologies, biochemical alterations and behaviors. Proteomic studies in this model showed that many of dysregulated proteins were metabolism-related and could be associated with AMPK signaling. I also found level of AMPKα1 was significantly deceased in the plasma of AD and mild cognitive impairment (MCI) patients as compared to healthy controls and the efficacy of AMPKα1 level in plasma to differentiate patients from controls was relatively high. In summary, this study found that AMPK signaling was dysregulated in a vervet monkey model of Alzheimer's disease, which could be used as a tool for development of therapeutics of Alzheimer's disease, and AMPKα1 was significantly decreased in the plasma of AD and MCI patients, indicating that AMPKα1 could serve as a biomarker for the early diagnosis of Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common form of dementia syndromes and one of the leading causes of disability and mortality in elderly in the world, especially in the developed countries (Mayeux R and Stern Y, 2012). There were about 5.7 million patients by now in the United States according to reports from Alzheimer's Association. As the aging population grows, the prevalence and incidence have increased dramatically in recent years. As expected, there will be more than 13.8 million patients with Alzheimer's disease in 2050 (Alzheimer's Association, 2018). Currently there is no cure or effective intervention for AD. Unfortunately, despite decades of intensive study, the etiology of Alzheimer's disease is still unclear, which hampers development of therapeutic targets and diagnostic biomarkers. The classic pathological hallmarks of Alzheimer's disease are senile plaques and neurofibrillar tangles, which consists of β-amyloid protein and hyperphosphorylated tau protein, respectively. However, besides these two pathological hallmarks, disruption of energy homeostasis, oxidative stress, dysfunction of mitochondria, protein synthesis and autophagy are also found in the brain of patients with Alzheimer's disease. One of the molecules that are linked to these pathologies is AMP-activated protein kinase (AMPK).

Introduction of AMPK

AMPK was first identified as a kinase that phosphorylated acetyl-CoA carboxylase in fatty acid synthesis and HMG-CoA reductase in cholesterol synthesis (Hardie DG, et al., 1989). AMPK is a master energy sensor of cells. The hydrolysis of ATP to ADP is the source of energy that drives all kinds of process in cells. AMPK can sense the ratio of AMP/ATP and regulates catabolic and anabolic pathway correspondingly to adjust the energy metabolism to an equilibrium (Hardie DG and Carling D, 1997). AMPK is a heterotrimeric protein complex consisting of 3 subunits: the α, β and γ. Each of the subunit has 2 or 3 isoforms (Carling D, et al., 2011). The α subunit is the catalytic

subunit of AMPK, which can phosphorylate many downstream proteins (Stapleton D, et al., 1996). The α subunit can also be phosphorylated by upstream regulators. The main phosphorylation site of the α subunit is the Thr172, which can lead to thousand-fold increase in catalytic activity when it is phosphorylated (Hawley SA, et al., 1996). This site can also be dephosphorylated by protein phosphatase, which leads to the deactivation of AMPK (Davies SP, et al., 1995). The α1-containing AMPK isoforms have higher catalytic activities, while the α2-containing AMPK isoforms are more sensitive to dephosphorylation by protein phosphatase (Rajamohan F, et al., 2016). The β and γ subunit are regulatory subunits of AMPK. The γ subunit is responsible for sensing the AMP/ATP ratio. When AMP or ADP binds to the γ subunit, the catalytic activity of the phosphorylated α subunit can increase about 2-5 times further, thus, AMP or ADP here acts as an allosteric activator (Carling D, et al., 2011). Additionally, the binding of AMP or ADP to the γ subunit can introduce conformational change to the kinase and prevent dephosphorylation of the Thr172 in the α subunit by protein phosphatase (Davies SP, et al., 1995). In fact, the amount of phosphorylation of AMPK is mainly determined by the rate of dephosphorylation, which in turn is determined by the ratio of AMP/ATP (Carling D, et al., 2011). The role of β subunit is not very clear at present. It has been proposed that β subunit may be an intracellular glycogen sensor, since the β subunit contains a domain, termed as glycogen-biding domain(GBD), which is present in many enzymes that metabolize glycogen (McBride A, et al., 2009). Myristoylation of the β subunit is necessary for AMP to promote Thr172 phosphorylation (Oakhill JS, et al., 2010). It has been showed that β2-containing AMPK isoforms are less sensitive to A769662, an AMPK angonist, as compared to β1-containing AMPK isoforms (Rajamohan F, et al., 2016).

Regulation of AMPK

There are two classic kinase identified in human that can phosphorylate α subunit of AMPK at Thr172. The first one is calcium/calmodulin-dependent protein kinase kinase-β(CaMKKβ). Since

CaMKKβ is activated by the increase of intracellular calcium concentration, AMPK can be indirectly activated by the rise of intracellular calcium, which can lead to tau phosphorylation (Mairet-Coello G, et al., 2013). The other one is the liver kinase B1(LKB1) (Krishan S, et al., 2014). Since these two kinases are linked to many other receptors and signaling pathways, AMPK can integrate information from different pathways to make an appropriate response.

As mentioned above, the activity of AMPK is largely determined by phosphorylation at Thr172. But this is not the only site which can regulate the activity of AMPK. In recent years, it's found that phosphorylation of AMPKα1/α2 at Ser487/491 can also regulate the activity of AMPK. For example, protein kinase B (Akt) and protein kinase C (PKC) can phosphorylate AMPKα1 at Ser487, which can then inhibit phosphorylation at Thr172, thus reduce the activity of AMPK (Hawley SA, et al., 2014; Heathcote HR, et al., 2016). The ribosomal S6 kinase (S6K), which is downstream effector of AMPK, can phosphorylate AMPKα2 at Ser491 (Dagon Y, et al., 2012). 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an agonist of AMPK, can stimulate AMPKα1/α2 phosphorylation at Ser487/491 (Stone JD, et al., 2012). These studies show that phosphorylation at sites other than the Thr172 can also regulate the activity of AMPK.

Downstreams of AMPK

AMPK can maintain energy homeostasis through multiple mechanisms. For example, AMPK regulates the metabolism of fatty acids. When the concentration of AMP increases in cells, AMPK is activated and inhibit fatty acids synthesis via phosphorylation of acetyl CoA carboxylase 1 (ACC1) (Hardie DG, Pan DA, 2012). ACC1 can catalyse the carboxylation of acetyl CoA to malonyl CoA (Bianchi A, et al., 1990), which in turn inhibits carnitine palmitoyl transferase 1 (CPT1) (Bremer J, 1963). CPT1 can facilitate transportation of activated long-chain fatty acids into the mitochondria for metabolism via β-oxidation pathway, resulting in increase of ATP concentration

(Bremer J, 1963). On the other hand, AMPK can inhibit sterol regulatory element-binding protein 1 (SREBP1) (Li Y, et al., 2011), a transcription factor which upregulates the expression of fatty acids synthase (FASN) and ACC1 (Hardie DG, 1989). These two enzymes are critical for fatty acids synthesis which requires ATP. Thus, by inhibiting these enzymes, AMPK can reduce the consumption of ATP and increase the production of ATP. AMPK can also phosphorylate and inhibit hydroxymethylglutaryl CoA reductase (HMGCR), which is a rate-limiting enzyme in cholesterol synthesis (Habegger KM, et al., 2012). Taken together, AMPK can maintain energy homeostasis through inhibiting anabolic processes and activating catabolic processes.

Furthermore, AMPK plays an important role in regulating protein synthesis through many downstream targets. First, AMPK can directly inhibit the mechanistic target of rapamycin complex 1 (mTORC1) by phosphorylating the mTORC1 subunit raptor to reduce protein synthesis, which is an ATP-consuming process (Gwinn DM, et al., 2008). mTORC1 is a cellular signaling hub and plays an important role in protein synthesis via its two established downstream effectors: eukaryotic translation initiation factor 4e-binding protein 1 (4eBP1) and p70 S6 kinase (S6K1) (Hay N and Sonenberg N, 2004; Holz MK, et al., 2005). The canonical pathways involved in regulation of mTORC1 include the insulin-phosphatidylinositol -3-kinase (PI3K)-protein kinase B (AKT) signaling. AKT can phosphorylate the tuberous sclerosis complex 2 (TSC2), which binds to the tuberous sclerosis complex 1 (TSC1) to form a dimer that inhibits mTORC1 activity (Dan HC, et al., 2002). Phosphorylated TSC2 binds to cytosolic anchoring protein 14-3-3, thus disrupting the TSC1/TSC2 dimer (Shaw RJ, 2009). Therefore, activation of the insulin/PI3K/AKT signaling pathway leads to upregulation of the mTORC1 and promote protein synthesis. AMPK can affect this signaling pathway by phosphorylating the TSC2 at a site different from that phosphorylated by AKT, which results in the formation of TSC1/TSC2 dimer, thus inhibiting mTORC1 (Shaw RJ, 2009). In addition to mTORC1, AMPK also regulates protein synthesis through mRNA translational factor eukaryotic

elongation factor 2 (eEF2). Briefly, AMPK phosphorylates and activates eukaryotic elongation factor 2 kinase (eEF2K), leading to eEF2 phosphorylation and consequently disruption of elongation step in mRNA translation (Seinberg G and Kemp B, 2009).

Moreover, AMPK is involved in regulation of autophagy. AMPK can phosphorylate the unc-51-like autophagy-activating kinase 1 (ULK1) at Ser317 and Ser777, which is an initiator of autophagy (Kim J, et al., 2011). ULK1 can also be phosphorylated by mTORC1 at Ser757, which leads to the inhibition of phosphorylation of ULK1 at Ser317 and Ser777 (Kim J, et al., 2011). Generally, AMPK can initiate autophagy by directly phosphorylating ULK1, and by indirectly inhibiting mTORC1, which can inhibit the activation of ULK1.

Role of AMPK in neurofibrillary tangles and senile plaques

Neurofibrillary tangles and senile plaques are the two main pathological hallmarks of Alzheimer's disease. It has been revealed that AMPK played significant roles in both of these two pathological processes. First, AMPK can phosphorylate tau protein directly at many sites when mouse cortical neurons were exposed to $A\beta_{1-42}$ (Thornton C, et al., 2011), while hyperphosphorylated tau can assemble to form paired helical filaments (PHF) and subsequently to neurofibrillary tangles (Alonso AD, et al., 2001; Mandelkow E, et al., 2007). Activated AMPK was found to accumulate in the cytoplasm of cortical neurons in many tauopathies including Alzheimer's disease and locate with tangle-like structures (Vingtdeux et al. 2011). Recently, it has been proved that AMPK can modulate tau phosphorylation and tau pathology *in vivo* (Domise M, et al., 2016). Consistent with these studies, histone deacetylase 2 (HDAC2), which is significantly increased in Alzheimer's disease, can induce tau phosphorylation through activation of AMPK (Liu D, et al., 2017). On the other hand, there are studies reporting that AMPK can reduce tau phosphorylation through downstream effectors. For example, AMPK can activate silent information regulator type 1

(SIRT1), which in turn reduces the acetylation of tau protein, while acetylation of tau blocks the ubiquitination and degradation of tau protein, thus eventually AMPK can reduce the accumulation of phosphorylated tau protein (Min SW, et al. 2010). AMPK can also inhibit GSK3β to reduce the phosphorylation of tau protein (Greco SJ, et al. 2009). It was found that this signaling pathway was controlled by adenylate kinase-1 (AK1) (Park H, et al., 2012).

Activation of AMPK can also attenuate amyloidosis. It has been showed that this effect can be achieved through different signaling pathways. For example, Activation of AMPK by agonists reduces the β-cleavage of the amyloid precursor protein (APP) in cultured rat cortical neurons, while knockout of AMPKα2 increased amyloid-β production (Won JS, et al. 2010). Reduced expression of phosphatase protein 2Cα (PP2Cα) leads to activation of AMPKα1/α2, which then reduces the expression of β-amyloid converting enzyme 1 (BACE1), a major APP cleavage enzyme, and subsequently reduces the production of amyloid-β (Lu J, et al. 2010). When SIRT1 is activated by AMPK, it can suppress the production of amyloid-β production by increasing the expression of α-secretase (Donmez G, et al., 2010). AMPK can also reduce amyloid-β production by activating autophagocytosis through the mTORC1 signaling, which can facilitate the clearance of amyloid-β protein (Vingtdeux V, et al., 2010). In contrary, other studies showed that activation of AMPK leads to the biogenesis of amyloid-β peptide. For example, metformin, which is an activator of AMPK, can increase the biogenesis of amyloid-β peptide via up-regulating the BACE1 expression (Chen YM, et al., 2009). Interestingly, amyloid-β can also transiently inhibit the activity of AMPK (Seixas da Silva GS, et al., 2017).

