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Swedish mutation within amyloid precursor protein modulates global gene expression towards the pathogenesis of Alzheimer's disease

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The Swedish mutation (K595N/M596L) of amyloid precursor protein (APP-swe) has been known to increase abnormal cleavage of cellular APP by Beta-secretase (BACE), which causes tau protein hyperphosphorylation and early-onset Alzheimer's disease (AD). Here, we analyzed the effect of APP-swe in global gene expression using deep transcriptome sequencing technique. We found 283 genes were down-regulated and 348 genes were up-regulated in APP-swe expressing H4-swe cells compared to H4 wild-type cells from a total of approximately 74 million reads of 38 base pairs from each transcriptome. Two independent mechanisms such as kinase and phosphatase signaling cascades leading hyperphosphorylation of tau protein were regulated by the expression of APP-swe. Expressions of catalytic subunit as well as several regulatory subunits of protein phosphatases 2A were decreased. In contrast, expressions of tau-phosphorylating glycogen synthase kinase 3β (GSK-3β), cyclin dependent kinase 5 (CDK5), and cAMP-dependent protein kinase A (PKA) catalytic subunit were increased. Moreover, the expression of AD-related Aquaporin 1 and presenilin 2 expression was regulated by APP-swe. Taken together, we propose that the expression of APP-swe modulates global gene expression directed to AD pathogenesis. [BMB reports 2010; 43(10): 704-709]

INTRODUCTION

The development of gene expression microarrays in the mid 1990s represented a significant technical achievement that permitted the systematic genome-wide evaluation of gene expression (1, 2). The ability of microarrays has led to important advances in a wide range of biological problems, including the identification of gene expression differences among diseased and healthy tissues, and new insights into developmental processes pharmacological responses, and of the evolution of gene regulation (3-6). Nonetheless, array technology has several limitations. For example, background levels of hybridization (i.e., hybridization to a probe that occurs irrespective of the corresponding transcript's expression level) limit the accuracy of expression measurements, particularly for transcripts present in low abundance. Furthermore, probes differ considerably in their hybridization properties (7).

Deep transcriptome sequencing (RNA-Seq) has recently emerged as a method enabling the study of RNA-based regulatory mechanisms in a genome-wide manner (8). RNA-Seq overcomes several shortcomings of microarray-based detection of transcripts, including probe cross-hybridization (9), restricted signal dynamic range, and low sensitivity and specificity, which often lead to difficulties in the detection of low abundance transcripts and discrimination between similar sequences. Sequence level transcript information has much greater power to distinguish between paralogous genes, better detection of low abundance transcripts, and allows replicable digital quantification based upon counting sequence reads (10-14). Furthermore, RNA-Seq can identify transcript sequence polymorphisms, RNA editing sites, and splicing variants, and there is no strict requirement for a reference genome sequence (15).

Alzheimer's disease (AD) is characterized by memory impairment caused by extracellular senile plaques, intracellular neurofibrillary tangles, and neuronal death. There have been extensive reports that the pathogenesis of this disease is closely related to abnormal proteolytic cleavage of amyloid precursor protein (APP), which leads to deposits of insoluble Aβ aggregates.

Recently, it has been reported that the APP intracellular domain (AICD) moves to nucleus and regulates transcriptional transactivation (16, 17). The nuclear localization of AICD was proposed to be interacting with adapter protein Fe65 and his-

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tone acetyl transferase Tip60. Several target genes of this transcriptional modulation include glycogen synthase kinase-3 beta, CD82, and APP (18-21). APP also controls aquaporin 1 gene expression by epigenetic control mechanism (22). The Swedish mutation of APP, replacement of Lys-595 and Met-596 with Asp and Lys respectively, enhances early-onset and propagation of AD, and leads to the cognitive impairments associated with AD (23).

In this study, we investigated the role of APP-swe mutation in the pathogenesis of AD with regard to transcriptional regulation using deep transcriptome sequencing technique. We demonstrated that the exogenous expression of APP-swe modulated global gene expression including AD-related phosphatases, kinases, presenilin2, and glycogen synthase kinase 3β.

