DMP in the putative promoter region of TLR9 in the superior temporal gyrus postmortem brain tissue in patients with Alzheimer's disease.

Yu Sun[1,2] and Qingqin S Li[1,2]

[1]Neuroscience Therapeutic Area, Janssen Research & Development, LLC, 1800 American Blvd, Pennington, NJ 08534
[2]Research Information Technology, Janssen Research & Development, LLC, 1800 American Blvd, Pennington, NJ 08534

Abstract

Innate immune response mediated by Toll-like receptors (TLRs) has been implicated to be the therapeutic targets for Alzheimer's disease (AD). TLR9 agonist cytosine-guanosine-containing DNA oligodeoxynucleotides (ODNs) have been shown to be effective in reducing AD pathology in three AD animal models. To interrogate the involvement of TLR9 in AD, we measured the difference in methylation level of CpG probes in the promoter regions of TLR9 and demonstrated that hypermethylation of cg11855705 (p=0.003) and hypomethylation of cg08721301 (p=0.03), and cg22484793 (p=0.03) in postmortem brain tissue from the superior temporal gyrus (STG) region in AD subjects comparing to cognitively normal control subjects. These results provided an independent line of evidence in the potential role of TLR9 in AD pathology.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease and represents the most common cause of dementia. Neuritic plaques (NPs) consisting of amyloid β-protein (Aβ) and neurofibrillary tangles (NFTs) comprised of hyper phosphorylated tau protein are two prominent neuropathological hallmarks of AD. Neuroinflammation has been implicated in AD disease processes [1,2]. Fibrillar Aβ deposits are closely associated with neuroinflammatory responses such as microglia activation and/or innate immune response. Toll-like receptors (TLRs) are a family of pattern recognition receptors in the innate immune system and have been implicated in clearance of Aβ deposits in the brain [3,4] and proposed to be therapeutic targets for AD [5]. In particular, activation of microglia with the Toll-like receptor 9 (TLR9) ligand boosted ingestion of Aβ *in vitro* [3]. Stimulating the innate immune system via TLR9 with cytosine-guanosine-containing DNA oligodeoxynucleotides (ODNs) in Tg2576 AD model transgenic mice reduced the cortical and vascular amyloid burden and Aβ42, Aβ40, and Aβ oligomer levels [4]. Furthermore, this treatment also reduced both Aβ and tau pathologies and levels of toxic oligomers and rescued cognitive deficits in 3xTg-AD mice, an animal model with both Aβ and tau related pathology [6]. CpG ODNs treatment was also effective in Tg-SwDI, an animal model with abundant vascular amyloid association with low levels of parenchymal amyloid deposits, with effects in reducing cerebral amyloid angiopathy (CAA) pathology and negating memory deficit [7]. Transcription of TLR9 is up-regulated in control subjects upon Aβ stimulation, while down regulated in AD patients [8]. However, there was also report of up-regulation of Tlr9 mRNAs in plaque material of aged APP23 transgenic mice compared to plaque-free tissue, while plaque-free tissue did not show an increased expression of any Tlr mRNAs compared to age-matched control mice [9]. TLR9 is also thought to play a role via sensing the methylation level of mitochondrial DNA released from damaged human

cells and triggering the inflammatory cytokine cascade [10]. Furthermore, homozygote GG genotype from single nucleotide polymorphism rs187084 in TLR9 was significantly associated with a decreased LOAD risk after adjusting for age, gender, and ApoE ε4 status (P=0.035) [11]. There was also a significant interaction between ApoE ε4 and rs187084, with a stronger effect of rs187084 observed among e4 carriers (P<0.001 in genotypic test and p=0.003 in allelic test). The GG genotype of the TLR9 rs187084 polymorphism was associated with a higher TLR9 expression in peripheral blood monocytes.

