Functional analysis of APOE locus genetic variation implicates regional enhancers in the regulation of both TOMM40 and APOE

Lynn M Bekris1,2, Franziska Lutz1 and Chang-En Yu1,2

Genetic variation within the apolipoprotein E gene (APOE) locus is associated with late-onset Alzheimer’s disease risk and quantitative traits as well as apoE expression in multiple tissues. The aim of this investigation was to explore the influence of APOE locus cis-regulatory element enhancer region genetic variation on regional gene promoter activity. Luciferase reporter constructs containing haplotypes of APOE locus gene promoters; APOE, APOC1 and TOMM40, and regional putative enhancers; TOMM40 intervening sequence (IVS)2-4, TOMM40 IVS6 poly-T, as well as previously described enhancers; multienhancer 1 (ME1), or brain control region (BCR), were evaluated for their effects on luciferase activity in three human cell lines. Results of this investigation demonstrate that in SHSY5Y cells, the APOE promoter is significantly influenced by the TOMM40 IVS2-4 and ME1, and the TOMM40 promoter is significantly influenced by the TOMM40 IVS6 poly-T, ME1 and BCR. In HepG2 cells, the TOMM40 promoter is significantly influenced by all four enhancers, whereas the APOE promoter is not influenced by any of the enhancers. The main novel finding of this investigation was that multiple APOE locus cis-elements influence both APOE and TOMM40 promoter activity according to haplotype and cell type, suggesting that a complex transcriptional regulatory structure modulates regional gene expression.

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INTRODUCTION

Multiple genetic variations within the apolipoprotein E gene (APOE) locus are strongly associated with late-onset Alzheimer’s disease (LOAD) risk.1 The ε4 allele of APOE is the strongest known genetic risk factor for LOAD.2–4 However, inheritance of the APOE ε4 is neither necessary nor sufficient to cause the disease,5,6 and the mechanism by which the ε4 allele functionally influences the risk and progression of LOAD remains unknown.

Other genetic elements in the APOE locus, such as APOE promoter polymorphisms, have been reported to be associated with LOAD, including single-nucleotide polymorphisms (SNPs) in position −491, −427, −219 (Th1/E47cs) and +113 as well as gene expression.7–11 Thus, it has been postulated that expression levels of the APOE gene product can also contribute to LOAD risk. In addition to the APOE promoter, other cis-regulatory elements of APOE have been characterized, including multienhancer 1 (ME1), which influences APOE regulation in macrophages and adipocytes,12,13 and a brain control region (BCR) that can modulate APOE expression in neurons and microglial.14

SNPs within the TOMM40 gene have robustly shown association with LOAD in multiple genome-wide association studies.15–18 The TOMM40 SNP, rs2075650, located within the intervening sequence (IVS) 2, has been associated with both LOAD risk17,19 and quantitative traits,20–23 such as age-at-onset and Aβ42 levels, suggesting that the TOMM40 region contributes to LOAD phenotypes. Our recent study has reported that IVS 2 SNPs within the TOMM40 gene, as well as other SNPs both proximal and distal to APOE, are associated with cerebrospinal fluid apoE levels24 and post-mortem brain apoE expression in Alzheimer’s disease (AD) hippocampus.25 Recently, it has been reported that a poly-T polymorphism (rs10524523) within IVS 6 of TOMM40 is associated with LOAD age-at-onset.26

A report on APOE locus linkage disequilibrium (LD) patterns suggests that strong LD with APOE ε4 exists both proximal (in a region partially spanning the TOMM40 gene) and distal to APOE (in the ME1 region).4 Interestingly, the association between TOMM40 and LOAD risk is not fully explained by LD between TOMM40 SNPs and the APOE SNP (rs429358) that defines ε4 status, suggesting that other APOE locus SNPs contribute to this association with LOAD.4
Given that multiple SNPs spanning a large region at the APOE locus are associated with LOAD risk, LOAD-specific quantitative traits, age-at-onset, LD and apoE expression levels, we hypothesized that a unique haplotype structure functionally influences the expression of multiple genes at the APOE locus. Thus, the aim of this investigation was to demonstrate that promoter activity of three APOE locus genes (TOMM40, APOE and APOC1) is influenced by previously described regulatory element enhancers (ME1, BCR) and putative regulatory element enhancers (TOMM40 IVS2-4, TOMM40 IVS6 Poly-T) differentially according to haplotype.