RESULTS

Transcriptome analysis of H4 cell line expressing APP-swe mutant

We investigated the transcriptomes of wild-type H4 and H4-swe cells by sequencing at a depth of roughly 74 million short reads per sample. Gene expression was estimated by calculating read density as 'reads per kilobase of exon model per million mapped reads' (RPKM). These estimates are typically performed using common gene annotation (e.g., RefSeq) with the entire annotated transcript representing the 'exon model'. To identify the APP-swe-regulated genes, we compared the number of reads mapped to transcripts. In each sample, approximately 20,000 known RefSeq genes were detected by one or more reads (Table 1). Many of the 20,000 mRNAs in each sample were represented by only a few reads. We observed an estimated 15,000 genes at a threshold of 20 reads per gene. We determined that the expression of 283 up-regulated and 348 down-regulated genes differed significantly between the control and APP-swe-transfected cells with the criteria of a fold change greater than 2 and transcript number larger than 3. Genes which were up-regulated by greater than 4-fold (Supplementary Table 1A) or down-regulated by greater than 7-fold (Supplementary Table 1B) in APP-swe-transfected cell compared with control cells were presented. The stable cell line expressing APP-swe mutant showed 1.5-fold increase in APP transcript compared to wild type cells expressing wild-type APP.

Gene Ontology (GO) analysis

We used the PANTHER (www.pantherdb.org) to categorize the up-and down-regulated genes identified in the current study. We performed GO analysis with expressed genes whose expression was up- and down- regulated by greater than 2-fold change in APP-swe-transfected H4 cells compared with wild-type H4 control cells.

Gene Ontology analysis revealed that genes involved in synthase and synthetase and nucleic acid binding were specifically enriched in the up-regulation category (Supplementary

Table 1. Transcriptome sequencing overview

Sample Name	H4	HASW
Total # of Reads	74,641,392	75,730,050
Average read length	38	38
Total base sequence	2,836,372,896	2,877,741,900
No. of genes observed with > = 1 read	20,252	20,190
No. of genes observed with > = 2 read	19,286	19,224
No. of genes observed with > = 20 read	14,866	14,937
No. of genes observed with > = 100 read	12,391	12,429

Total base mapped to 25,627 known Refseq. Genes

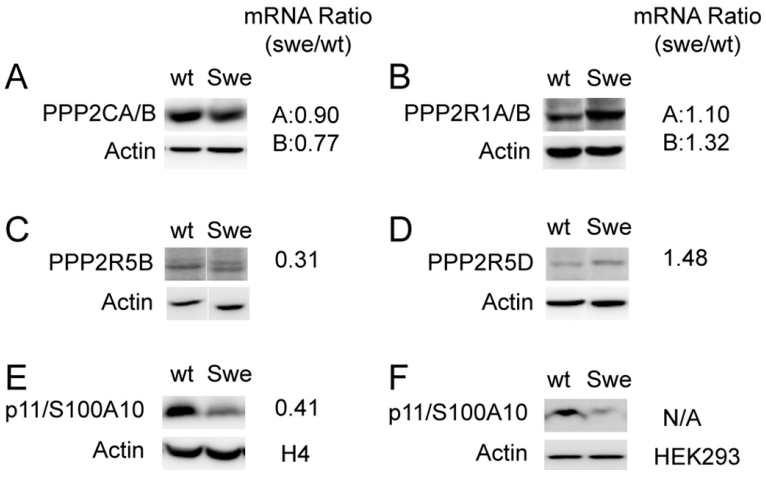

Fig. 1. Validation of transcriptome data with western blotting. Expression levels of PP2A catalytic C subunit alpha and beta PPP2CA/B (A), structural A subunit alpha and beta PPP2R1A/B (B), and two regulatory B subunits PPP2R5B (C) and PPP2R5D (D) were analyzed by Western blotting in H4 wt or H4-swe. The expression of p11/S100A10 was measured in lysates from H4 wt, H4-swe (E), HEK293 wt or HEK293-swe (F) cell lines. mRNA ratio represented fold difference of each mRNA transcript number of APP-swe cells compared with wt cells.