Despite the genetic contribution in both familial and sporadic AD, data from monozygotic twins discordant in AD development and onset [12-14] suggest that non-genetic factors play a key role. Epigenetic mechanism includes DNA methylation and covalent histone modification, which generate heritable changes in gene activity and expression without changing DNA sequence. Methylation level are often examined in three ways 1) global methylation level; 2) methylation level of CpG sites in candidate genes; and 3) epigenome wide association analysis (EWAS) with promising but sometimes inconsistent results. The epigenetic state is age, disease, disease stage, tissue, and cell type dependent which could contribute to the inconsistent results. Dysregulation of histone acetylation has been implicated in learning and memory, onset of age-associated memory impairment and the pathogenesis of neurodegenerative diseases. In an AD animal model, severe amyloid pathology correlated with a dysregulation of histone acetylation in the forebrain [15]. Learning and memory consolidation deficits and/or AD pathology reversal effects were demonstrated for histone deacetylases (HDAC) inhibitors such as phenylbutyrate [16,17], sodium valproate [18], sodium butyrate [15,18-20], or vorinostat [18], suberoylanilide hydroxamic acid [19-21], trichostatin A [22] and environmental enrichment [23] in AD and/or brain-injured animal models. The recovery of learning/memory function correlated with elevated histone acetylation

[23], induced sprouting of dendrites, increased number of synapses [23], and increased expression of genes implicated in associative learning.

NP- and NFT-vulnerable brain regions such as superior temporal gyrus (STG) and middle temporal gyrus (MTG) regions of postmortem brains have been examined previously for global methylation level, at selected candidate locus, or at methylome wide [24-26]. In this study, we interrogated the difference in the STG methylation level of CpG probes in the putative regulatory regions of TLR9 to provide additional line of evidence in the involvement of TLR9 in AD.

Material and Methods

Samples from the STG region of postmortem brains from 91 Alzheimer's Disease patients and 60 healthy controls with normal cognition function were obtained from Banner Sun Health Research Institute under its brain donation program [27]. Genomic DNA and total RNA, including miRNA were simultaneously purified from the brain tissues samples using AllPrep DNA/RNA/miRNA Universal Kit (QIAGEN) following standard protocol. Genomic DNA was subject to genome wide methylation analysis using Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA). All data generation were conducted by laboratory personnel blinded as to the clinical phenotype.

Quality control of the epigenetic data was performed using R package ChAMP [28]. Probes that did not perform well (with detection p-value ≥ 0.01 in one or more samples, or with bead count >3 in at least 5% of samples), probes with known SNP sites, non-cg probes, probes align to multiple locations on the genome, as well as probes located on the sex chromosomes were filtered out. The final dataset contained 753,038 probes and 151 samples.

The methylation levels were then normalized using Dasen method from R package WaterMelon [29]. The proportion of NeuN+ cells (primarily neurons) was estimated using the estimate Cell Counts function in R package minfi [30]. We used R package sva [31] to detect hidden batch effect in the samples and included the top five surrogate variables generated in addition to sex and age as covariates in the linear regression model to identify differentially methylated probes between AD patients and cognitively normal controls. For this study, only probes from TLR9 region was examined and no multiple testing correction was made.

Results

Post-mortem samples used in the epigenetic analysis were described in Table 1 with the age of AD patients slightly older than the age of cognitively normal patients. TLR9 is transcribed from the reverse complement strand of the human genome, there are nine CpG probes from Infinium Methylation EPIC BeadChip within 1500 base pair upstream of transcript start site (TSS) of TLR9 (e.g. upstream of the transcriptional start). We observed a hypermethylation of CpG site cg11855705 (p=0.003) and hypomethylation of cg08721301 (p=0.03), and cg22484793 (p=0.03) (Figure 1 and Table 2) in the putative promoter region of TLR9 in AD subjects in the superior temporal gyrus (STG) region of postmortem brains from AD patients comparing to cognitively normal patients.

Discussion

Innate immunity seems to play opposing roles during the AD progression, e.g. activated microglia and reactive astrocytes exert neuroprotection mediated through Aβ phagocytosis in the early stage, whereas they fail in Aβ clearance and therefore exert detrimental effects including neuroinflammation and neurodegeneration as the disease progresses. This may be the reason behind opposing directionality in expression level observed in various experimental settings. In this study, the net methylation consequence of the three CpG sites is unknown as hypermethylation of cg11855705 presumably would result in a predicted lower expression of TLR9 transcript level and consistent with the peripheral results reported by Fiala et al. and Wang et al., while hypomethylation of cg08721301 and cg22484793 presumably would result in a predicted higher

Table 1. Samples used in the epigenetic analysis.