The main novel finding of this investigation was that genomic regions within TOMM40 influence both TOMM40 and APOE, but not APOC1 promoter activity, suggesting that these two genes may be co-regulated. Furthermore, APOE locus enhancers influence both TOMM40 and APOE promoters according to haplotype and cell type, implicating a complex system of gene regulation that is specific to genetic content and cellular microenvironment.

**MATERIALS AND METHODS**

**APOE locus LD structure and trans-acting factor site prediction**

The pattern of pairwise LD was measured for the APOE locus by D’ metrics of chromosome 19: 50,080–50,150 kb. Haplotypes were constructed using the algorithm implemented in Haploview (version 4.2) for SNPs available with a minor allele frequency > 0.01 for the Haploview Caucasian of Northern and Western European descent sample (CEU)27 (Figure 1).

Trans-acting factor sites were predicted for APOE locus promoters and enhancers using UCSC human genome browser ENCODE data for histone marker sites and DNase I hypersensitivity sites28,29 (Figure 2).

**Generation of regulatory haplotype reporters**

Genomic DNA was obtained from the University of Washington (UW) Alzheimer’s Disease Research Center after approval by the human subject Institutional Review Boards of UW and Veterans Affairs Puget Health Care System. Our regions of interest were PCR amplified and DNA sequenced from genomic DNA of 32 Caucasian subjects. See supplement for list of primer sequences. Two to three haplotypes for each regulatory genetic region of interest were chosen according to sequenced SNP content and frequency (Table 1). Variants of TOMM40, APOE or APOC1 promoter core region haplotypes were inserted 3′ to the luciferase gene of the pGL4.10[ luc2] vector (Promega, Madison, WI, USA) to produce promoter-only constructs. To produce promoter–enhancer constructs, haplotype variants of TOMM40 IVS2-4, TOMM40 IVS6 poly-T, ME1 or BCR genomic regions were inserted into the promoter constructs 3′ to the luciferase gene. The In-Fusion PCR Cloning System (Clontech, Mountain View, CA, USA) was used for all the cloning procedures. After propagating the recombinant DNA in *Escherichia coli* host cell, the reporter constructs were isolated and purified by ion-exchange column (Qiagen, Valencia, CA, USA). Inserts of all constructs were fully DNA sequenced to validate the correct genetic contents.

**Cell culture**

Human neuroblastoma SHSY5Y cells (ATCC, Manassas, VA, USA) were grown in 44.5% Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY, USA) with 44.5% F12 (Gibco), 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (100 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin) at 37 °C in a 5% CO₂ atmosphere. Human hepatocytoma HepG2 cells (ATCC) and U118 astrocytoma cells (ATCC) were grown in 89% Dulbecco’s modified Eagle’s medium (Gibco), 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (100 μg ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin) at 37 °C in a 5% CO₂ atmosphere. Cells were passaged at 5×10⁴ per well into 96-well tissue culture plates 48 h before transfection.
Luciferase reporter construct transfection and assay

SHSYSY and HepG2 cells were transiently transfected for 48 h with promoter–enhancer reporter constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. All transfection assays included a TOMM40, APOE and APOC1 promoter-only (no enhancer) luciferase pGL4.10[luc2] haplotype reporter constructs as basal expression controls and promoter-less luciferase pGL4.10[luc2] as a negative control. In addition, the renilla pGL4.75[hRluc/CMV] was co-transfected as an internal control. All transfection assays included duplicate constructs, and transfection assays were performed in triplicate. Failed transfection reactions were duplicated, leading to 6–8 replicate transfections for each haplotype reporter construct.

SHSYSY, HepG2 and U118 transiently transfected cells were harvested after 48 h using the Dual Glo Luciferase Assay System (Promega), which allows for high throughput analysis of firefly luciferase (pGL4.10[luc2]) constructs in the first step. In the second step, after quenching the firefly luminescence and activating the renilla luciferase (pGL4.75[hRluc/CMV]), luminescence from the internal control was analyzed. Luciferase luminescent counts per second (CPS) were measured using a Wallac Victor2 1420 Multilabel Counter (Wallac, Waltham, MA, USA), with renilla luciferase luminescence counts per second (CPS) for each transfected construct, per manufacturers instructions.