Table 2A). Down regulated genes were enriched in categories involved in defense/immunity protein and select calcium binding protein (Supplementary Table 2B).

Validation of the data with western blot

To validate results of the RNA-Seq analysis, we performed Western blots for several proteins which included protein kinase C alpha isoform, PP2A structural A subunit, PP2A catalytic C subunit, PPP2R5D and PPP2R5B regulatory B subunits, and S100A10 protein. Up-regulated genes in transcriptome sequencing, PPP2R1A (PP2A structural subunit A alpha isoform), PPP2R1B (PP2A structural subunit A beta isoform), and PPP2R5D (PP2A regulatory B56delta subunit) showed increased expressions with Western blotting. Down-regulated genes; PPP2CA (PP2A catalytic C subunit alpha isoform), PPP2CB (PP2A catalytic C subunit beta isoform), and PPP2R5B (PP2A regulatory B56beta subunit) showed decreased expressions with Western blotting. S100A10, one of S100 gene family, showed decreased expressions in H4-swe cells and HEK293 which expressed APP-swe (HEK293-swe) genes (Fig. 1). These results indicated that the expression of APP-swe mutant in cells alters gene transcription including transcription factors.

Genes related to Alzheimer's disease pathogenesis

The Familial Alzheimer's disease (FAD) Swedish mutation in amyloid precursor protein has been know as an initiator of Alzheimer's disease pathogenesis by increasing Aβ peptide accumulation in brain. Along with a mutation within amyloid protein, several important genes have been identified in brains with AD. Presenilin (PSEN) interacts with gamma-secretase, and makes an unusual protease that processes AD-causing Aβ peptide (19, 25). Glycogen synthase kinase 3β regulates tau phosphorylation, and overexpressing GSK3β causes AD-like tau protein hyperphosphorylation. Protein phosphatase type 2A (PP2A) also regulates tau phosphorylation by direct or indirect mechanisms (24, 25). Recently, aquaporin 1 expression was reported to be down-regulated by knocking down the expression of APP or presenilin 2 (18). Interestingly, these genes related to AD pathogenesis were regulated by expressing APP-swe in H4 cells. Presenilin 2 (1.57-fold), AQP1 (0.2-fold) glycogen synthase kinase 3β (1.35-fold), and tau phosphorylating cyclin-dependent kinase 5 (CDK5) (1.89-fold) were up-regulated in H4-swe cells (Table 2). Moreover, the expression level of PP2A, a phosphatase directed to tau protein, was affected by expressing APP-swe. Expression of catalytic C subunit alpha and beta was reduced by 0.9- and 0.77-fold respectively, while regulatory B subunits varied in their expression from 0.31- to 1.48-fold. The expression of PPP2R2A B subunit (B alpha), which has been known as a major regulatory subunit for tau dephosphorylation was reduced by 0.82-fold. APP-swe expressing H4 cells and HEK293 cells showed an increase in basal phosphorylation level of tau protein. We analyzed the phosphorylation level of three phosphorylation sites of tau protein; Ser202/Thr205, Thr231, and Ser422, all of which were reported as to be dephosphorylated by PP2A. The basal phosphorylation level of Ser422 and Thr231 was increased in H4-swe compared to H4 wild-type cell line, and Ser422 phosphorylation was increased in APP-swe expressing HEK293 cells (Fig. 2). These results indicate that the regulation of gene expression by APP-swe was directed to Alzheimer's disease pathogenesis in which kinases, protease, and phosphatases play a critical role.

