-6×10^{-35}) minor allele frequency in Ashkenazi controls than in any of the European controls. Within the outbred European group alone (discovery plus replication), there was no evidence for heterogeneity at rs9922369 (Breslow-Day $P = 0.75$) and support for association was stronger (OR 1.39, $P = 4.7 \times 10^{-6}$) than in the unrestricted meta-analysis. The results of association analyses for each individual sample are given in **Supplementary Table 3**.

We also applied an imputation approach[10] using a frequentist additive model. The imputed and array data for the loci for which we have nominally significant independent support in the follow up samples are given in **Supplementary Figure 3a–e** online. Imputation did not provide clearly superior additional evidence for association. We observed a secondary region of high statistical significance with the imputed data near *ZNF804A* (**Supplementary Fig. 3a**), but this was not supported by array SNPs or by haplotype analysis based on array SNPs ($P_{min} = 0.01$); provisionally, we assume this to be a false positive.

Genome-wide imputation revealed two additional loci where one or more SNP showed association at $P < 10^{-5}$: chromosome 10 at 17.0 Mb (rs11594134, $P = 2.76 \times 10^{-8}$) and chromosome 15 at 60.4 Mb (rs464356, $P = 4.77 \times 10^{-6}$) (**Supplementary Fig. 3f,g**).

ZNF804A maps to chromosome 2 at 185.1–185.5 Mb (**Supplementary Fig. 3a**). The array association signal was restricted to *ZNF804A*, suggesting that this is the most likely schizophrenia susceptibility gene in the region. The encoded protein is uncharacterized and has no known function, but it contains predicted zinc ion and DNA binding domains, suggesting that it may have a possible role as a regulator of gene expression. Further discussion of the other loci with strong independent support in the replication samples ($P < 0.0005$) is provided in the **Supplementary Note**, but we note that none implicate clear functional candidates on the basis of current understanding of pathophysiology. The identification of risk alleles and genetic mechanisms should therefore provide new insights into schizophrenia pathogenesis.

Our study demonstrates that despite the lack of biological validating criteria for diagnosis, schizophrenia is amenable to the same genetic approaches as other common disorders, and like most other disorders, the effect sizes are small (**Table 1**). Assuming that our UK case-control sample has no unique characteristics that enhance our ability to detect risk loci, our findings strongly suggest that further GWA analyses of larger samples will identify many additional specific genetic risk factors with the potential to shed light into the pathophysiology of one of the most enigmatic major causes of human morbidity. Collection and analysis of large enough samples to provide convincing association signals should now be a priority.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

M.C.O'D., M.J.O., and N.C. directed this study and the collection of the UK sample. M.C.O'D. and M.J.O. took primary responsibility for drafting the manuscript assisted by N.N. and H.W. Collaborative scientific direction, replication sample collections, diagnoses and construction of case-control material were led by A.C., M.G., and D.W.M. (Ireland), D.R. (Munich, Germany), W.M., M.R., M.M.N., S.C., J.S. and P.P. (Bonn, Germany), G.K. (Bulgaria), L.H. and G.F. (China); N.I. (Japan) and S.S. and A.D. (Israel). J.L.M., C.C.A.S. and H.-T.L. were responsible for procedures related to calling Affymetrix genotypes. Replication genotyping was performed and analyzed by N.N., H.W., T.P., L.C., L.G. and S.D. (Cardiff); C.V. and P.Hoffmann (Bonn); Y.S. (China); S.S. (Israel); and M.I. (Japan). I.N. developed the database for the GWA project in which the data were stored, the primary analyses were performed and the results visualized. V.M. and P.Holmans supervised the association statistics to which N.N. additionally contributed. M.H. and N.C. were responsible for most of the quality control procedures. Additional scientific coordination of clinical sample collection and diagnosis was performed by S.Z., E.M.Q., A.M.H., H.-J.M., I.G. and T.G.S. All authors discussed the results and approved the manuscript.