Relative quantitation of haplotypes expression levels and statistical analysis

To measure the enhancer effect on promoter activity, luciferase activities of promoter-only constructs served as baseline controls (set at 1.0) and were compared with promoter–enhancer constructs. First, a ratio of F/R CPS was calculated as firefly (F) luciferase construct CPS per renilla (R) luciferase internal control CPS for each transfected construct, per manufacturers instructions (Dual Glo Luciferase Assay System, Promega). Haplotype expression levels relative to promoter was calculated for each promoter–enhancer reporter construct (that is, F/R APOE-promoter haplotype 1-ME1 haplotype 2/F/R APOE-promoter haplotype 1). This data were then analyzed using an analysis of variance to compare haplotypes (SPSS Version 13, Armonk, NY, USA). All statistical tests within specific promoter–enhancer haplotype groups were computed by Bonferroni correction for multiple comparisons.

RESULTS

APOE locus genomic signature

To evaluate the LD structure of the 64-kb APOE locus, SNPs spanning from the TOMM40 5’ region to the BCR were analyzed using Haploview. The promoter and enhancer regions of interest in this investigation: TOMM40, APOE and APOC1 promoter regions; ME1 and BCR regulatory element enhancers; TOMM40 IVS2-4, TOMM40 IVS6 Poly-T putative regulatory element enhancers, are either in strong or moderate LD with each other or in the case of the BCR, are not in LD with other selected genetic elements (Figure 1). Except for the TOMM40 IVS6 poly-T, all the promoter and enhancer regions of interest are consistently overlapped with the ENCODE tracks in the UCSC human genome browser. These ENCODE tracks include DNase I hypersensitivity sites, histone mark H3K4Me3 sites for promoter and H3K4Me1 sites for enhancers (Figure 2a).

Regulatory haplotype reporter constructs

Putative regulatory haplotype content was identified by SNP genotyping of human subjects and amplified from subject genomic DNA. Putative regulatory haplotype variants were then verified by sequencing, and cloned into the luciferase reporter constructs as shown in Figure 2b. Thus, a large panel of luciferase reporter clones were generated that included eight promoter-only constructs (three promoters with a total of eight variants, Table 1) and 80 promoter–enhancer haplotype constructs (8 promoter haplotypes plus 10 enhancer haplotypes; Table 1). Luciferase gene activities of reporter

Figure 2 APOE locus putative promoter-enhancer regions. Regional genes include:TOMM40, APOE, APOC1, APOC4, and APOC2. TOMM40 promoter, APOE promoter, APOC1 promoter haplotype regions are shown as red bars. Putative enhancer haplotype regions; TOMM40 IVS2-4, TOMM40 IVS6 Poly-T and previously described enhancer haplotype regions; ME1 and BCR are shown as black bars. The APOE locus containing regulatory haplotype regions tested is located at chr19:50,893,899-50,148,827 as indicated on the UCSC Genome Browser (created by the Genome Bioinformatics Group of UC Santa Cruz, assembly March 2006 (NCBI 36/hg18): http://genome.ucsc.edu/index.html. ENCODE promoter (H3K4Me3) and enhancer (H3K4Me1) associated histone marks from multiple cell lines (H3K4Me3) overlap with regulatory haplotype reporter construct sites. ENCODE DNase1 hypersensitivity cluster results overlap with regulatory haplotype reporter construct sites (a). Regulatory haplotype luciferase reporter construct maps include promoter haplotype only constructs and promoter-enhancer haplotype construct map (b).
constructs were measured in the three human cell lines that included a neuronal cell line (SHSY5Y), a hepatocyte cell line (HepG2) and an astrocyte cell line (U118). No statistically significant differences were observed among APOC1 promoter–enhancer haplotypes (data not shown). In contrast, statistically significant differences in luciferase gene expression were observed for TOMM40 and APOE promoter–enhancer haplotypes in both SHSY5Y and HepG2 cells, but not U118 cells (Figure 3).