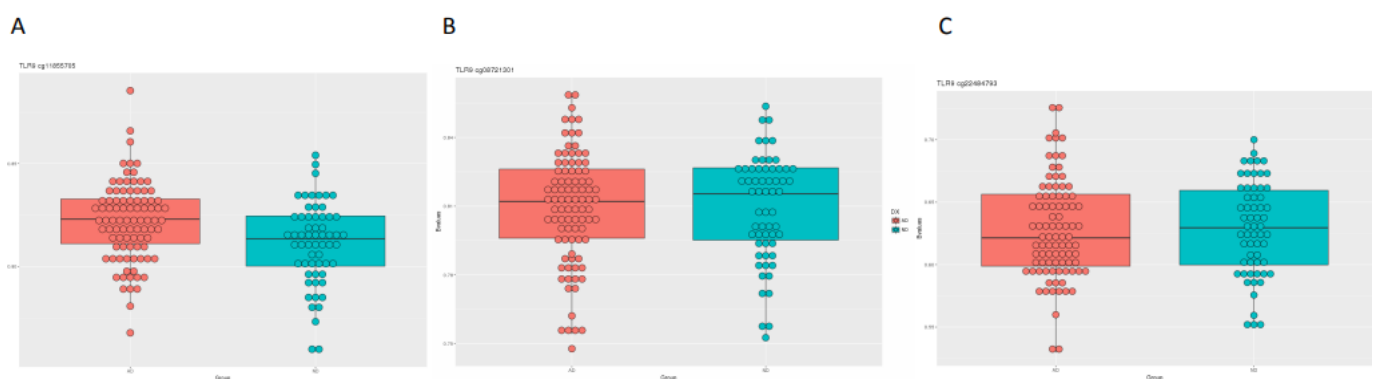| Variables	Alzheimer's Disease (n=91)	Cognitively Normal (n=60)	P-value
Age (years, mean ± SD)	83.93 ± 8.84	80.65 ± 6.88	0.005*
Gender (male, %)	44 (48.4%)	37 (61.7%)	0.13**
* Wilcox rank sum test; ** Fisher exact test			

Figure 1. *Differential methylation positions (DMP) upstream of TLR9 in AD vs. cognitively normal subjects (A) cg11855705 (B) cg08721301 (C) cg22484793.*

Table 2. DMP in AD subjects vs. controls.

chr	pos	strand	Name	Relation_to_ Island	UCSC_RefGene_ Group	logFC	AveExpr	t	p value	adj P Val	B
chr3	52260373	+	cg11855705	Open Sea	TSS200	-0.12	2.17	-3.07	0	0.21	-1.63
chr3	52260227	+	cg08721301	Open Sea	TSS200	0.09	2.1	2.16	0.03	0.45	-3.67
chr3	52261325	-	cg22484793	Open Sea	TSS1500	0.11	0.76	2.16	0.03	0.45	-3.67
chr3	52260349	-	cg01395047	Open Sea	TSS200	0.04	0.85	1.52	0.13	0.64	-4.72
chr3	52260965	-	cg05778154	Open Sea	TSS1500	-0.05	2.37	-1.37	0.17	0.69	-4.91
chr3	52260671	+	cg18452449	Open Sea	TSS1500	0.03	1.64	0.97	0.33	0.8	-5.34
chr3	52260280	-	cg17716965	Open Sea	TSS200	-0.01	1.38	-0.48	0.63	0.92	-5.66
chr3	52260432	-	cg16302310	Open Sea	TSS1500	-0.01	2.26	-0.2	0.84	0.97	-5.75
chr3	52261446	-	cg26207095	Open Sea	TSS1500	0	1.76	0.08	0.93	0.99	-5.76

expression of TLR9 transcript level and consistent with the Frank et al. where Tlr9 mRNAs in plaque material of aged APP23 transgenic mice was upregulated compared to plaque-free tissue and control tissues.

Conclusion

Overall, the study provides an independent line of evidence from epigenetic mechanism in the potential role of TLR9 in AD in a brain region vulnerable to AD pathology. Further replication evidence will be needed.

Acknowledgement

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*Correspondence to:

Qingqin S. Li
Neuroscience Therapeutic Area
Janssen Research and Development, LLC
1800 American Blvd
Pennington, New Jersey
USA
Tel: 6097304576
E-mail: qli2@its.jnj.com