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THE PRODUCTION RATIOS OF AICDε51 AND Aβ42 BY INTRAMEMBRANE PROTEOLYSIS OF βAPP DO NOT ALWAYS CHANGE IN PARALLEL

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RUNNING TITLE

Variety of ε-cleavage of βAPP

Abstract

Background: During intramembrane proteolysis of βAPP by presenilin (PS)/γ-secretase, ε-cleavages at the membrane-cytoplasmic border precede γ-cleavages at the middle of the transmembrane domain. Generation ratios of Aβ42, a critical molecule for Alzheimer disease (AD) pathogenesis, and the major Aβ40 species may be associated with ε48 and ε49 cleavages, respectively. Medicines to down-regulate Aβ42 production have been investigated by many pharmaceutical companies. Therefore, the ε-cleavages, rather than the γ-cleavage, may be more effective upstream targets for decreasing the relative generation of Aβ42. Thus, one may evaluate compounds by analyzing the generation ratio of the AICD species (ε-cleavage-derived), instead of that of Aβ42.

Methods: Cell-free γ-secretase assays were performed to observe *de novo* AICD production. Immunoprecipitation/MALDI-TOF MS analysis was performed to detect the N-termini of AICD species. Aβ and AICD species were measured by ELISA and immunoblotting techniques.

Results: Effects on the ε-cleavage by AD-associated pathological mutations around the ε-cleavage sites (*i.e.*, βAPP V642I, L648P and K649N), were analyzed. The V642I and L648P mutations caused an increase in the relative ratio of ε48 cleavage as expected from previous reports. Cells expressing the K649N mutant, however, underwent a major ε-cleavage at the ε51 site. These results suggest that ε51, as well as ε48 cleavage, is associated with Aβ42 production. Only AICDε51, though, and not Aβ42 production, dramatically changed with modifications to the cell-free assay conditions. Interestingly, the increase in the relative ratio of the ε51 cleavage by the K649N mutation was not cancelled by these changes.

Conclusion: Our current data indicate that the generation ratio of AICDε51 and Aβ42 do not always change in parallel. Thus, to identify compounds that decrease the relative ratio of Aβ42 generation, measurement of the relative level of Aβ42-related AICD species (*i.e.*, AICDε48 and AICDε51) might not be useful. Further studies to reveal how the ε-cleavage precision is decided are necessary before it will be possible to develop drugs targeting ε-cleavage as a means for decreasing Aβ42 production.

Key words: Alzheimer Disease, βAPP, γ-cleavage, ε-cleavage, presenilin/γ-secretase, "dual-cleavage" mechanism, AICDε51

INTRODUCTION

The transmembrane domain of β-Amyloid Protein Precursor (βAPP) is proteolysed by presenilin (PS)/γ-secretase [1]. Analysis of the resultant products has revealed that the proteolysis proceeds by at least two-distinct cleavages. The "ε-cleavage" liberates its intracellular domain (*i.e.*, AICD) into the cytoplasm, while the "γ-cleavage" releases Alzheimer disease (AD)-associated Amyloid β-protein (Aβ) [2-6].

There are some variations in both the γ- and ε-cleavages of βAPP [6-8]. The major N-termini of AICD species consist of leucine-49, valine-50 and leucine-52 (Aβ-numbering), while the major C-termini of Aβ species are comprised of valine-40 and alanine-42. (Figure 1A) [6]. Among these, highly aggregatable Aβ42 is the major component of senile plaques in AD brains [9].

Are there any relationships between the ε- and γ-cleavages? How do these cleavages occur? Ihara and colleagues have tried to address these questions and recently revealed that ε-cleavage precedes γ-cleavage in *in vitro* γ-secretase assays [10]. βAPP-CTF stubs, βAPP membrane-tethered remnants following β-cleavage, first undergo ε-cleavage [10]. The ε-cleavage liberates AICD from the membrane and produces a membrane-bound 48/49 amino-acid-long Aβ species that undergoes further C-terminal truncation by PS/γ-secretase [11]. Stepwise cleavages remove every three amino-acid residues from the C-terminus of the long Aβ species, which finally secretes Aβ40/42 [12-14]. For example, mutant PS causes increased both ε48 and γ42 cleavages [8]. Thus, the γ-cleavage seems to occur in an ε-cleavage-dependent manner [10]. Moreover, these results indicate that the production process for pathological Aβ42 is distinct from that of Aβ40 [15]. That is, the major ε49 cleavage causes the production of Aβ40, while a minor ε48 cleavage causes production of pathological Aβ42 [14].