### Table 1  APOE locus haplotype description

| SNP         | Location | Alleles | MAF   | Promoter haplotypes |
|-------------|----------|---------|-------|---------------------|
|             |          |         |       | 1  | 2  | 3   |
| TOMM40      |          |         |       | G  | A  | G   |
| promoter    |          |         |       | C  | C  | T   |
| rs6857      | 17660472 | G/a     | 0.109 | G  | A  | G   |
| rs71352238  | 17662554 | T/c     | 0.087 | C  | C  | T   |
| APOE        |          |         |       | 1  | 2  | 3   |
| promoter    |          |         |       | A  | T  | A   |
| rs449647 (-491) | 17676782 | T/a   | 0.146 | A  | T  | A   |
| rs769446 (-427) | 17676846 | T/c   | 0.038 | C  | T  | T   |
| rs40509 (-219) | 17677054 | C/t   | 0.492 | T  | C  | C   |
| rs440446 (113) | 17677385 | G/c   | 0.380 | C  | G  | G   |
| APOC1       |          |         |       | 1  | 2  |     |
| promoter    |          |         |       | −CGTT | +CGTT |     |
| rs11568822 HpaI site | 17685858 | CGTT | 0.166 |     |     |     |

| Enhancer haplotypes | 1 | 2 |
|---------------------|---|---|
|                     | G | A |
| rs2075660           | 17663837 | Alg | 0.130 |
| rs157581            | 17663932 | Alg | 0.285 |
| rs34095326          | 17664062 | G/a | 0.039 |
| rs34045544          | 17664127 | G/c | 0.114 |
| rs11556505          | 17664362 | C/T | 0.123 |
| rs157582            | 17664437 | G/a | 0.276 |
| rs59007384          | 17664883 | G/t | 0.223 |
| TOMM40 IVS6         |          | Poly-T 16–35 | — | 16T | 24T | 35T |
| rs10524523          | 17671267 | Poly-T | 0.061 |
| rs417357            | 17671337 | C/t | 0.061 |
| S_17671434 novel    | 17671434 | C/t | — |
| rs11609985          | 17671630 | C/t | 0.460 |
| ME1                 |          |         |       | 1  | 2  | 3   |
| rs445925            | 17683858 | G/a | 0.138 | G  | G  | G   |
| rs10414043          | 17683931 | G/a | 0.131 | A  | G  | G   |
| rs72562200          | 17684153 | G/t | 0.131 | T  | G  | G   |
| rs483082            | 17684996 | G/t | 0.258 | T  | G  | G   |
| rs59525138          | 17684509 | C/t | 0.304 | C  | T  | C   |
| rs584007            | 17684696 | Alg | 0.387 | G  | G  | A   |
| BCR                 |          |         |       | 1  | 2  |     |
| rs11083752          | 17722581 | G/a | 0.448 | G  | A  |     |
| rs7248162           | 17722904 | T/c | 0.451 | T  | C  |     |
| rs7247227           | 17722977 | Alg | 0.460 | A  | G  |     |
| rs7247551           | 17722984 | G/a | 0.365 | A  | G  |     |

Abbreviations: BCR, brain control region; MAF, minor allele frequency; ME1, multienhancer 1; SNP, single-nucleotide polymorphism.

Allele composition of each haplotype genomic DNA fragment inserted into luciferase reporter constructs. Location in contig: NT_011109.16.

Haplotype specific expression level in SHSY5Y cells

Significant differences in luciferase gene expression were found among two APOE promoter–enhancer haplotypes and three TOMM40 promoter–enhancer haplotypes in the SHSY5Y human neuronal cell line (Figures 3 and 4).

A total of six APOE promoter–TOMM40 IVS2-4 haplotype variants were tested and compared with their promoter-only counterparts. Significant differences in luciferase gene expression were found.
between four haplotype pairs (Figure 4a; significant levels relative to 1.0 as noted with asterisk). These differences were not observed for similar TOMM40 promoter haplotypes expressed in SHSY5Y cells, indicating that an enhancer/silencer effect of this TOMM40 IVS2-4 region is specific to the APOE promoter in SHSY5Y cells (Figure 4).
A total of nine APOE promoter-ME1 haplotypes were tested. Expression levels of APOE promoter-ME1 haplotypes were higher than the APOE promoter-only construct, where one haplotype (APOE promoter 2-ME1-1) showed significantly higher expression (Figure 4b; significant levels above 1.0 noted with asterisk). We also tested nine TOMM40 promoter-ME1 haplotype variants. Significant differences in luciferase gene expression were observed in this setting where the TOMM40 promoter 1-related haplotypes have higher expression levels than the other haplotypes (Figure 4d). All TOMM40 promoter-ME1 haplotypes levels were lower than the TOMM40 promoter-only construct (levels below 1.0; significantly lower levels are noted with asterisks). These results confirm the previously described enhancer activity of ME1 on APOE promoter activity\(^2,13\) and suggest an opposite silencer effect of ME1 on TOMM40 promoter activity.