Table 2. Expression levels of genes related to Alzheimer's disease pathogenesis

Gene symbol	Gene name	Folds difference (H4swe/H4wt)
CDK5	Cyclin dependent kinase 5	1.897888
GSK3A	Glycogen synthase kinase 3 alpha	1.192308
GSK3B	Glycogen synthase kinase 3 beta	1.350174
PRKACA	PKA catalytic subunit alpha	0.988075
PRKACB	PKA catalytic subunit beta	1.514196
AQP1	Aquaporin 1	0.195865
PSEN2	Presenilin2	1.573918
APBB1	Fe65 APP-interacting protein	2.917772
PPP2CB	PP2A Catalytic subunit beta	0.773054
PPP2R1B	PP2A structural subunit beta	1.328273
APBB1	Fe65 APP-interacting protein	2.917772
PPP2R2A	PP2A regulatory B subunit	0.825934
PPP2R2C	PP2A regulatory B subunit	0.412371
PPP2R3A	PP2A regulatory B subunit	0.630915
PPP2R5B	PP2A regulatory B subunit	0.310479
PPP2R5D	PP2A regulatory B subunit	1.485033

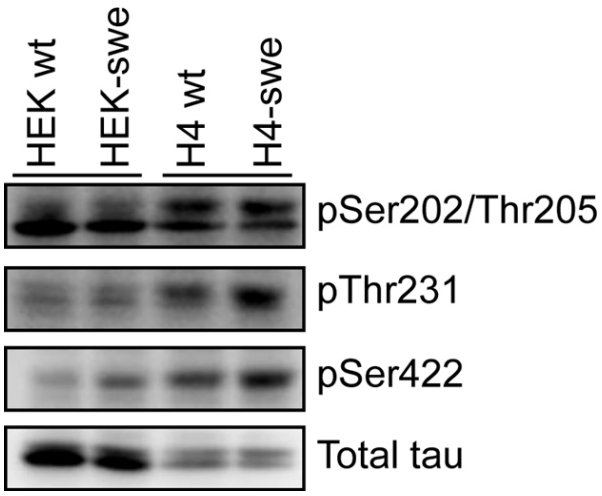

Fig. 2. Phosphorylation of tau protein was increased in APP-swe expressing cells. Basal tau phosphorylation on PP2A target residues Ser202/Thr205, Thr231, and Ser422 was analyzed in wt or APP-swe expressing cells. Lysates from each cell were probed with its respective phospho-specific antibody.

DISCUSSION

Here, we describe a new approach for sequence analysis of transcriptome, which involves generating random primed cDNA libraries, followed by sequencing using Illumina GAIIx. Analysis of those 74 million randomly derived sequence reads aligned to the human genome showed that the approach can be used to study gene expression.

The processed cytosolic fragment of APP has been reported to affect expression of several genes by moving to the nucleus. AICD interacts with Fe65, Jip1b, MINT1, and Tip60 to make a transcription factors for several genes. The expression of AICD-interacting proteins was increased, for example in Fe65 (2.99-fold). KAI1/CD82 was reported as a target gene of APP intracellular domain (AICD) (20, 21), and its expression was increased in APP-swe by 1.43-fold. Besides the direct role of AICD as a transcription factor, epigenetic regulation including DNA methylation and histone modification might explain the global gene expression by APP-swe mutant. Recent studies have shown that an expression of S100 protein family is regulated by DNA methylation (26), and demethylating agent improved cognitive function in an animal model.

Gene ontology analysis revealed that proteins in the transcription factor category were largely affected by APP-swe. Up-regulated and down-regulated transcription factors were second-most affected genes, and 32 transcription factors were up-regulated and 39 transcription factors were down-regulated in H4 cells expressing APP-swe. These changes in transcription factor expression might explain the huge difference between H4 wt and H4-swe cell.