AMPK as a linkage between energy metabolism dysfunction and Alzheimer's disease

It has been shown that patients with type 2 diabetes mellitus had higher risk to develop Alzheimer's disease and *vice versa* (Yaffe K, et al., 2004; Janson J, et al., 2004). Thus, many

investigators speculated that there might be a linkage between energy metabolism dysfunction and Alzheimer's disease. While AMPK plays an important role in energy metabolism, it has been hypothesized that AMPK might also play an important role in Alzheimer's disease, and this has been supported by many studies.

First, AMPK is involved in the insulin resistance in the brain of patients with Alzheimer's disease. A large cohort clinical study has showed that middle-aged subjects without dementia, diabetes or stroke who had hyperinsulinemia at baseline had a greater decline in cognitive function over a 6-year follow-up as compared to subjects without hyperinsulinemia (Young SA, et al. 2006). It was also found that insulin signaling pathway was impaired in the brain of patients with Alzheimer's disease (Liu Y, et al., 2011; Willette AA, et al., 2015). Even in the early stage of the disease, gene expression profile of molecules related to insulin signaling was altered in a transgenic mouse model (Pedros I, et al., 2014). These studies are all evidence of insulin resistance in Alzheimer's disease. Insulin resistance can modulate the activity of AMPK through overactivation of Akt, leading to phosphorylation of AMPK at Ser485 while not Thr172 (Kim B, et al., 2015). On the opposite side, by inhibiting AMPK, Amyloid-β oligomers can decrease the surface levels of glucose transporters (GLUT) in hippocampal neurons, which leads to insulin resistance (Seixas da Silva GS, et al., 2017). Insulin resistance can increase amyloidosis by reducing insulin-PI3K-AKT signaling (Liu Y, et al., 2011), which negatively regulates the GSK-3β activity (Cross DA, et al. 1995). While Inhibitors of GSK-3β can decrease the expression of amyloid-β protein through activation of AMPK (Cai ZY, et al., 2012), thus AMPK serves as a mediator in this process. However, paradoxically, metformin, a regularly prescribed antidiabetic drug which can reduce insulin resistance and an activator of AMPK, increases the risk of developing Alzheimer's disease for those patients with diabetes mellitus (Imfeld P, et al., 2012). Taken together, insulin resistance can increase the expression of amyloid-β, but the role of AMPK in insulin resistance needs to be elucidated.

Second, AMPK plays a role in the mitochondrial dysfunction of Alzheimer's disease. Since neurons rely heavily on ATP synthesis by mitochondria, they are quite sensitive to mitochondrial dysfunction. AMPK can increase mitochondrial biogenesis to protect neurons (Lage R, et al., 2008). This has also been indicated in Alzheimer's disease. Activation of AMPK can ameliorate Alzheimer' disease-like pathology and spatial memory impairment via protecting the mitochondrial function in a streptozotocin-induced Alzheimer's disease rat model (Du LL, et al., 2015). Many substances can promote neuronal survival and mitochondrial biogenesis through the activation of AMPK signaling, such as pioglitazone and melatonin (Cersosimo E, et al., 2009; Kwon KJ, et al. 2010). Though it was reported that metformin can increase the biogenesis of amyloid-β peptides (Chen YM, et al., 2009), it can exert neuroprotective effects on human neural stem cells against amyloid-β-induced mitochondrial dysfunction through activation of AMPK (Chiang MC, et al., 2016). Thus, activation of AMPK may have a positive influence on the mitochondrial function in Alzheimer's disease.

Third, AMPK can exert its influence in oxidative stress and inflammation. Oxidative stress might be the earliest event in Alzheimer's disease (Nunomura A, et al., 2001), and inflammation is found to be ubiquitous in the brain of patients with Alzheimer's disease (Akiyama H, et al., 2000). Activation of AMPK can reduce the oxidative stress (Hotamisligil GS, 2010; Wang S, et al., 2010), and then subsequently suppress downstream targets, such as NF-κB (Li XN, et al., 2009). AMPK can aslo directly activate many inflammatory regulators, such as SIRT1 and FOXO proteins, which can inhibit NF-κB signaling (Canto C, et al., 2009; Greer EL, et al., 2007). These inflammatory regulators have multifaceted functions, such as inhibiting amyloidosis, triggering autophagy and reduce oxidative stress (Donmez G, et al., 2010; Lee IH, et al., 2008). But activation of AMPK by amyloid-β can lead to a translational block, which can activate the c-Jun N-terminal kinase 3 (JNK3) through the ER stress. Once activated, JNK3 can perpetuate metabolic stress and induce

secondary reactions, which has been found in human cases and transgenic mouse models (Yoon SO, et al., 2012). Thus, activation of AMPK plays complicated role in oxidative stress and inflammation, thus further investigations are needed.

Synaptic plasticity and AMPK in Alzheimer's disease

Since AMPK can regulate protein synthesis which is vital to the synaptic plasticity, much attention has been paid to the role of AMPK in synaptic plasticity. Inhibition of AMPK signaling can alleviate impairments in hippocampal synaptic plasticity induced by amyloid-β either by using pharmacological or genetic approaches. This effects are mediated by the downstream effector eukaryotic elongation factor 2 (eEF2) and its kinase eEF2K (Ma T, et al., 2014). Dysregulation of the AMPK downstream targets, such as elongation factor 1A (EF1A) is also found to correlate with synaptic plasticity impairments in Alzheimer's disease (Beckelman B, et al., 2016). On the contrary, other studies suggest that activation of AMPK can improve synaptic plasticity. For example, Deficiency in adiponectin, a cytokine secreted by adipocyte, can lead to neuronal apoptosis and reduced synaptic protein synthesis through inactivation of AMPK (Ng RC, et al., 2016). Recently, it's found that osmotin, a plant protein homolog of adiponectin, could reduce amyloidosis and improve pre-and post-synaptic function through the adiponectin receptor 1/AMPK/SIRT1 signaling (Shah SA, et al., 2017). A study which was conducted in wildtype mice found that application with the AMPK agonist AICAR could improve cognition and motor coordination in both young and old mice (Kobilo T, et al., 2014). These facts indicate that the role of AMPK in synaptic plasticity might be condition-dependent.

Autophagy and AMPK in Alzheimer's disease

It was found that autophagic vacuoles accumulated significantly in hippocampus and prefrontal cortex in patients with Alzheimer's disease, indicating impaired autophagy function in the brain

of patients with Alzheimer's disease (Nixon RA and Yang DS, et al., 2011). Later study revealed that amyloid-β-induced autophagy was mediated by the receptor for advanced glycation end products (RAGE)-calcium-CaMKKβ-AMPK signaling (Son SM, et al., 2012). Blockade of protein phosphatase 2A (PP2A) can inhibit autophagy and cause intraneuronal accumulation of ubiquitinated proteins through the AMPK-mTORC1 signaling (Magnaudeix A, et al., 2013). Chronic hypoxia has similar effects in a transgenic mouse model (Liu H, et al., 2015). Long-term caloric restriction can increase fibroblastic growth factor 21 (Fgf21) which then activates the AMPK-mTORC1 signaling to exert neuroprotective effects through autophagy (Ruhlmann C, et al., 2016). Cilostazol, which can increase intracellular cAMP levels, can modulate the autophagic degradation of amyloid-β in neurons through the SIRT1-LKB1-AMPK signaling (Park SY, et al., 2016). Activation of AMPK by resveratrol, a natural compound, leads to activation of autophagocytosis through the mTORC1 signaling, which then increase the clearance of amyloid-β (Vingtdeux V, et al., 2010). AMPK can also directly activate ULK1 to increase autophagic flux, which in turn reduce tau aggregates and memory impairments in a transgenic mouse model (Kim YD, et al., 2017). Thus, many signals can modulate autophagy, while the AMPK-mTORC1 signaling is in the center of these pathways.

Summary

As a master energy sensor of cells, AMPK is involved in many pathological processes of Alzheimer's disease, such as amyloidosis, tangle formation, disruption of energy homeostasis, oxidative stress, dysfunction of mitochondria, protein synthesis and autophagy. All of these pathological processes contribute to the progression of Alzheimer's disease. But the roles of AMPK in some of the processes are still elusive, and further investigations are needed. In conclusion, AMPK may play an important role in the pathology of Alzheimer's disease and may serve as a therapeutic target of Alzheimer's disease.

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Chapter 1 Isoform-specific dysregulation of AMP-activated protein kinase signaling in a non-human primate model of Alzheimer's disease

Introduction

Despite decades of intensive study, the etiology of Alzheimer's disease (AD) is still unclear and there is no effective cure for this disease. Many studies showed promising results in the transgenic mouse model, which is the most widely used animal model for Alzheimer's disease, but failed in clinical trials (Guell-Bosch J, et al., 2016). This might be because those transgenic mouse models recapitulate the pathophysiology of familial Alzheimer's disease, while most of the patients are sporadic. Due to the gap between transgenic mouse model and human AD patient, it is important to develop alternative animal models that mimic Alzheimer's disease. Many studies have shown that aged non-human primates can exhibit AD-like pathologies and behaviors (Dam D and Deyn P, 2017). Furthermore, the phyletic relationship between human and non-human primates is much closer. Thus, non-human primates might be a better model for sporadic Alzheimer's disease and can be used as a tool for development of therapeutics of Alzheimer's disease.

Energy metabolic dysfunction has been found to be a distinct characteristic in Alzheimer's disease (Kandimalla R, et al., 2017). Alzheimer's disease had been called "type 3 diabetes" (Monte S and Wands J, 2008). AMP-activated protein kinase (AMPK) is one of the important molecules involved in energy metabolism. It functions like an energy gauge, which can sense the ratio of AMP/ATP and regulates catabolic and anabolic signaling pathways to adjust the energy metabolism to an equilibrium (Hardie DG and Carling D, 1997). Besides this fundamental physiological function, AMPK also plays important roles in protein synthesis, autophagy and lipid metabolism (Krishan S, et al., 2014). AMPK has 3 subunits, the α, β and γ. The α subunit is the catalytic subunit and has 2 isoforms, α1 and α2. The activity of AMPK is determined by phosphorylation at Thr172 at α

subunit (Carling D, et al., 2011). Many studies have evidenced that AMPK is involved in the pathogenesis of Alzheimer's disease (Salminen A, et al., 2011). AMPKα isoform dysregulation might be a key underlying mechanism for Alzheimer's disease. Drugs that target AMPKα have shown promising results in transgenic mouse model and cultured neurons. However, whether this strategy can also be applied in human is not clear. Here, I explored AMPKα isoform dysregulation in a non-human primate model of Alzheimer's disease.

Materials and Methods

Animals

9 aged female vervet monkeys (*Chlorocebus aethiops sabaeus*) were included in this study. They were from 19.5 to 23.4 years old, roughly equal to 60s-70s of human beings. These monkeys were from Vervet Research Colony located at Wake Forest University Primate Center. The Vervet Research Colony is a colony of Caribbean origin vervet monkeys originally founded at University of California, Los Angeles in 1975 and relocated to Wake Forest University in 2008. All the monkeys were raised in social groups which reflected the natural social composition of vervet groups in wild. All the monkeys had standard commercial monkey chow and water. All procedures were conducted in accordance with state and federal laws and approved by the Institutional Animal Care and Use Committee (IACUC).

Behavior assessments

Behavior assessments were conducted about 1 mouth before the sacrifice of these monkeys. For each monkey, 4 observations were carried out and each observation lasted about 1 hours. The behaviors assessed included alert, moving, climbing and jumping. For each behavior, the rate (number of the behavior per hour) and time percentage (time spent on the behavior during observation) were recorded.

Tissue collection

Monkeys were sacrificed according to the guidelines of IACUC. Just before sacrifice, CSF was collected by inserting a 22-gauge needle into the cisternal space and stored at -70°C to determine Aβ42 level. Brains were rapidly removed and sectioned into coronal slabs which were about 2cm long, 5mm wide and 3mm thick and stored at -80°C. Slabs from left or right frontal corti, hippocampi or cerebellums were included in this study for western blot. Tissues from frontal cortex, temporal cortex, parietal cortex, occipital cortex, insular cortex, striatum, thalamas, hippocampus, cerebellum, midbrain, pons and medulla were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned into 5µm slice and mounted on positively charged microscope slides for Aβ and tau immunohistochemistry. The slides were kept at -20°C.