A total of nine TOMM40 promoter-IVS6 poly-T haplotype variants were tested. The expression level of TOMM40 promoter 1-IVS6 16T haplotype was significantly higher than other haplotypes (Figure 4c). Six TOMM40 IVS6 poly-T haplotypes had significantly lower expression levels compared with the TOMM40 promoter-only construct (asterisk). These results suggest that the TOMM40 IVS6 poly-T (or rs10524523) locus has measurable enhancer/silencer activity and that the direction of its effect depends on the specific haplotype content of the TOMM40 promoter (Figure 4c).

A total of six TOMM40 promoter-BCR enhancer haplotype variants were tested. Significant differences were found between the TOMM40 promoter 1-BCR-1 haplotype and the other haplotypes (Figure 4e). The majority of TOMM40 promoter-BCR haplotypes showed significantly lower expression levels compared with the TOMM40 promoter-only construct (significant levels below one noted with asterisk), suggesting a silencer effect of BCR on the TOMM40 promoter in SHSY5Y cells (Figure 4e).

**Haplotype specific expression level in HepG2 cells**

Expression levels of the same eight promoter-only constructs and 80 promoter–enhancer haplotype constructs were also tested in the HepG2 human neuronal cell line (Table 1). Significant differences were found among three separate TOMM40 promoter–enhancer haplotypes, differences that were not observed among APOE promoter–enhancer haplotypes (Figures 3 and 4).

Among the six haplotype variants of TOMM40 promoter-TOMM40 IVS2-4, significant expression differences in HepG2 cells were found between the TOMM40 promoter 1-TOMM40 IVS2-4 2 haplotype and two other TOMM40 promoter-TOMM40 IVS2-4 haplotypes. All TOMM40 promoter-TOMM40 IVS2-4 haplotypes, except TOMM40 promoter 1-TOMM40 IVS2-4-2, have significantly lower expression levels relative toTOMM40 promoter-only construct (asterisk), suggesting that TOMM40 IVS2-4 has a silencer effect in HepG2 cells (Figure 4f).

Significant differences in expression levels in HepG2 cells were also found between TOMM40 promoter-ME1 haplotype variants. All TOMM40 promoter 1-ME1 haplotype levels were lower than the TOMM40 promoter-only counterpart in HepG2 cells (asterisks), suggesting a silencer effect that is significantly lower for the TOMM40 promoter 1-ME1-1 and TOMM40 promoter 2-ME1-1 haplotypes compared with the TOMM40 promoter 3-ME1-1 haplotype (Figure 4g).

Significant differences in expression levels in HepG2 cells were found between multiple TOMM40 promoter-BCR haplotypes (Figure 4h). All TOMM40 promoter-BCR haplotypes have significantly lower expression compared with the TOMM40 promoter-only construct in HepG2 cells (asterisk), suggesting a silencer effect HepG2 cells that is significantly lower for the BCR-2 haplotype compared with the BCR-1 haplotype (Figure 4h).

**DISCUSSION**

The core region of APOE locus consists of a large haplotype block that contains several genes, including TOMM40, APOE and APOC1 (Figure 1).\(^4\) Genetic markers in all three genes have consistently been shown to be associated with LOAD in multiple studies.\(^2,14\) Current consensus is that the ε4 allele of APOE is the only true effector of LOAD, and positive association signals of other markers are merely the effect of LD with the ε4 allele. The TOMM40 gene contains a poly-T repeat within intron 6 (TOMM40 IVS6 poly-T rs10524523) that has been recently reported to be associated with AD age-at-onset,\(^26\) and SNPs in the TOMM40 intron 2–4 region (TOMM40 IVS2-4) are associated with LOAD risk, quantitative trait loci,\(^20,23\) and apoE expression levels.\(^24,25\) In addition, the association between TOMM40 SNPs and LOAD cannot be fully explained by LD between TOMM40 SNPs and APOE ε4 alone\(^26\) and APOE locus enhancers, ME1 and BCR are associated with expression levels.\(^12,14\) However, the functional influence of genetic variation within APOE locus enhancers has not been previously characterized. In this investigation, we hypothesized that promoter activity of three APOE locus genes (TOMM40, APOE and APOC1) is functionally influenced by genetic variation within previously described regulatory elements (ME1, BCR) and putative regulatory elements (TOMM40 IVS2-4, TOMM40 IVS6 Poly-T) differentially according to haplotype. The main novel finding was that enhancers within TOMM40 influence both TOMM40 and APOE promoter activity in a haplotype and cell-type-specific manner.