The expression of S100 protein family including S100A2, S100A4, S100A6, and S100A10 has been demonstrated to be largely dependent on CpG methylation (26). We tested the global DNA methylation pattern with H4 wt and H4-swe cell lines. Result showed that H4-swe cells generally expressed a hypo-methylation pattern compared to H4 wt cells, and S100A10 promoter CpG was hypo-methylated (H4: 0.25 vs H4-swe: 0.11 in average methylation index) indicating that promoter CpG methylation was discordant with the expression pattern of S100A10 protein (supplement data). The methylation index of KAI-1/CD82 (H4 wt: 0.027 vs H4-swe: 0.036), APP (H4 wt: 0.041 vs H4-swe: 0.057), presenilin 2 (H4 wt: 0.43 vs H4-swe: 0.56), and aquaporin (H4 wt: 0.66 vs H4-swe: 0.52) showed that the expression changes by APP-swe was discordant with promoter methylation. It is notable that the expression of APP-swe plus APP wild-type is increased only by 1.7-fold, and there were huge changes in global gene expression including several AICD-target genes. We did not check the actual expression stoichiometry between APP-wt and APP-swe, and the expression of APP-swe seems sufficient based on the large alterations in gene expression. These results suggested that direct modulation by AICD-mediated transcription and/or indirect modulation by regulating the expression of transcription factors might be major factors that affect the global gene expression by APP-swe.

Our transcriptome sequencing results showed the expression of genes involved in Alzheimer's disease was affected by APP mutation that leads to Alzheimer's disease pathogenesis. However, the precise mechanism of APP-mediated regulation of global gene expression remains to be elucidated.

MATERIALS AND METHODS

Cell culture and transfection

Human glioblastoma H4 cells and APP-swe expressing stable cell line H4-swe (K670N/M671L) were cultured in DMEM (GIBCO #11995) containing 10% fetal bovine serum (GIBCO #16000), 100 Units/ml penicillin, 2 mM glutamine and 100 μg/ml streptomycin (GIBCO #15140). The media for H4-swe cells are supplemented with 500 μg/ml geneticin (GIBCO #10131-027) to maintain APP-swe expression.

RNA preparation

Total RNA was extracted from cell lines using RNeasy Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). One microgram of total RNA was converted to cDNA using Superscript II reverse transcriptase and oligo-$(dT)_{12\text{-}18}$ primer (Invitrogen).

RNA-Sequencing

Total RNA (5 μg) was subjected to two rounds of hybridization to oligo (dT) beads (Dynal). The resulting mRNA was then used as template for cDNA synthesis. The mRNA was randomly fragmented to between 200 and 700 bp by focused acoustic shearing (Covaris Inc.) and converted to first strand cDNA using Superscript III (Invitrogen), followed by second-strand cDNA synthesis using Escherichia coli DNA pol I (Invitrogen). The double stranded cDNA library was further processed by Illumina Genomic DNA Sample Prep kit. The procedure involved end repair using T4 DNA polymerase, Klenow DNA polymerase and T4 Polynucleotide kinase followed by a single adenosine base addition using Klenow 3' to 5' exo- polymerase, and was ligated with Illumina's adaptor oligo mix using T4 DNA ligase. Adaptor-ligated library was size selected by separating on a 2% agarose gel and cutting out the library smear at 500 bp. The library was PCR amplified for 18 cycles using phusion polymerase and purified by Qiaquick PCR Purification Kit (Qiagen). The library was quantified by Quant- iT picogreen dsDNA Assay Kit (Invitrogen) following the manufacturer's protocol. We prepared Genome Analyser paired- end flow cell on the supplied Illumina cluster station and generated 38-bp paired-end sequence reads on the Illumina Genome Analyser *IIx* platform following the manufacturer's protocol. Primary data analysis including image analysis, base-calling and alignment was carried out with the Illumina pipeline.

Western blotting

For analysis of protein expression, 25 μg of protein and low-range molecular weight standards were electrophoresed on a 12% acrylamide SDS-PAGE gel and immunoblotted onto polyvinylidene diflouride (PVDF) membranes (Immobilon-P; Millipore, Bedford, Massachusetts). The membranes were blocked for 1 h at room temperature (7.5% nonfat dry milk in 137 mM NaCl, 25 mM Tris, 3 mM KCl, 25 mM Tris-HCl, 0.2% Tween 20 and phosphatase inhibitor cocktail (1 : 100)) and incubated with primary antibody (1 : 1,000) for 2 hours in room temperature. Antibody binding was detected using a goat anti-mouse horseradish peroxidase-linked IgG (1 : 10,000; Bio Rad, Hercules, California) and ECL immunoblotting detection system (Amersham Pharmacia, Piscataway, New Jersey).

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