Measurement of Aβ42 level in CSF

Aβ42 level in CSF were measured using enzyme-linked immunosorbent assay (ELISA) (Luminex, Invitrogen) according to manufacturer's instructions. In brief, 200 µl of working wash solution (WWS) was added into each well of a 96 well filter plate (Multiscreen, Millipore) to pre-wet the plate and then aspirated after incubation for 30 secs at room temperature. The diluted bead solution was vortexed for 30 secs and then sonicated for 30 secs. 25 µl of the diluted bead solution was added into each well and the beads were washed twice with 200 µl of WWS. Then the filter plate was placed on paper towels to remove residual liquid. 50 µl of 1X detector antibody was added into each well followed by 25 µl of assay diluent and 25 µl of sample. 50 µl of standard dilutions were added into standard curve wells. The plate was covered with aluminum foil and incubated at room temperature for 3 hours on an orbital shaker. After aspirating liquid in wells, beads were washed twice with 200 µl of WWS. 100 µl of R-phycoerythrin (RPE) conjugate was added into each well and the plate was incubated at room temperature for 2 mins on an orbital

shaker. Plate was read in the LiquiChip workstation (Qiagen). Aβ42 level was calculated based on the standard curve generated by the standard dilutions.

Western blot

Tissues from frontal corti, hippocampi and cerebellums were sonicated in cold lysis buffer with proteinase and phosphatase inhibitor. Protein concentration in each sample was determined by BCA protein assay. Samples containing equal amounts of protein lysates and loading buffer were boiled at 100℃ for 5 min. Then samples were loaded to 4-12% or 16.5% Tris-glycine SDS-PAGE gels (Mini-Protean, Bio-Rad) for electrophoresis. After transfer, nitrocellulose membranes were blocked for 10min in blocking buffer (Superblock, Thermo Scientific) and then probed with primary antibodies for Aβ (1:500, 6E10, Covance), P-AMPKα1 (1:1000, abcam), AMPKα1 (1:1000, abcam), P-AMPKα2 (1:1000, abcam), AMPKα2 (1:1000, abcam) and GAPDH (1:1000, Cell Signaling) in 5% nonfat milk TBS-T buffer overnight at 4℃. After washing for 3 times with TBS-T buffer, the membranes were probed with secondary antibodies (1:5000, Bio-Rad) in 5% nonfat milk TBS-T buffer at room temperature for 1 hour. After washing for 3 times with TBS-T buffer, the membranes were developed in ECL substrate (Bio-Rad) at room temperature for minutes and imaged using ChemiDoc MP imaging system (Bio-Rad). Densitometric analysis was conducted using ImageJ and data of those probed proteins was normalized to GAPDH.

Immunohistochemistry

Slides from brain regions mentioned above were baked at 60℃ for 30 min first, then deparaffinized in xylene and rehydrated through a graded alcohol series. For antigen retrieval, slides were boiled in citrate buffer at pH 6.0 for 10 mins. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 15 mins. Slides were incubated in a humid chamber in primary antibody for NeuN (1:400, Cell Signaling), Aβ (1:5000, 6E10, Covance) and PHF-tau

(1:4200, AT8, Pierce) overnight at 4℃. Then slides were incubated in biotinylated secondary antibody (1:200, Vector Labs) for 30 mins at room temperature followed by incubation in ABC reagent (Vectastain, Vector Labs) for another 30 mins at room temperature. Primary and secondary antibodies and ABC reagent were diluted in 1% goat serum. Slides were developed in diaminobenzidine solution (ImmPACT, Vector Labs) for minutes depending on the primary antibodies and counterstained in hematoxylin solution for 30 secs. Negative controls were incubated in 1% goat serum without primary antibodies. Slides then were dehydrated in a grated alcohol series and xylene, cover-slipped and dried overnight. Images were taken by a Keyence BZ-X710 microscope.

To assess the Aβ plaque burden of each animal, erea within each brain region with the highest Aβ plaque density was identified, then a single 100X field was taken on that erea, Aβ plaque density in each brain region was semiquantified as follows. Regions without Aβ plaque density was scored as 0, regions with Aβ plaque density >0 but <6 plaques/mm2 was scored as 1, regions with Aβ plaque density >6 but <20 plaques/mm2 was scored as 2, regions with Aβ plaque density >20 plaques/mm2 was scored as 3. Then scores from all the regions were summarized to represent the Aβ plaque burden of the animal.

To assess neurodegeneration of each animal, five consecutive 40X fields were taken from CA1 and CA3 erea respectively in NeuN staining slide of each animal. Neurons were counted from the field and neuron density was calculated by dividing the number of neurons with the erea of the field.

Proteomic study

Hippocampal tissues of six monkeys were dissected in the cold PBS and lysed immediately in 500 µL of PBS with protease/phosphatase inhibitor using a Bead Mill Homogenizer (Bead Ruptor, Omni International). 500 µL of 2X radioimmunoprecipitation (RIPA) buffer was added and the mixture

was incubated on ice for 30 minutes. Tubes were centrifuged at 18,000 x g for 10 minutes then supernatant was transferred to a clean tube. Protein concentration was measured by BCA analysis and 100 µg of protein was subjected to tryptic digestion.

Reducing alkylation was performed in the presence of 10 mM dithiothreitol and 30 mM iodoacetamide. Four times the sample volume of cold (-20 °C) acetone was added to the tube which was then incubated at -20 °C overnight. Protein was pelleted by centrifugation at 14,000 x g for 10 minutes. After removal of supernatant, the pellet was dried by evaporation of residual acetone for 10 minutes at room temperature, and then was suspended in 50 mM ammonium bicarbonate. The protein suspension was incubated with trypsin at 1:50 enzyme-to-substrate ratio at 37 °C overnight. The resulting peptides were cleaned up by desalting using a C18 spin column. Purified peptide mixture is prepared in 5% (v/v) ACN containing 1% (v/v) formic acid for LC-MS/MS analysis.

The LC-MS/MS system consisted of a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific) and a Dionex Ultimate-3000 nano-UPLC system (Thermo Scientific) employing a Nanospray Flex Ion Source (Thermo Scientific). An Acclaim PepMap 100 (C18, 5 µm, 100 Å, 100 µm x 2 cm) trap column and an Acclaim PepMap RSLC (C18, 2 µm, 100 Å, 75 µm x 50 cm) analytical column were used for the stationary phase. Good chromatographic separation was observed with a linear gradient consisting of mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) where the gradient was from 5% B at 0 min to 40% B at 170 min. MS spectra were acquired by data dependent scans consisting of MS/MS scans of the twenty most intense ions from the full MS scan with dynamic exclusion option which was 10 seconds.

Spectra were searched using Sequest HT algorithm within the Proteome Discoverer v2.1 (Thermo Scientific) in combination with the mouse UniProt protein FASTA database (annotated 16,747 entries, December 2015). Search parameters were as follow: FT-trap instrument, parent mass error tolerance of 10 ppm, fragment mass error tolerance of 0.02 Da (monoisotopic), variable modifications of 16 Da (oxidation) on methionine and fixed modification of 57 Da (carbamidomethylation) on cysteine.

The numbers of peptide spectrum matches in each group were averaged and fold change was calculated as mean of AD-like group / mean of normally aged group. P value was calculated using the student's t-test. Proteins with fold change >1.15 and P<0.08 were considered upregulated and proteins with fold change <0.85 and P<0.08 were considered downregulated. PANTHER (Protein Annotation Through Evolutionary Relationship) program V10.0 (http://www.pantherdb.com) was used to classify these upregulated or downregulated proteins according to their molecular function or roles in biological processes. Then STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) program V10.0 (http://string-db.org) was used to perform functional interaction networks between upregulated or downregulated proteins and AMPKα. Prediction methods were neighborhood, coexpression, gene fusions, experiments, cooccurrence, databases and text mining using medium confidence (0.4).

Statistics

t test was performed for continuous variables between two groups. Linear regression was performed to determine the correlation between Aβ42 levels and Aβ plaque burden. All the statistical analyses were conducted using GraphPad 5.0. P<0.05 was considered significant.

Result

The characteristics of the monkeys were summarized in Table I. They were all female. Average age was 22.1 ± 1.5 years. Average BMI was 60.5 ± 7.5 kg/m^2. Diabetic status and the hemisphere where brain tissues were from were indicated in the table.

Table I. Characteristics of the monkeys.

ID	Sex	Age (years)	Body weight (kg)	BMI (Kg/m^2)	Diabetic Status	Hemisphere
1342	F	23.4	5.53	64.39		Left
1092	F	21.4	6.58	72.80		Left
1223	F	23.4	5.20	50.35		Right
1349	F	21.4	4.19	48.39		Right
1079	F	20.4	4.99	60.95		Left
1474	F	23.4	4.22	60.52		Right
1316	F	22.6	4.32	61.17	Possible	Right
1077	F	19.5	4.80	66.30		Left
1127	F	23.4	4.43	60.02	Possible	Left

To identify which monkeys are AD-like and which monkeys are normally aged, I first examined Aβ and P-tau pathologies, which are the two pathological hallmarks of Alzheimer's disease. Typical Aβ plaques could be found in neocortex while not in hippocampus, as shown in Fig. 1A, 1B and 1C. The Aβ plaque burden varied in different monkeys, as summarized in Table II. Neurofibrillary tangles were absent, but PHF (Paired Helical Filament) could be found in neocortex and hippocampus in all the animals (Fig. 1D, 1E, 1F).

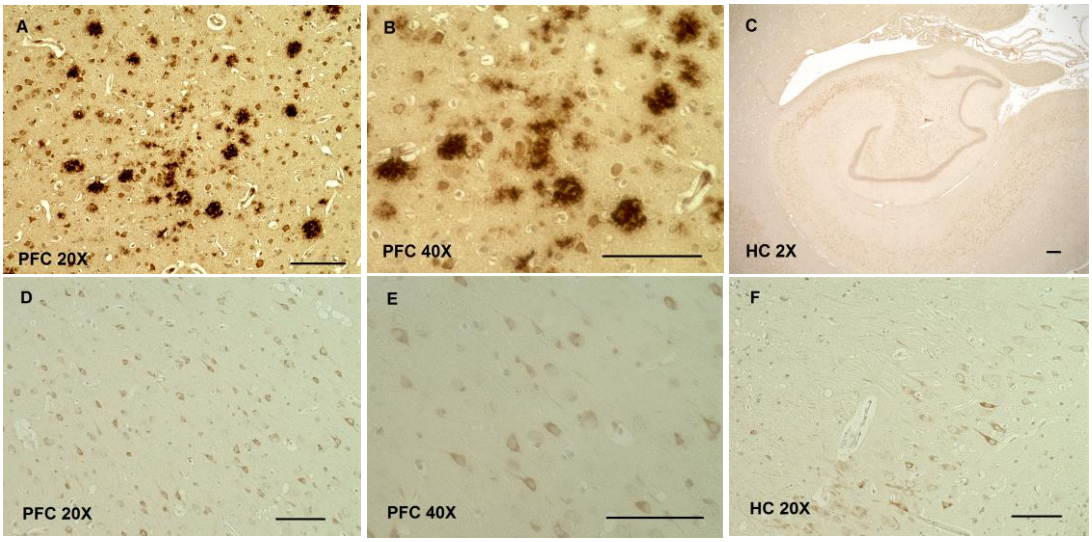


Figure 1. Aβ and P-tau pathologies of the monkeys. A and B show Aβ plaques in 20X and 40X magnification in prefrontal cortex. C shows no plaque in hippocampus. D and E show PHF in 20X and 40X magnification in prefrontal cortex. F shows PHF in hippocampus. Scale bars are 100µm in 20X and 40X images, and 300µm in 2X image. (Abbreviation: PFC Prefrontal Cortex, HC Hippocampus)

Table II. Pathological characteristics of the monkeys.

ID	P-tau pathology (Neurofibrillary tangle)	Aβ pathology (Aβ plaque burden)
1342	N/A	15
1092	N/A	10
1223	N/A	13
1349	N/A	1
1079	N/A	2
1474	N/A	11
1316	N/A	16
1077	N/A	2
1127	N/A	8

Aβ levels in CSF were also measured by ELISA in these monkeys. Aβ levels in CSF were significantly correlated with Aβ plaque burden (Fig. 2). Aβ levels were lower in those with high Aβ plaque burden, which was similar to the pattern seen in human patients.