Significant differences in reporter assay expression levels were found in three TOMM40 promoter–enhancer and two APOE promoter–enhancer haplotype variants in SHSY5Y neuronal cells (Figures 3 and 4). Significant differences were also found in three different TOMM40 promoter–enhancer haplotypes in HepG2 hepatocyte cell line (Figures 3 and 4). However, no significant differences were found in all APOC1 promoter–enhancer haplotypes and all promoter–enhancer haplotypes transfected into U118 astrocyte cells (Figure 3). Specifically, the TOMM40 promoter 1-TOMM40 IVS6 16T haplotype showed increased expression in the SHSY5Y neuronal cell line compared with six other haplotype variant counterparts (Figure 4c). Interestingly, six TOMM40 IVS6 poly-T haplotypes (asterisks) had significantly lower expression levels compared with the TOMM40 promoter haplotype alone (levels below one) implicating TOMM40 promoter activity inhibition by all TOMM40 IVS6 poly T haplotypes except the TOMM40 promoter 1-TOMM40 IVS6 poly-T 16T haplotype (Figure 4c). There was no significant difference between APOE promoter-TOMM40 IVS6 poly-T or APOC1 promoter-TOMM40 IVS6 poly-T haplotypes in any of the cell types tested, which suggests that the TOMM40 IVS6 poly-T 16T haplotype impacts TOMM40 expression, but not APOE or APOC1 expression. However, as others have reported promoter activity outside of the APOE core promoter sequence used in the present study,\(^8,30\) the possibility remains that the TOMM40 IVS6 poly-T haplotype influences the APOE promoter outside of the core promoter region tested in this study.

The TOMM40 IVS6 poly-T haplotype-specific expression was only significant in the neuronal SHSY5Y cell line, not HepG2 or U118 cell lines, suggesting that this haplotype may be particularly important in neurons. Interestingly, the TOMM40 IVS6 poly-T 16T haplotype contains a short version of poly-T repeat that has recently been associated with a later AD age-at-onset.\(^26\) This LOAD association, together with our result of higher haplotype expression, suggests that the short version (16T) of TOMM40 IVS6 poly-T combined with a...
specific TOMM40 promoter haplotype may be a modifier of AD risk through increased TOMM40 expression not APOE expression.

All three of the TOMM40 promoter 1-MEI haplotypes showed significant differences compared with other TOMM40 promoter-MEI haplotypes in SHSY5Y cells (Figure 4d). In contrast, this TOMM40 promoter 1 pattern was not seen in HepG2 cells where the most significant increase in expression was seen between TOMM40 promoter 3-MEI-1 and the other TOMM40 promoter haplotypes, indicating a cell-type-specific expression pattern for ME1 that is also haplotype specific (Figure 4g). In addition, the APOE promoter 2-MEI-1 haplotype showed a marginally significant increase in expression (Figures 3 and 4b). This is consistent with previous reports of increased expression in the presence of a ME1 transgene12,13,31 and is a novel finding in that genetic variation within ME1 can further modulate expression levels. There was no increased expression of the ME1 haplotypes in the U118 astrocyte cell line, which is in contrast to a previous report that shows apoe expression is increased in transgenic APOE-ME1 mouse astrocytes in the brain.31 It can be speculated that this inconsistency is due to either human versus mouse or in vitro versus in vivo differences between studies. But, as none of the reporter constructs showed significant differences in the U118 astrocyte cell line, it may represent a lack of APOE locus regulatory element activity in this cell line. The APOE promoter-ME1 showed marginally significant differences between haplotypes in SHSY5Y cells, but differences did not remain significant after correction of multiple comparisons (Figures 3 and 4b). However, most APOE promoter-MEI haplotype expression was increased above the APOE promoter-only baseline of 1.0, significantly APOE promoter 2-MEI-1, suggesting that ME1 has a general enhancing effect on APOE promoter activity. Notably, all TOMM40 promoter 1-MEI haplotype levels were lower than the TOMM40 promoter-only haplotypes in both SHSY5Y neuronal cells and HepG2 cells (Figures 4d and g; levels below 1.0; significantly lower levels are noted with asterisks). The TOMM40 promoter-MEI results suggest that ME1 inhibits TOMM40 expression in contrast to enhancing APOE expression.