Modulation of γ-secretase function to specifically inhibit Aβ42 production is one of the promising strategies for developing drugs to modify the disease course of AD [16]. Given the possible correlation between the ε- and γ-cleavages, we think that targeting the up-stream ε-cleavages will be a novel and more efficient method for developing Aβ42-lowering drugs. To test if precision of the ε-cleavage can be used as a novel target for drug development, we investigated the ε-cleavage pathway, particularly ε51 cleavage, which has previously not been well-characterized [7].

RESULTS

The βAPP K649N Belgian mutant increased both the relative ratio of AICDε51 and Aβ42 production in a cell-free γ-secretase assay.

To test if the ε51 cleavage precedes the γ42 cleavage, we analyzed the effects of three βAPP mutants (V642I [17], L648P [18], and K649N [19]) around the ε-site. The L648P and K649N mutants (βAPP695 numbering) are located downstream of the ε51 site, and the V642I mutant is located upstream of the ε48 site (Figure 1A). Each of the three mutants is familial AD-associated and, therefore, increases the relative ratio of Aβ42 production. We raised K293 cells stably expressing each of the mutants, prepared the crude membrane fractions [20] and performed the cell-free γ-secretase assays [7,21].

As shown in Figure 1B, the K649N βAPP mutant caused marked increase in the relative ratio of AICDε51 production. However, the other two mutants caused completely different effects on the cleavage. The L648P mutant produced a barely detectable level of AICDε51, while in the V642I mutant cells, the ratio of AICDε51 production was comparable to that of wild-type (wt) expressing cells. It is of note that, instead of increased AICDε51 production, these V642I and L648P mutants substitutively increased the relative ratio of AICDε48 production. Next we measured Aβ species secretion by the stable cells in conditioned media using ELISA (Figure 1C). As expected, we observed a significant increase in the ratio of Aβ42 to total Aβ secretion in the conditioned medium of the mutant cells. This data indicates that the K649N mutant increased the ratio of Aβ42 production through up-regulation of the ε51 cleavage, while the V642I and L648P mutants increased Aβ42 production through the ε48 cleavage. Based on these results, we suggest that not only the ε48 but also the ε51 cleavage precedes Aβ42 production, possibly by sequential three amino-acid C-terminal truncation [14] (Figure 1D).

Incubation in higher pH does not cancel the K649N βAPP mutant effects.

We previously found that the precision of ε-cleavage changes depending on the buffer pH [7,21]. The relative ratio of AICDε51 production is the most sensitive to such changes. Therefore, we next determined whether the relative ratio of AICDε51 and/or Aβ42 production by the K649N mutant is affected by changing the buffer pH during the cell-free assay. As expected, incubation in the higher pH (pH 7.4 vs pH 6.0) buffer decreased the relative ratio of AICDε51 generation in both the K649N mutant and wt βAPP membrane fraction. However, the pH effect was not so strong as to cancel the AICDε51 up-regulation effect by the K649N mutant (Figure 2A). We further analyzed the pH effects on the increase in the relative ratio of Aβ42 production by the mutant

(Figure 2B). Surprisingly, the assay pH elevation did not cause any changes in the relative ratio of Aβ42 generation. Therefore, unlike the effects of the K649N mutant on the ε51- and γ42-cleavages, the elevation of the buffer pH causes a decrease in the relative ratio of AICDε51 production but does not cause any changes in Aβ42 production. The data suggests that two distinct mechanisms may contribute to the determination of the relative ratio of AICDε51 production.

Alkali pre-treatment of the crude membrane fraction cancels the effect of higher pH cell-free incubation on ε-cleavage.

Since the ε51 cleavage occurs at the membrane-cytosol interface, we considered that membrane-bound substances might induce the pH-dependent effects on AICDε51 production. Many substances detach from the membrane upon treatment with alkali solution [22]. To test this theory, we washed the wt βAPP membrane fraction in a pH 11 solution (see "Materials and Methods") then conducted the cell-free assay at pH 6.0. The relative ratio of AICDε51 production markedly decreased (Figure 3A), while that of the Aβ42 did not (Figure 3B). The phenomena are reminiscent of the effects of raising the pH of the incubation buffer (see Figure 2). Thus, we further considered that the decrease in the AICDε51 production resulting from the use of a higher incubation buffer pH might also be due to detachment of substances from the membrane. When the membrane fraction was incubated in a pH 7.4 buffer after alkali treatment, we could no longer observe the pH-dependent incubation buffer effects on the AICDε51 ratio (Figure 3C). Collectively, though incubation at lower pH buffer increased in the AICDε51 ratio (Figure 2A), the effects was cancelled by the alkali pre-treatment (Figure 3A). These results suggest that substances removed by the alkali treatment might induce the changes in the relative ratio of AICDε51 production.