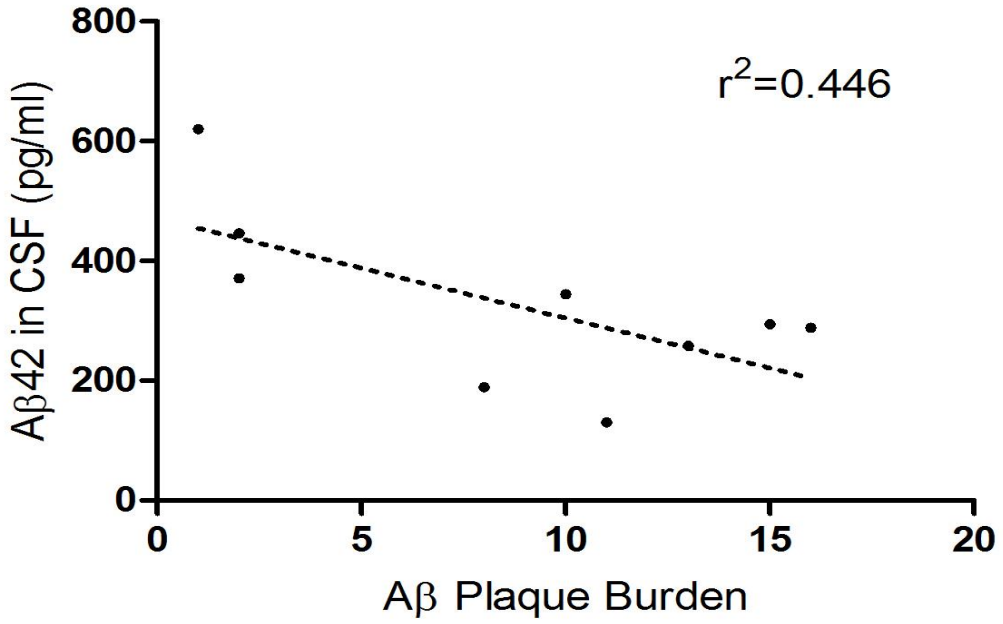


Figure 2. Relationship between Aβ42 levels in CSF and Aβ plaque burden. Aβ level in CSF is significantly correlated with Aβ plaque burden ($P<0.05$).

To further characterize these monkeys, I explored Aβ dimers in the brain of these monkeys. Aβ dimers were only found in the monkeys with the highest Aβ plaque burden, while those with intermediate or lowest Aβ plaque burden had no Aβ dimers in their brains (Fig. 3A). I didn't find Aβ dimers in the hippocampus of these monkeys (Fig. 3B).

Thus, I categorized the monkeys into three groups, one with highest Aβ plaque burden (n=3), one with intermediate Aβ plaque burden (n=3) and one with lowest Aβ plaque burden (n=3). Those with highest Aβ plaque burden were considered as AD-like, based on brain pathologies, CSF biomarkers, and levels of Aβ dimer. Those with lowest Aβ plaque burden were considered as normally aged controls. The status of those with intermediate Aβ plaque burden was not determined here.

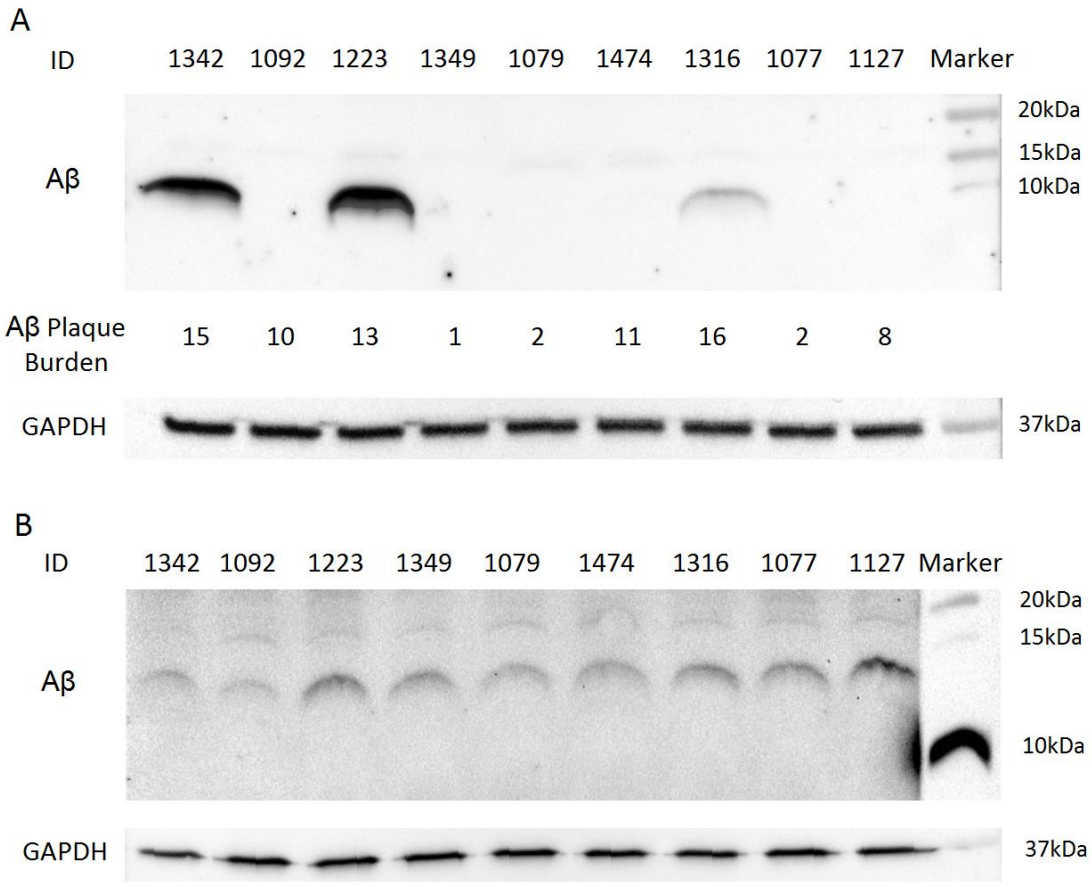
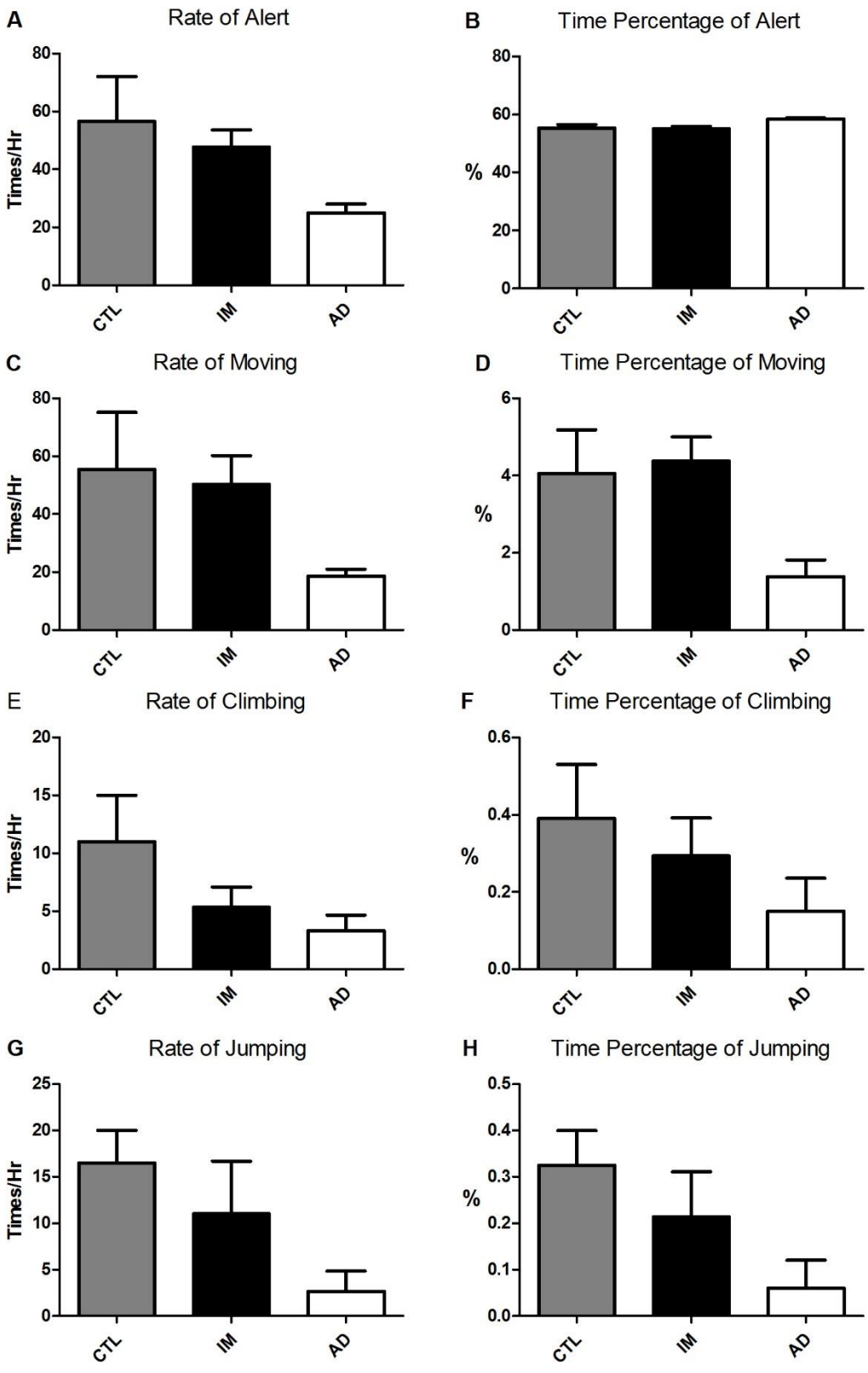


Figure 3. Aβ dimer in PFC and hippocampus. A shows Aβ dimers can be found in the PFC of three monkeys with the highest Aβ plaque burden. B shows lack of Aβ dimer in the hippocampi of these monkeys.

Next, I did behavioral assessments to evaluate the cognitive status of these monkeys and to verify our categorization. Rates of alert, moving, climbing and jumping were decreased in a tiered way in monkeys with intermediate and highest Aβ plaque burden as compared to those with lowest Aβ plaque burden (Fig. 4A, 4C, 4E, 4G). The same pattern could be found in time percentage of moving, climbing and jumping, except alert (Fig. 4B, 4D, 4F, 4H). Though the decreases were not significant due to small animal number in each group, finding in behavioral assessments also supported the categorization I proposed above.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G
Rate of Jumping
Times/Hr
25
20
15
10
5
0
CTL
IM
AD
H
Time Percentage of Jumping
%
0.5
0.4
0.3
0.2
0.1
0.0
CTL
IM
AD

Figure 4. Behavioral assessment of the monkeys in 3 groups. CTL represents the group with lowest Aβ plaque burden, which are thought to be normally aged controls, IM represents the group with intermediate Aβ plaque burden, while AD represents the group with highest Aβ plaque burden, which are thought to be AD-like. Rate means how many times the monkey did the behavior in an hour. Time percentage means how much time the monkey spent to do the behavior in a given time. The behaviors assessed are indicated in each figure. No significant difference for any of behavioral assessment is found among these groups (P>0.05).

I also determined neurodegeneration in the hippocampi of those monkeys. Neuron density in CA1 of hippocampus was significantly decreased in the AD-like monkeys as compared to those normally aged monkeys (Fig. 5A). Neuron density in CA3 of hippocampus was not significantly different between the two groups (Fig. 5B).

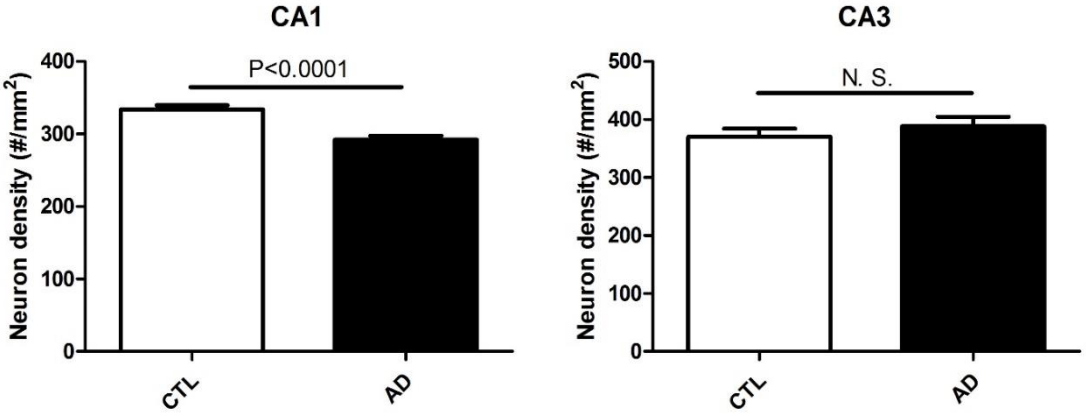


Figure 5. Neuron density in the hippocampi of AD-like and normally aged monkeys. A shows neuron density is decreased in CA1 of hippocampi of AD-like monkeys as compared to normally aged monkeys (P<0.0001). B shows no difference is found in CA3 of hippocampi between the two groups (P>0.05).

After categorizing these monkeys, I investigated AMPKα signaling in AD-like and normally aged monkeys by western blot. AMPKα1 activity was not different in PFC, hippocampus or cerebellum between the two groups (data not shown). AMPKα2 activity was not altered in PFC or cerebellum, but significantly different in hippocampus between the two groups. Phosphorylation of AMPKα2 was significantly increased in the hippocampi of AD-like monkeys (Fig. 6A & 6C), while total AMPKα2 levels were not different between the two groups (Fig. 6B & 6C).