The TOMM40 promoter is influenced by the silencer effect of BCR in both SHSY5Y and HepG2 cells (Figures 4e and h). All TOMM40 promoter-BCR haplotypes have significantly lower expression compared with the TOMM40 promoter-only haplotypes (significant levels below 1 noted with asterisk) in both HepG2 cells (Figure 4h) and SHSY5Y cells (Figure 4e) except the TOMM40 promoter 1-BCR-1 haplotype in SHSY5Y cells (Figure 4e). Moreover, in HepG2 cells the difference in expression between the BCR-1 and BCR-2 haplotypes is significant regardless of the TOMM40 promoter haplotype, suggesting that genetic variation in the BCR may influence TOMM40 expression according to cell type independent of TOMM40 promoter haplotype. Furthermore, the lack of influence by the BCR on the APOE promoter haplotypes in HepG2 cells is consistent with a previous report describing apoe expression in mice expressing apoe and BCR transgenes as constrained to the brain and not present in the liver.14 Why BCR reportedly influences brain apoe levels14 and not the APOE promoter haplotypes in SHSY5Y neuronal cells remains to be determined.

The APOE promoter 2 haplotype is significantly influenced by the TOMM40 IVS2-4-2 haplotype with increased expression compared with the other TOMM40 IVS2-4 haplotypes in the SHSY5Y cells (Figure 4a). These results are consistent with results from our previous studies where similar genetic variation within TOMM40 IVS2-4 (rs59007384 G allele) is associated with higher cerebrospinal fluid and post-mortem brain apoe expression.24,25 The TOMM40 promoter-1 haplotype is also influenced by the TOMM40 IVS2-4-2 haplotype, showing higher expression compared with other haplotypes in HepG2 cells (Figure 4f). These results suggest that genetic variation within the TOMM40 IVS2-4 region impacts expression of APOE locus genes in a cell-type-specific manner. Interestingly, a SNP (rs2075650) within the TOMM40 IVS2-4 region is significantly associated with AD quantitative traits, such as Aβ42, in genome-wide association studies26–28,33 as well as survival into old age, further implicating the TOMM40 IVS2-4 region in a biological effect.

Evidence presented here implicates APOE locus genetic variation within regional enhancers as contributors to regional promoter activity. These results are further supported by ENCODE data that show promoter (H3K4Me3) and enhancer (H3K4Me1) associated histone marks overlap with the regulatory haplotype reporter construct sites presented here (Figure 2).19,20 In addition, ENCODE DNase1 hypersensitivity cluster results overlap with regulatory haplotype region reporter construct sites, suggesting trans-acting factor binding at these APOE locus regulatory haplotype regions (Figure 2).20,21 Thus, it may be further speculated that both TOMM40 and APOE expression are influenced by regional regulatory haplotypes that in turn biologically impact AD pathogenesis.

In conclusion, functional characterization of APOE locus haplotype expression suggests that a complex transcriptional regulatory structure is modulated by distinct haplotypes composed of multiple and distantly located SNPs. The main novel finding of this investigation is that regions within TOMM40 influence both TOMM40 and APOE promoter activity in vitro depending on haplotype and cell type. These TOMM40 regions include a recently described poly-T in intron 6 that is associated with AD age-at-onset,26 and an intron 2–4 region that has been associated with AD risk19,33 and AD quantitative traits in multiple genome-wide association studies.20–23 This functional study suggests that non-APOE ε4 allele SNPs contribute to a promoter–enhancer haplotype structure that influences both TOMM40 and APOE gene regulation, suggesting that the genetic association between AD and TOMM40, found in multiple genetic studies, may be due to an AD modifier effect by TOMM40 that is haplotype and cell type specific.

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