Alkali pre-treatment of the crude membrane fraction did not cancel the effects of the K649N mutant on the ε-cleavage.

As shown in Figure 1, the K649N βAPP mutation causes up-regulation of both the AICDε51 and Aβ42 ratio, while alkali pre-treatment causes down-regulation of only the AICDε51 ratio (Figure 3). These data indicate that changes in the AICDε51 ratio caused by the mutation and by the treatment occur by two distinct processes. A further experiment was conducted to confirm whether the K649N mutation cause a change in the relative ratio of AICDε51 production through the effect of the alkali treatment (Figure 4A). Following treatment of the K649N mutant membrane fraction in the alkali solution, the cell-free assay was performed at pH 6.0. As shown in Figure

4A, even after the alkali treatment, the K649N mutant membrane produced a relatively higher level of AICDε51 than that of the wt fraction (Figure 3A). Moreover, the elevated Aβ42 ratio was not changed by the pre-treatment (Figure 4B).

DISCUSSION

In the present study we determined that there are at least two factors that change the precision of ε-cleavage: (i) a process induced by a pathological βAPP mutation and (ii) another process induced by possibly unidentified substances removed from the membrane fraction by alkali pre-treatment. In the case of βAPP mutations, the relative ratio of ε51 and ε48 production increases in parallel with the ratio of AD-associated Aβ42.

It has been reported that ε-cleavage precedes γ-cleavage and γ-cleavage seems to occur in an ε-cleavage-dependent manner [10]. Considering these reports and our own preliminary results, it seemed possible that measurement of the relative ratio of AICDε48/AICDε51 production might help develop Aβ42-lowering anti-AD drugs. Further study revealed, however, that the relative level of AICDε51 production is drastically affected by the removal of unidentified substances from the membrane as a result of alkali pre-treatment. Interestingly, the alkali pre-treatment did not cause any changes in the relative ratio of Aβ42 generation. These results indicate that changes in the precision of ε-cleavage do not always cause parallel alterations in the precision of γ-cleavage, even though ε-cleavage occurs upstream of the γ-cleavage. Therefore, although measuring the levels of AICD species is a potentially attractive new target for developing Aβ42 lowering compounds, challenges still must be overcome before screening methods for such compounds can be established. For example, the paradoxical mechanism discussed above must first be understood before an assay in which the ε-cleavage precision accurately reflects the γ-cleavage precision can be developed.

How does alkali pre-treatment result in a decreased ratio of AICDε51 production? One may consider the presence of unknown substances which (i) transiently associate with the PS/γ-secretase and affect its intramembrane cleavage precision, or (ii) truncate a couple of N-terminal amino-acid residues of AICD produced by the ε-cleavage. The second possibility is reminiscent of ACE activity to truncate the C-terminus of Aβ42 [23]. Of course, the possibility that alkali pre-treatment might change the character of PS/γ-secretase itself also cannot be excluded.

CONCLUSION

Our current data suggest that the precision of ε-cleavage do not always changes in parallel with the precision of γ-cleavage, even though ε-cleavage occurs upstream of the γ-cleavage. Thus, to measure the levels of AICD species might be an attractive new target for developing Aβ42 lowering compounds, there still remain some

challenges.

MATERIALS AND METHODS

Cell culture and cDNA constructs

cDNAs of βAPP V642I, L648P and K649N mutants were generated by PCR-based mutagenesis using a Quickchange mutagensis kit (Stratagene) or KOD plus (Toyobo) with wt βAPP695 cDNA as a template. K293 cells were transfected and cultured as previously described [24].