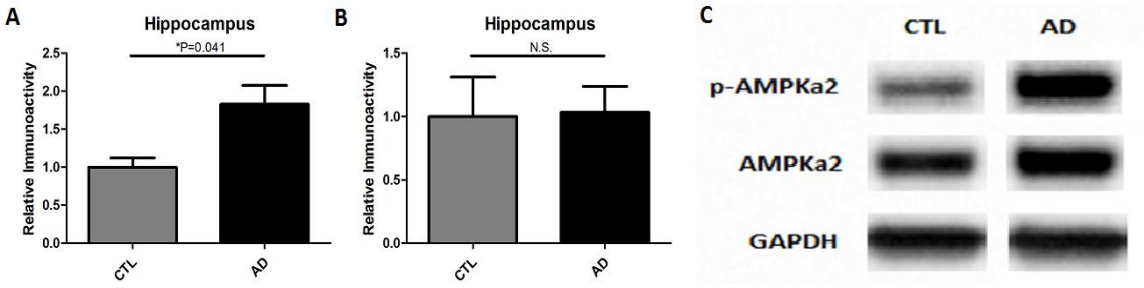


Figure 6. AMPKα2 signaling in the hippocampus of AD-like and normally aged monkeys. A shows P-AMPKα2 is increased in AD-like monkeys ($P<0.05$). B shows AMPKα2 is not different between the two groups ($P>0.05$). C shows the representative bands of western blot.

Next, I did proteomic study using hippocampal tissues of AD-like and normally aged monkeys. Totally, 2708 proteins were detected in these samples. Of them, 31 proteins were up-regulated (Table III), 46 proteins were down-regulated (Table IV) in the hippocampus of AD-like monkeys as compared to normally aged monkeys. Those up-regulated or down-regulated proteins were classified according to molecular function or biological process using the online program PANTHER. Up-regulated proteins were classified into 6 categories with 31 hits according to molecular function (Fig. 7A). The categories with the most hits were binding and catalytic activity. If classified according to the biological process, up-regulated proteins could be classified into 9 categories with 50 hits (Fig. 7B). The categories with the most hits were cellular and metabolic process. Down-regulated proteins were classified into 4 categories with 29 hits according to

molecular function (Fig. 7C). The category with the most hits was catalytic activity. If classified according to the biological process, down-regulated proteins could also be classified into 9 categories with 56 hits (Fig. 7D). The categories with the most hits were also cellular and metabolic process.

Table III. Up-regulated proteins in the hippocampus of AD-like monkeys.

Protein Name	Gene Name	Accession #	MW (kDa)	mean #PSM CTL	mean #PSM AD	Fold (AD/CTL)
Dynamin-2	DNM2	P50570	98.0	11.67	13.67	1.17
Ras-related protein Ral-A	RALA	P11233	23.6	6.00	7.33	1.22
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	IDH3A	P50213	39.6	13.33	16.33	1.23
Dynein light chain 2, cytoplasmic	DYNLL2	Q96FJ2	10.3	5.67	7.00	1.24
Ras-related protein Rap-1A	RAP1A	P62834	21.0	6.67	8.67	1.30
Guanine nucleotide-binding protein G(q) subunit alpha	GNAQ	P50148	42.1	9.67	13.00	1.34
Profilin-1	PFN1	P07737	15.0	8.33	11.67	1.40
GTP-binding nuclear protein Ran	RAN	P62826	24.4	4.67	7.00	1.50
Cell adhesion molecule 4	CADM4	Q8NFZ8	42.8	6.67	10.00	1.50
Transaldolase	TALDO1	P37837	37.5	3.00	4.67	1.56
S-phase kinase-associated protein 1	SKP1	P63208	18.6	8.33	13.33	1.60
Translationally-controlled tumor protein	TPT1	P13693	19.6	5.00	8.33	1.67
Sorting nexin-1	SNX1	Q13596	59.0	1.33	2.67	2.00
Nuclear transport factor 2	NUTF2	P61970	14.5	2.67	5.67	2.13
Ubiquitin-conjugating enzyme E2 D3	UBE2D3	P61077	16.7	1.67	3.67	2.20
Receptor expression-enhancing protein 5	REEP5	Q00765	21.5	1.33	3.33	2.25
AP-1 complex subunit mu-1	AP1M1	Q9BXS5	48.6	1.33	3.00	2.25
Voltage-gated potassium channel subunit beta-2	KCNAB2	Q13303	41.0	1.67	4.00	2.40
Keratin, type II cytoskeletal 2 epidermal	KRT2	P35908	65.4	3.00	8.33	2.78

Tumor protein D52	TPD52	P55327	24.3	1.00	3.00	3.00
Glutathione S-transferase omega-1	GSTO1	P78417	27.5	1.00	3.33	3.33
Chondroitin sulfate proteoglycan 5	CSPG5	O95196	60.0	0.67	2.33	3.50
Neural Wiskott-Aldrich syndrome protein	WASL	O00401	54.8	0.67	2.33	3.50
Leucine-rich PPR motif-containing protein, mitochondrial	LRPPRC	P42704	157.8	1.00	4.67	4.67
IQ and AAA domain-containing protein 1	IQCA1	Q86XH1	95.3	0.33	1.67	5.00
Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	Q13151	30.8	0.33	1.67	5.00
L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	AASDHPPT	Q9NRN7	35.8	0.33	2.00	6.00
Very-long-chain enoyl-CoA reductase	TECR	Q9NZ01	36.0	0.33	2.00	6.00
Protein phosphatase 1E	PPM1E	Q8WY54	84.9	0.33	2.00	6.00
E3 ubiquitin-protein ligase CHIP	STUB1	Q9UNE7	34.8	0	1.67	∞
Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-12	GNG12	Q9UBI6	8.0	0	1.33	∞

Abbreviation: MW molecular weight, #PSM number of peptide spectrum matches, CTL control, AD Alzheimer's disease.

Table IV. Down-regulated proteins in the hippocampus of AD-like monkeys.

Protein Name	Gene Name	Accession #	MW (kDa)	mean #PSM CTL	mean #PSM AD	Fold (AD/CTL)
Glutamate decarboxylase 1	GAD1	Q99259	66.9	2.33	0.00	0.00
Calcium-binding protein 39	CAB39	Q9Y376	39.8	1.67	0.00	0.00
Calpain-13	CAPN13	Q6MZZ7	76.6	1.33	0.00	0.00
Histidine triad nucleotide-binding protein 3	HINT3	Q9NQE9	20.3	1.33	0.00	0.00
Vesicle-associated membrane protein 1	VAMP1	P23763	12.9	1.33	0.00	0.00
Disintegrin and metalloproteinase domain-containing protein 23	ADAM23	O75077	91.9	2.33	0.00	0.00
V-type proton ATPase subunit G 1	ATP6V1G1	O75348	13.7	2.33	0.33	0.14

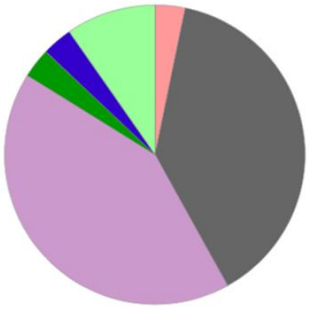
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	NDUFA11	Q86Y39	14.8	2.33	0.33	0.14
COP9 signalosome complex subunit 8	COPS8	Q99627	23.2	2.00	0.33	0.17
Sodium/potassium-transporting ATPase subunit beta-3	ATP1B3	P54709	31.5	2.00	0.33	0.17
Vesicular inhibitory amino acid transporter	SLC32A1	Q9H598	57.4	3.00	0.67	0.22
Mitochondrial pyruvate carrier 2	MPC2	O95563	14.3	4.33	1.00	0.23
Proteasome subunit beta type-5	PSMB5	P28074	28.5	4.33	1.00	0.23
KH domain-containing, RNA-binding, signal transduction-associated protein 1	KHDRBS1	Q07666	48.2	2.67	0.67	0.25
Inorganic pyrophosphatase 2, mitochondrial	PPA2	Q9H2U2	37.9	2.33	0.67	0.29
Contactin-associated protein-like 2	CNTNAP2	Q9UHC6	148.1	3.00	1.00	0.33
Prolyl endopeptidase-like	PREPL	Q4J6C6	83.9	7.67	2.67	0.35
Histidine triad nucleotide-binding protein 2, mitochondrial	HINT2	Q9BX68	17.2	3.67	1.33	0.36
ATP synthase subunit delta, mitochondrial	ATP5D	P30049	17.5	2.67	1.00	0.38
Heterogeneous nuclear ribonucleoprotein M	HNRNPM	P52272	77.5	2.67	1.00	0.38
Mitochondrial import inner membrane translocase subunit Tim8 A	TIMM8A	O60220	11.0	3.33	1.33	0.40
Methylglutaconyl-CoA hydratase, mitochondrial	AUH	Q13825	35.6	2.33	1.00	0.43
40S ribosomal protein S16	RPS16	P62249	16.4	2.33	1.00	0.43
Interferon-inducible double-stranded RNA-dependent protein kinase activator A	PRKRA	O75569	34.4	6.67	3.00	0.45
Acylpyruvase FAHD1, mitochondrial	FAHD1	Q6P587	24.8	3.33	1.67	0.50
Vacuolar protein sorting-associated protein 29	VPS29	Q9UBQ0	20.5	2.67	1.33	0.50
Secretogranin-2	SCG2	P13521	70.9	6.67	3.33	0.50
ATP-dependent RNA helicase DDX1	DDX1	Q92499	82.4	3.67	2.00	0.55

Synaptic vesicle membrane protein VAT-1 homolog-like	VAT1L	Q9HCJ6	45.9	9.67	5.33	0.55
DnaJ homolog subfamily A member 1	DNAJA1	P31689	44.8	4.67	2.67	0.57
Phosphatidylinositol transfer protein alpha isoform	PITPNA	Q00169	31.8	4.00	2.33	0.58
NADH-cytochrome b5 reductase 3	CYB5R3	P00387	34.2	5.00	3.00	0.60
Putative heat shock protein HSP 90-beta-3	HSP90AB3P	Q58FF7	68.3	20.67	13.00	0.63
Gamma-synuclein	SNCG	O76070	13.3	5.67	3.67	0.65
Destrin	DSTN	P60981	18.5	5.00	3.33	0.67
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	NDUFV1	P49821	50.8	19.33	13.33	0.69
MICOS complex subunit MIC60	IMMT	Q16891	83.6	23.33	17.00	0.73
ATP-citrate synthase	ACLY	P53396	120.8	11.67	8.67	0.74
2-iminobutanoate/2-iminopropanoate deaminase	HRSP12	P52758	14.5	7.33	5.67	0.77
Protein NipSnap homolog 1	NIPSNAP1	Q9BPW8	33.3	10.33	8.00	0.77
Malate dehydrogenase, cytoplasmic	MDH1	P40925	36.4	37.33	29.33	0.79
Phosphate carrier protein, mitochondrial	SLC25A3	Q00325	40.1	20.33	16.00	0.79
ATP synthase subunit O, mitochondrial	ATP5O	P48047	23.3	8.00	6.33	0.79
Microtubule-associated protein 6	MAP6	Q96JE9	86.5	14.67	11.67	0.80
Fructose-bisphosphate aldolase C	ALDOC	P09972	39.4	80.67	67.00	0.83
Ras-related protein Rab-14	RAB14	P61106	23.9	14.33	12.00	0.84

Abbreviation: MW molecular weight, #PSM number of peptide spectrum matches, CTL control, AD Alzheimer's disease.

To identify how AMPKα was related to these up-regulated or down-regulated proteins, I conducted protein-protein interaction analysis using the online program STRING. Protein-protein interaction analysis showed that AMPKα could be related to most of the up-regulated or down-regulated proteins through a network, indicating that AMPKα had wide connections with other signaling pathways and played an important role in the pathogenesis of Alzheimer's disease.

A Molecular Function



Up-Regulated Proteins

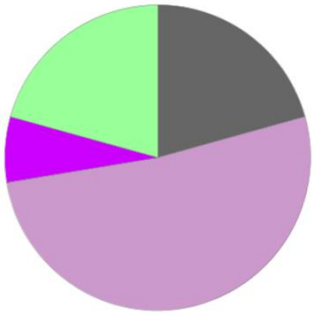
- antioxidant activity (GO:0016209)
- binding (GO:0005488)
- catalytic activity (GO:0003824)
- receptor activity (GO:0004872)
- signal transducer activity (GO:0004871)
- transporter activity (GO:0005215)

B Biological Process

Up-Regulated Proteins

- biological adhesion (GO:0022610)
- biological regulation (GO:0065007)
- cellular component organization or biogenesis (GO:0071840)
- cellular process (GO:0009987)
- developmental process (GO:0032502)
- localization (GO:0051179)
- metabolic process (GO:0008152)
- multicellular organismal process (GO:0032501)
- response to stimulus (GO:0050896)

C Molecular Function



Down-Regulated Proteins

- binding (GO:0005488)
- catalytic activity (GO:0003824)
- structural molecule activity (GO:0005198)
- transporter activity (GO:0005215)

D Biological Process

Down-Regulated Proteins

- biological regulation (GO:0065007)
- cellular component organization or biogenesis (GO:0071840)
- cellular process (GO:0009987)
- developmental process (GO:0032502)
- localization (GO:0051179)
- metabolic process (GO:0008152)
- multicellular organismal process (GO:0032501)
- reproduction (GO:0000003)
- response to stimulus (GO:0050896)

Figure 7. Classification of the up-regulated and down-regulated proteins. A shows classification of up-regulated proteins according to molecular function. B shows classification of up-regulated proteins according to biological process. C shows classification of down-regulated proteins according to molecular function. D shows classification of down-regulated proteins according to biological process.

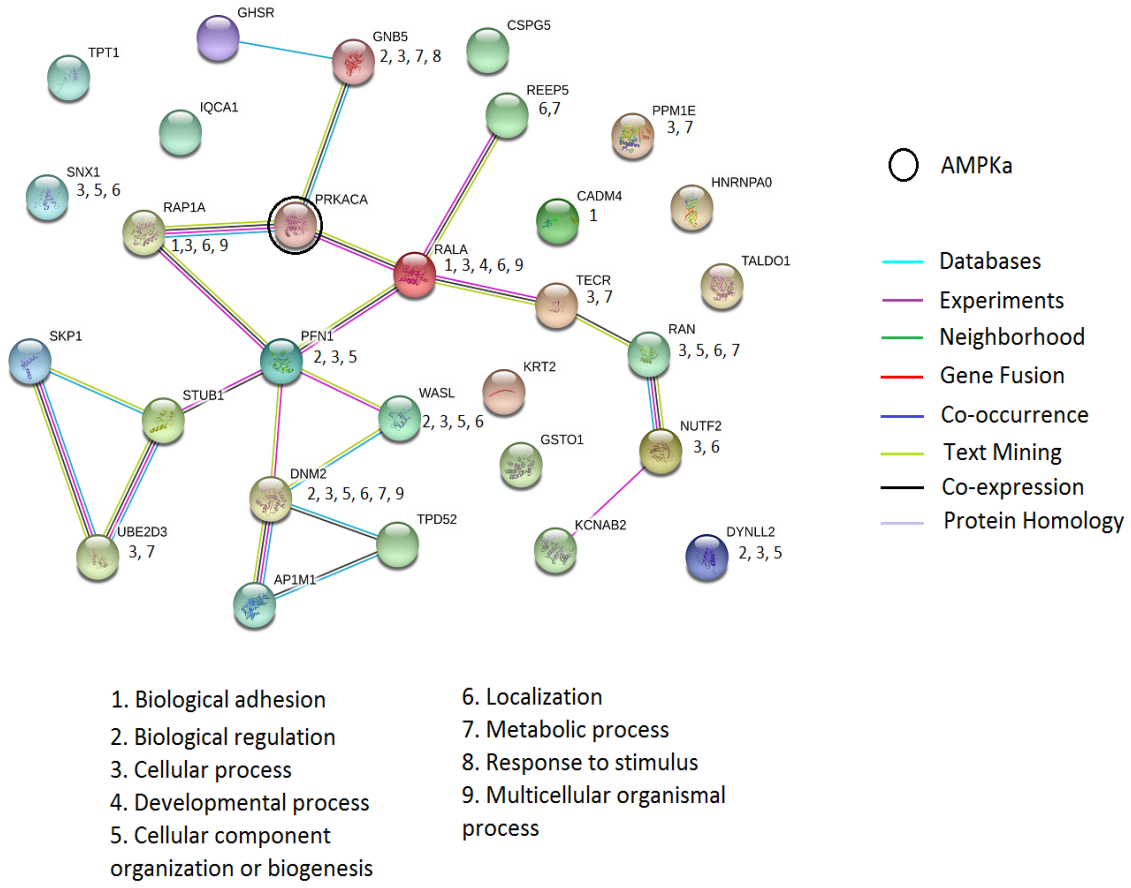


Figure 8. Interaction between AMPKα and the up-regulated proteins. Each node represents a protein and is marked by the name of gene which encodes the protein. AMPKα is circled in black. Figures next to nodes represent different biological processes that the proteins are involved in. Lines in color represent different evidences for each identified interaction.

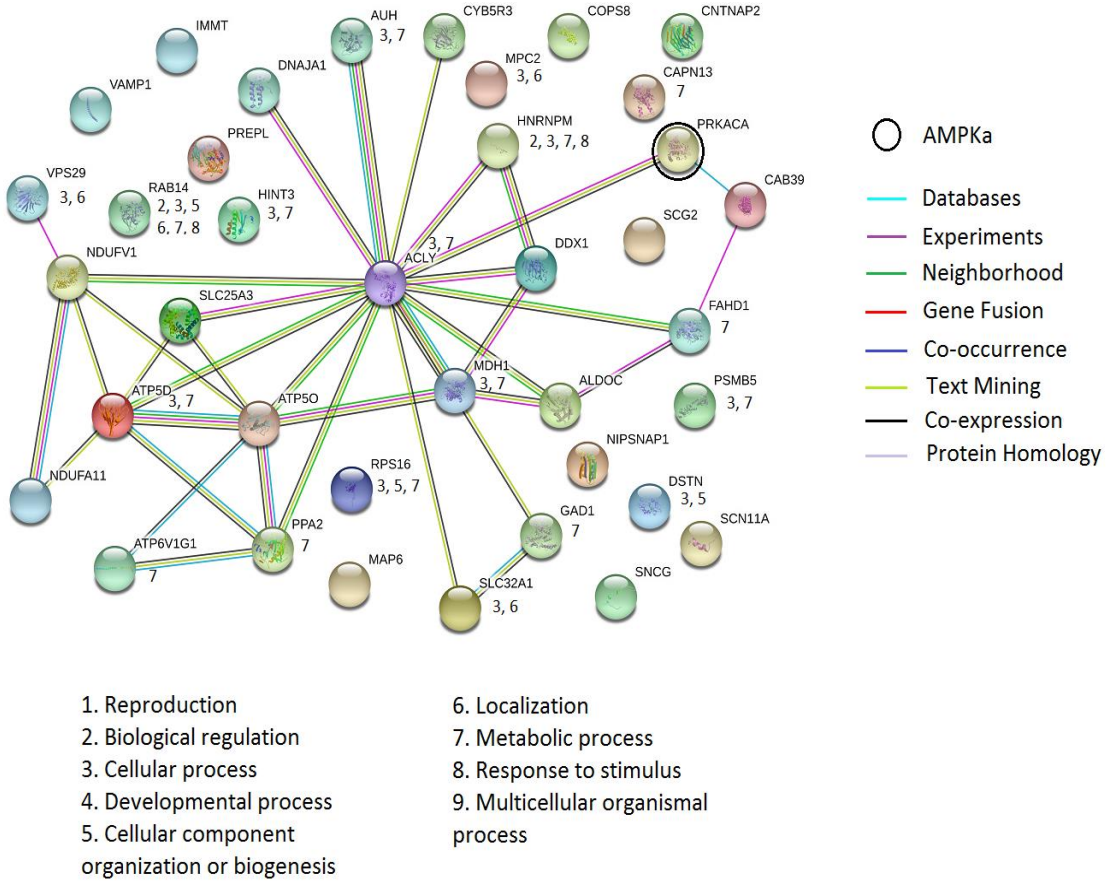


Figure 9. Interaction between AMPKα and the down-regulated proteins. Each node represents a protein and is marked by the name of gene which encodes the protein. AMPKα is circled in black. Figures next to nodes represent different biological processes that the proteins are involved in. Lines in color represent different evidences for each identified interaction.

Discussion

Non-human primates have long been valuable models for the study of human diseases due to their close phyletic relationships with human beings. Many studies carried out in different species of non-human primates had found AD-like pathologies in the brain of these animals when they got old, which resembled the pattern of sporadic Alzheimer's disease in human (Walker L and Jucker M, 2017). In this study, I used vervet monkey, which is an Old World Monkey, as the animal

model, and found AD-like pathologies in their brain. Interestingly, the apolipoprotein E (APOE), which has 3 different alleles in human, is fixed in vervet monkey with a gene sequence corresponding to human APOE4 allele, which will significantly increase the risk of developing Alzheimer's disease in human (Fainman J, et al., 2007).

To identify which monkeys were AD-like and which monkeys were normally aged, I adopted an approach which was similar to that used in the diagnosis of human AD patients (McKhann G, et al., 2011). I combined pathological, biochemical and behavioral assessments to differentiate them. First, Aβ plaque burdens of those monkeys were measured and they varied in those monkeys. I inclined to think those with higher Aβ plaque burden were AD-like while those with lower Aβ plaque burden were normally aged. However, as we know, high Aβ plaque burden is not equal to Alzheimer's disease (Aizenstein H, et al., 2008). Therefore, Aβ42 levels in CSF were measured, which was quite consistent with Aβ plaque burden. Interestingly, Aβ dimer assay revealed heterogeneity of the monkeys with higher Aβ plaque burden. Aβ dimers could only be found in the three monkeys with highest Aβ plaque burden, while not found in the three monkeys with intermediate Aβ plaque burden. What's more, the amounts of Aβ dimers were not correlated with Aβ plaque burden, again indicating that Aβ plaque burden is not equal to the severity of Alzheimer's disease. Aβ dimers and other oligomers have been proved to be the toxic forms of Aβ and are more closely related to the pathogenesis of Alzheimer's disease (Shankar G, et al., 2007; Walsh D and Selkoe D, 2007). Thus, in this study, those with positive Aβ dimer were considered AD-like. The status of those with intermediate Aβ plaque burden was confusing since their Aβ dimer was negative, thus their status was not further addressed in this study.

As in clinical studies, pathological and biochemical alterations should be evaluated in the context of cognitive impairment. However, there was not cognitive assessment in these monkeys. Thus, behavioral assessments were carried out to surrogate cognitive assessment. I found that rates

and time percentages of alert, moving, climbing, jumping and hanging decreased in AD-like monkeys as well as in those with intermediate Aβ plaque burden. Interestingly, rates and time percentages of these behaviors in those with intermediate Aβ plaque burden were also intermediate, indicating that those monkeys were in a status between AD and normal aging. Maybe that's why I couldn't determine their status in this study.

Another concern is that I found typical Aβ plaques in the neocortex of the vervet monkeys but not in the hippocampus, and I only found PHF while not neurofibrillary tangles in the brain of these monkeys. These two points have been demonstrated in different species from different studies (Heilbroner P and Kemper T, 1990; Sani S, et al., 2003; Toledano A, et al., 2012; Heuer E, et al., 2012). It seems that these might be common differences between non-human primates and human beings. Though I didn't find Aβ plaques in hippocampus, I did find neurodegeneration there, which was evidenced by PHF staining and neuron loss. Thus, hippocampus is also affected in this animal model. I found AMPKα2 signaling was dysregulated in the hippocampus, while not in PFC of the monkeys, that's might because AMPK signaling dysregulation is more significant in hippocampus.

As I mentioned in the introduction part, AMPK plays an important role in energy metabolism. Intriguingly, many up-regulated or down-regulated proteins in the proteomic study were related to energy metabolism, indicating that there might be a relationship between AMPK and these proteins. In fact, in the protein-protein analysis, I found that most of those up-regulated or down-regulated proteins could be related to AMPK through a network. Those findings imply that AMPK might be in a pivotal position in the pathogenesis of Alzheimer's disease.

Our study showed that aged vervet monkeys could serve as an animal model for sporadic Alzheimer's disease and AMPK signaling was dysregulated in this model. Thus, this model can be

further used to study the mechanisms of AMPK signaling in Alzheimer's disease and to develop drugs to treat Alzheimer's disease.

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Chapter 2 AMPKα isoforms as a potential biomarker for mild cognitive impairment and Alzheimer's disease

Introduction

Alzheimer's disease (AD) is one of the leading causes of disability and mortality in elderly in the world, especially in the developed countries (Mayeux R and Stern Y, 2012). There are about 5.7 million patients by now in the United States according to reports from Alzheimer's Association. As the aging population grows, the prevalence and incidence have increased dramatically in recent years. As expected, there will be more than 13.8 million patients with Alzheimer's disease in 2050 (Alzheimer's Association, 2018). Accumulating evidences indicate that early diagnosis and intervention of AD may offer the best hope for the patients (Guell-Bosch J, et al., 2016). There are several biomarkers that are currently recommended as diagnostic criteria including Aβ42, Tau and P-Tau in CSF, PiB imaging and MR imaging (McKhann G, et al., 2011). However, those biomarkers are not so sensitive in patients with minor cognitive impairment (MCI) as compared to their performance in AD patients (Olsson B, et al., 2016). Another concern for these biomarkers is that accessibility in primary institutions, remote areas or undeveloped countries is not satisfied. Thus, more feasible and sensitive biomarkers, especially those can be applied in MCI patients, are needed.

Multiple studies linked Alzheimer's disease with energy metabolic dysfunction. Energy metabolic dysfunction takes place in the early stage of Alzheimer's disease, may be the first sign of Alzheimer's disease, thus molecules involved in energy metabolism could be used as a biomarker for the early diagnosis of Alzheimer's disease (Pedros I, et al., 2014). One of the key molecules that play important role in metabolism is the AMP-activated Protein Kinase (AMPK). AMPK is a master energy sensor at cellular level. It senses the ratio of AMP/ATP and regulates catabolic and anabolic pathways to adjust the energy metabolism to an equilibrium (Hardie DG and Carling D,

1997). Besides this fundamental physiological function, AMPK also plays important roles in protein synthesis, autophagy and lipid metabolism (Krishan S, et al., 2014). AMPK has 3 subunits, the α, β and γ. The α subunit is the catalytic subunit and has 2 isoforms, α1 and α2 (Carling D, et al., 2011). It has been found that AMPK is involved in the pathogenesis of Alzheimer's disease (Salminen A, et al., 2011). Here, I carried out this pilot study to investigate whether AMPKα could serve as a biomarker for the early diagnosis of Alzheimer's disease.

Methods and Materials

Subjects

All the subjects were enrolled from clinical studies that were conducted in Wake Forest Baptist hospital, North Carolina, USA. MCI and AD patients were diagnosed according to the recommendation from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Patients who had major diseases like cancer, stroke, heart disease, psychiatric disease, etc., were excluded from our study. Controls are age-matched elderly without complaints in memory loss and history of major diseases. Demographic informations were collected by trained staffs. Neuropsychological test batteries were conducted by trained nurses. Fast bloods were drawn at the time of enrollment with anticoagulant and then centrifuged to separate plasma. Plasmas were stored at -80°C. CSFs were collected and Levels of Aβ42, Tau and phosph-Tau in CSF were determined at the same time. MRI scanning were carried out to measure the thicknesses of related corti and volumes of hippocampus and entorhino cortex using the program FreeSurfer (Schwarz C, et al., 2016). All the subjects and/or their guardians were fully informed of the study and signed the consent form.

Protocol

10 MCI patients, 10 AD patients and 10 controls were enrolled in our study. Levels of AMPKα1 and AMPKα2 in plasma were determined by ELISA kits following the manufacture's instruction (AMPKα1 ELISA kits were purchased from abcam, Cambridge, MA, USA (ab181422), AMPKα2 ELISA kits were purchased from LSBio, Seattle, WA, USA (LS-F10706)). All the samples were duplicated. Outliers were dropped from analysis and would be indicated.

Statistics

Category variables were presented as frequency (percentage) and continuous variables were presented as mean ± standard deviation. Fisher exact test was performed for category variables. 1-way ANOVA with post-hoc Tukey test or Kruskal-Wallis test was performed for continuous variables based on the distribution of the variables if there were more than two groups and t test was performed for continuous variables if there were two groups. Grubbs' test was performed to determine outlier. ROC analysis was performed to calculate AUC, cut-off point, sensitivity and specificity. Linear regression was performed to determine the correlation between levels of AMPKα and other biomarkers. Due to loss to follow-up, data of neuropsychological tests, CSF biomarkers and MRI scanning were incomplete. Those without the follow-up data were excluded from linear regression analysis. All the statistical analyses were conducted using GraphPad 5.0. $P<0.05$ was considered significant.

Result

Demographic and clinical characteristics of those subjects are shown in Table I. No differences were found in gender, age, year of education or diabetes incidence among groups. Apolipoprotein (APOE) ε4 frequency is significantly higher in AD patients. Mini-Mental State Examination (MMSE)

and Category Fluency test are significantly lower in AD patients, while Logical Memory test II (LMII) is significantly lower in both MCI and AD patients. Aβ level in CSF is lower in MCI and AD patients, but not significantly different. P-Tau and total Tau levels in CSF are significantly increased in AD patients. AD signature cortical thicknesses (ADSCT), an unweighted average of thicknesses of corti that specifically atrophy in AD, including bilateral Entorhinal, Inferior Temporal, Middle Temporal, Inferior Parietal, Fusiform, and Precuneus regions, are not significantly different among groups. The same is found in hippocampus and entorhinal cortex volume which have been corrected to the whole cerebral volume, though the trends are declined in MCI and AD patients. In general, results of neuropsychological tests, CSF and imaging biomarkers are consistent with MCI and AD characteristics, though there are variations in some of the indices.

Table I. Demographic and Clinical Characteristics of the subjects.

	Control(n=10)	MCI(n=10)	AD(n=10)	statistics
Male(%)	50.0	50.0	50.0	N.S.
Age(year)	66.5±9.1	68.0±10.5	67.5±7.3	N.S.
Education(year)	16.0±2.3	14.6±2.5	14.1±2.0	N.S.
Diabetes(%)	70.0	80.0	40.0	N.S.
APOE ε4(%)	22.2	5.6	40.0	P=0.0467
MMSE	28.8±1.3	28.5±1.7	23.7±4.4	P=0.0093
LMII	26.0±6.0	17.0±6.5	6.0±6.3	P<0.0001
Category Fluency	26.1±3.8	20.3±5.0	15.4±6.2	P=0.0013
Aβ42 in CSF	543.0±121.5	511.7±193.2	389.0±175.6	N.S.
P-Tau in CSF	33.3±32.2	29.1±10.6	70.8±35.5	P=0.0065
Total Tau in CSF	58.2±34.9	44.5±11.2	109.6±51.2	P=0.0209
ADSCT	2.66±0.07	2.54±0.20	2.51±0.21	N.S.
HC Volume_corrected	0.0027±0.0002	0.0028±0.0005	0.0025±0.0005	N.S.
ERC Volume_corrected	0.0011±0.0001	0.0011±0.0002	0.0010±0.0002	N.S.

Abbreviations: APOE Apolipoprotein E, MMSE Mini-Mental State Examination, LMII Logical Memory test II, ADSCT Alzheimer's disease Signature Cortical Thicknesses, HC Hippocampus, ERC Entorhinal Cortex.

Levels of AMPKα1 and AMPKα2 in different groups are shown in Figure 1. I outlier of AMPKα1 level each in MCI and AD groups is excluded from analysis. Level of AMPKα1 in plasma is

significantly decreased in MCI patients as compared to that in control patients (Fig.1A). Level of AMOKα1 is also decreased in AD patients as compared to that in control group, but not significantly (Fig.1B). When MCI and AD groups are combined, level of AMPKα1 is significantly decreased in the combined group (Fig. 1C). No significant differences are found between control and MCI or AD group (Fig. 1D). When MCI and AD groups are combined, there is still no difference (Fig. 1E&F).

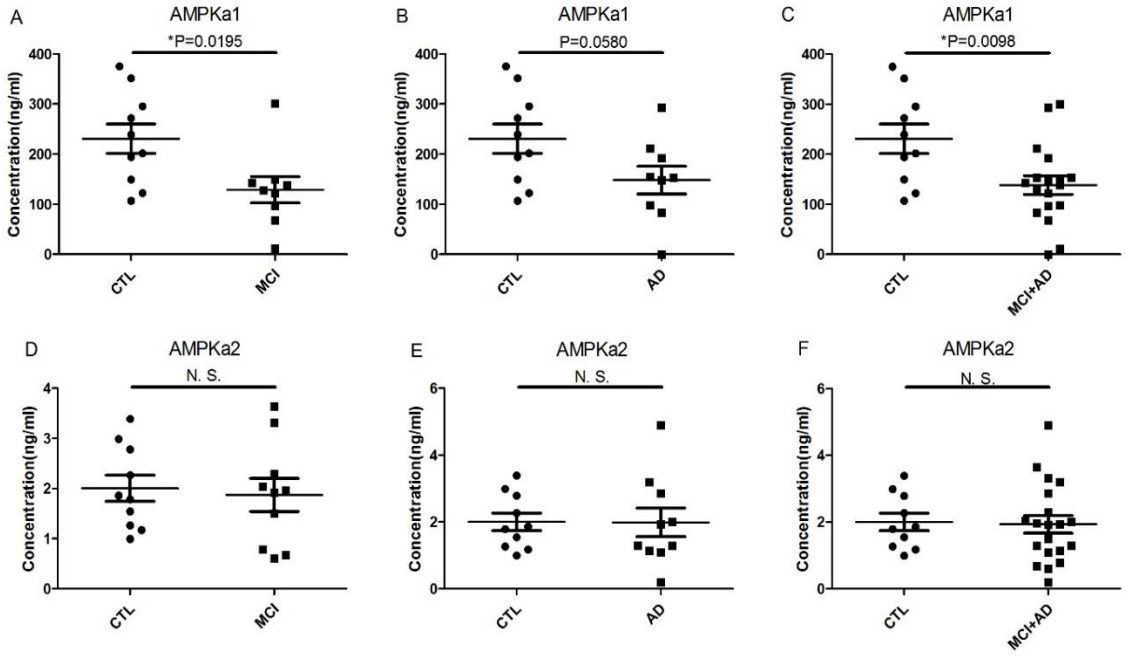

Figure 1. Levels of AMPKα1 and AMPKα2 in different groups. A-C shows comparison of AMPKα1 level between control and MCI, AD or combined group. AMPKα1 level is significantly decreased in MCI patients (p=0.0195), almost significantly decreased in AD patients (P=0.0580) and significantly decreased in combined patients (P=0.0098). D-F shows comparison of AMPKα2 level between control and MCI, AD or combined group. There is no significant difference between these groups (P>0.05).

To evaluate the efficacy and best cut-off point of AMPKα1 as a biomarker, ROC analysis is carried out in MCI, AD and combined patients respectively. ROC analysis in MCI patients shows AUC is 0.8000 and the best cut-off point is 172.1mg/ml with the corresponding sensitivity of 88.89% and specificity of 70.00% to differentiate MCI patients from controls (Fig.2A). ROC analysis in AD patients shows AUC is 0.7444 and the best cut-off point is 193.2mg/ml with the corresponding sensitivity of 77.78% and specificity of 70.00% to differentiate AD patients from controls (Fig. 2B). When MCI and AD pateints are combined as one group, ROC analysis in combined group shows AUC is 0.7722 and the best cut-off point is 193.2mg/ml with the corresponding sensitivity of 83.33% and specificity of 70.00% to differentiate patients from controls (Fig. 2C).

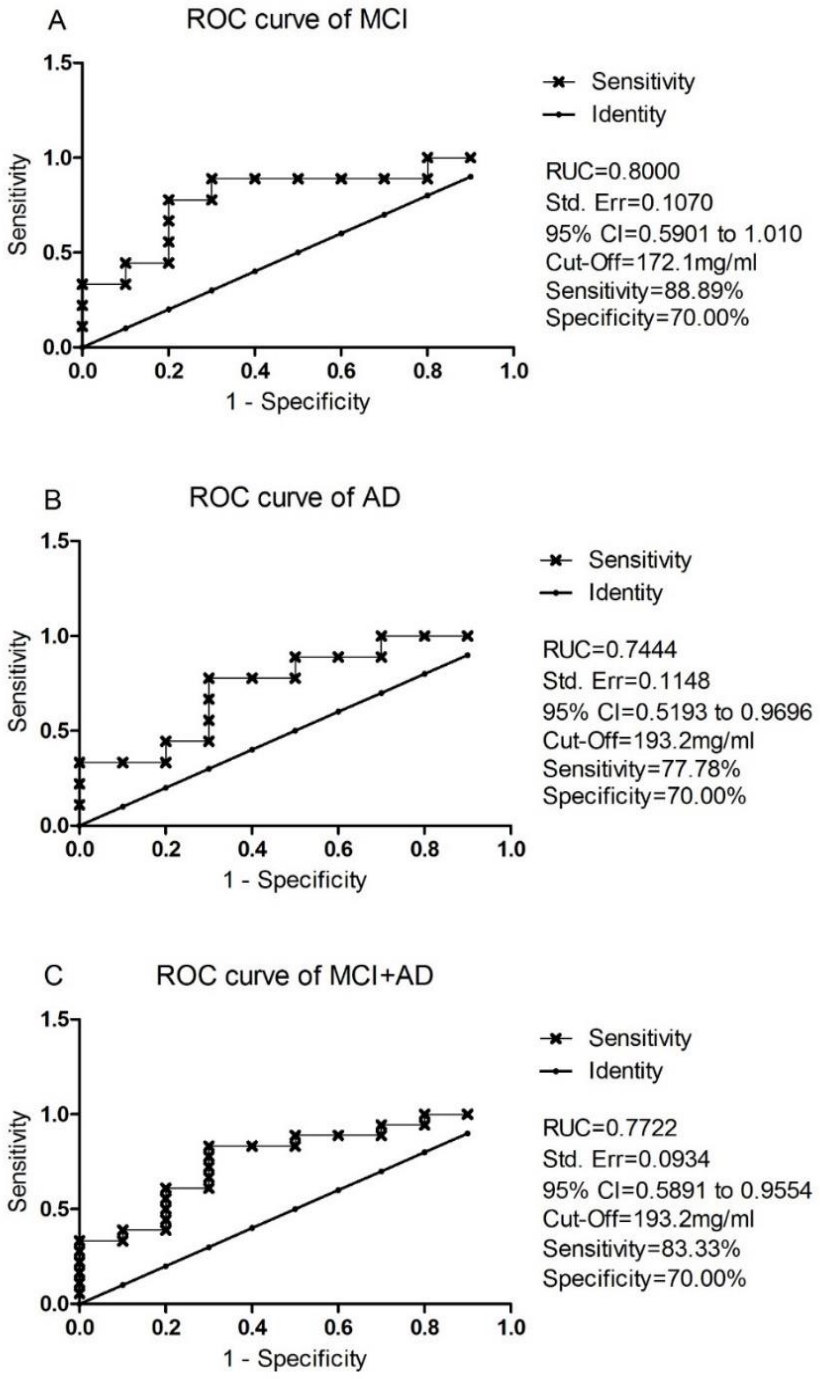

Figure 2. ROC analysis of AMPKα1 as a biomarker. A is ROC analysis in MCI patients, B is ROC analysis in AD patients, and C is ROC analysis in patients without differentiating them into MCI or AD.

Subjects without neuropsychological, CSF or imaging data are excluded from analysis. Level of AMPKα1 is not correlated with CSF biomarkers, including Aβ42 (n=24, Fig. 3A), P-Tau (n=24, Fig. 3B) and total Tau (n=23, Fig. 3C). Level of AMPKα1is correlated with ADSCT (n=25, Fig. 3D), but not correlated with hippocampus volume (n=25, Fig. 3E) or entorhinal cortex volume (n=25, Fig. 3F). Level of AMPKα1 is not correlated with MMSE (n=27, Fig. 3G), logical memory test II (n=21, Fig. 3H) or category fluency test (n=22, Fig. 3I) either.

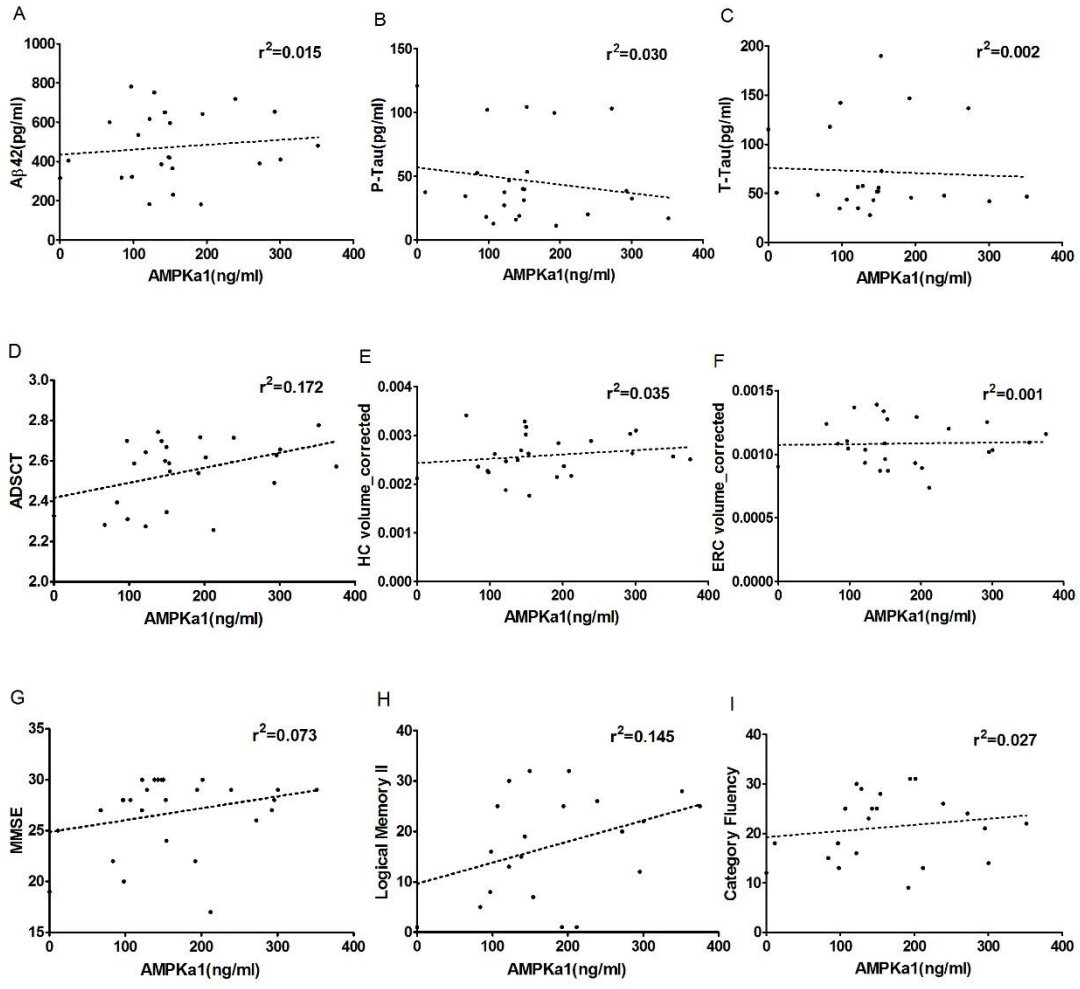

Figure 3. Correlations between AMPKα1 level and other biomarkers. Level of AMPKα1 is not correlated with Aβ42 (A, P>0.05), P-Tau (B, P>0.05) or total Tau (C, P>0.05) in CSF. Level of AMPKα1 is correlated with ADSCT (D, P<0.05), but not correlated with hippocampus volume (E,

P>0.05) or entorhinal cortex volume (F, P>0.05). Level of AMPKα1 is not correlated with MMSE (G, P>0.05), logical memory test II (H, P>0.05) or category fluency test (I, P>0.05) either.

Discussion

Early diagnosis of Alzheimer's disease remains a challenge by now, since many biomarkers that are well established in late-stage of Alzheimer's disease are not significantly altered in early stage of Alzheimer's disease, which also have been indicated in our study. For example, Aβ42, P-Tau and total tau in CSF are not significantly altered in MCI patients (Olsson B, et al., 2016). However, this is a very important issue in Alzheimer's disease. Many clinical trials which were conducted in late-stage AD patients failed, which raised the hypothesis that Alzheimer's disease should be treated as early as possible, since the pathologies of Alzheimer's disease might be only reversible in its early stage (Guell-Bosch J, et al., 2016). Many even tried to define pre-clinical stage of Alzheimer's disease, the stage before patients have symptoms, since it's speculated Alzheimer's disease could only be cured in this stage (Sperling R, et al., 2011). These concerns highlight the importance of biomarkers that can define the early even pre-clinical stage of Alzheimer's disease. Mountains of studies have shown that energy metabolic dysfunction might be the earliest signs of Alzheimer's disease, which makes those molecules that are involved in metabolic process ideal candidates as biomarkers for the early diagnosis of Alzheimer's disease (Kandimalla R, et al., 2017). Thus, I investigated the dysregulation of AMPKα isoforms in MCI and AD patients, which is a hub in energy metabolic signaling network, and found indeed the AMPKα1 isoform is deceased in MCI and AD patients. Another concern in the study of biomarker is accessibility. CSF or imaging biomarkers are relatively less accessible, which prevents their applications. From this perspective, biomarkers derived from blood have advantages over CSF or imaging biomarkers. Based on these two concerns, I suggest AMPKα1 might be a promising biomarker for the early diagnosis of Alzheimer's diseae.

There are also some limitations in our study. First, the number of subjects is relatively small. But even with 10 subjects in each group, I could find level of AMPKα1 in MCI patients is significantly lower as compared to controls. In AD patients, the decease is not significant, this can be explained by the small sample size. In fact, when MCI and AD patients are combined, the significance of difference between patients and controls becomes even distinct. I believe, as the sample size increases, the difference between patients and controls will become significant and stable. Second, the sensitivity and specificity of AMPKα1 as a biomarker is not so high. But considering the sensitivity of AMPKα1 in MCI is almost 90% and corresponding specificity is 70%, AMPKα1 is still a valuable biomarker and can be easily accessed.

Another concern is that level of AMPKα1 is not correlated with other biomarkers, like CSF or imaging biomarkers. This could be explained from two aspects. First, some of the biomarkers are not so sensitive in MCI patients, like Aβ42, P-Tau and total tau in CSF. While AMPKα1 is sensitive in MCI patients. Thus, this disparity of performance leads to the poor correlation between them. Second, some of the biomarkers' performance is not so good in our study, like the imaging biomarkers. Though, they show declined trends in MCI and AD patients, however, the differences are not significant, which might be due to small sample size and great variation. This also leads to the poor correlation between them.

In general, our study shows AMPKα1 is a valuable biomarker with high accessibility and can be used in the early diagnosis of Alzheimer's disease. To further validate the value of AMPKα1 in the diagnosis of Alzheimer's disease, larger clinical study is needed.

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Curriculum Vitae

Education

Bachelor in Medicine (2003-2008)

Nanjing University, Nanjing, Jiangsu, China

Undergraduate Research Advisor: Haifeng Shao, MD

Thesis Title: Catheter-related bloodstream infection caused by nontuberculous mycobacterium

Master in Medicine (2008-2010)

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Master Research Advisor: Yingdong Zhang, MD

Thesis Title: NINJ2 gene polymorphism and stroke

Master in Biomedical Science (2016-2018)

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Clinical Training

Internship (2008-2009)

Medicine Nanjing Drum Tower Hospital, Nanjing, Jiangsu, China

Residency (2010-2013)

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Fellowship (2013-2016)

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Work Experience

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Teaching Experience

Instructor, Neurology (2012-2016)

School of Medicine, Nanjing University, Nanjing, Jiangsu, China

- Lectured, supervised intern medical student

Instructor, Neurology (2012-2016)

Fourth School of Clinical Medicine, Nanjing Medical University, Nanjing, Jiangsu, China

- Lectured, supervised intern medical student

Lab Instructor, Introduction to Neuroscience (2017)

Department of Biology, Wake Forest University, Winston-Salem, NC, USA

- Lectured, supervised labs, developed and graded assignments

Student Director, Scientific Outreach Course (2018)

Wake Forest Graduate School of Arts & Sciences, Winston-Salem, NC, USA

- Taught basic neuroanatomy and neuroscience to K-12 students

Publications

Wang X, Zhou X, Ma T, et al. AMPKa1 is decreased in the plasma of patients with mild cognitive impairment. (In preparation)

Wang X, Zhou X, Ma T, et al. AMPKa2 is dysregulated in the hippocampus of a non-human primate model of Alzheimer's disease. (In preparation)

Wang X, Yin J, Huo X, et al. A comparative study of combination of MRS and ADC and combination of PWI and DWI in the evaluation of penumbra after acute cerebral infarction. *Journal of Clinical Neurology.* 2015, 28(6):401-403.

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Posters and Abstracts

Wang X, Yin J, Huo X, et al. A comparative study of combination of MRS and ADC and combination of PWI and DWI in the evaluation of penumbra after acute cerebral infarction. Chinese Association of Neurology Annual Meeting, Xiamen, Fujian, China (2014)

Invited Oral Presentations

AMPK signaling dysregulation in a non-human primate model of Alzheimer's disease and in AD patients

Neuroscience Graduate Program Seminar Series, Wake Forest University, Winston-Salem, NC, USA (2018)

A comparative study of combination of MRS and ADC and combination of PWI and DWI in the evaluation of penumbra after acute cerebral infarction

Association of Neurology Annual Meeting, Jiangsu Province, China (2012)

Honors and Awards

People's Scholarship	Nanjing University	2004
Outstanding Graduate Award	Nanjing University	2009
Outstanding Staff Award	Nanjing Brain Hospital	2013

Leadership

Chief Residency Physician (2015-2016)

Department of Neurology, Nanjing Brain Hospital, Nanjing, Jiangsu, China

- In charge of resident and intern physicians, managed medical affairs

Secretary of Department (2012-2016)

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- Coordinated with dean of the department to administer the department