Membrane preparation

The crude membrane fraction was prepared as previously described with a slight modification [7,21]. In the present study, the homogenization buffer contained 0.25 M sucrose and 50 mM HEPES (pH 7.4) containing a protease inhibitor cocktail (Roche). To prepare the alkaline pre-treated membrane, the membrane fraction was suspended in a 50 mM bicarbonate buffer (pH 11.0) and incubated at 4 °C for 1 h. The suspension was then centrifuged at 100,000 × g for 1 h followed by washing once with a 50 mM Mes buffer (pH 6.0).

Cell-free γ-secretase assay

The cell-free γ-secretase assay was performed as previously described with a modification [7,21]. The reaction buffer in the present study contained a 150 mM citrate buffer (pH 6.0), 50 mM MES (pH 6.0), 167 mM NaCl and a protease inhibitor mixture comprised a 5x complete protease inhibitor cocktail (Roche), 0.5 mM DIFP (WAKO), 1 μg/ml TLCK (Sigma-Aldrich), 10 μg/ml antipain (Peptide Institute), 10 μg/ml leupeptin (Peptide Institute), 5 mM 1,5 phenanthroline (Sigma-Aldrich), 10 μM amastatin (Peptide Institute), 10 μM bestatin (WAKO), 1 μM thiorphan (Sigma-Aldrich), 10 μM phosphoramidon (Peptide Institute) and 1 μM pepstatin A (Peptide Institute). To prepare the pH 7.4 buffer, 50 mM HEPES (pH 7.4) was used instead of the citrate and MES buffers.

Immunoprecipitation/MALDI MS (IP-MS) analysis

IP-MS analysis followed by cell-free incubation was carried out as previously described [7,21,25]. The heights of the MS peaks and molecular weights were calibrated using angiotensin and bovine insulin β-chain as standards (Sigma-Aldrich).

ELISA analysis for Aβ

Aβ40 and Aβ42 levels in conditioned media were quantified by ELISA (WAKO).

Immunoblotting of Aβ

SDS-solubilized proteins were separated by SDS-PAGE using an 8 M Urea gel [24] and transferred to a nitrocellulose membrane. Immunoblotting of Aβ species using 82E1 (IBL) was performed as previously described [26].

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The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Effect of familial AD-associated βAPP mutations around the ε-cleavage site.

A, Schematic diagram of intramembrane cleavage sites of βAPP and the familial AD mutations used in the present study. The amino acid sequence around the juxta membrane region of human βAPP is described (Aβ numbering). Filled inverted triangles indicate the cleavage sites. Substituted amino acids of the familial AD mutations are indicated in open rectangles. The site of each mutant is also indicated using APP695 numbering.

B, Mass spectra of *de novo* AICD species in the cell-free assay. Crude membrane fractions obtained from wt βAPP and the indicated βAPP mutant cells were used.

C, Relative secreted Aβ42 to Aβ40 ratio in the conditioned media of wt βAPP and the indicated βAPP mutant cells. The asterisks indicate statistical significance (*P<0.05, **P<0.001, one-way analysis of variance (ANOVA) and Tukey-Kramer method). Error bars indicate standard error of the mean (SEM).

D, Hypothesis for explaining increased γ42 cleavage in each mutant βAPP (upper panels) and differential production of Aβ40 and Aβ42 (lower panels).

Figure 2. Effect of cell-free incubation pH levels on the precision of ε/γ-cleavages.

A, Mass spectra of AICD generated in the cell-free assay performed at the indicated pH (upper and middle panels). Peak heights of AICDε49 and ε51 were measured and the ratios of AICDε49 to ε51 were calculated (lower panel). The asterisks indicate statistical significance (*P<0.05, **P<0.001, one-way ANOVA and Tukey-Kramer method). Error bars indicate SEM.

B, Levels of Aβ generated at the indicated pH. Levels of Aβ40 and 42 were measured by western blotting and the Aβ42 to 40 ratios calculated. The asterisks indicate statistical significance. Error bars show SEM.

Figure 3. Effect of alkali pre-treatment on the precision of ε/γ-cleavages of wt βAPP.

A, Mass spectra of AICD generated in the cell-free assay with and without alkali pre-treatment. Peak heights of AICDε49 and ε51 were measured and the AICDε49 to ε51 ratios calculated. The asterisk indicates statistical significance (*P<0.05, paired t-test). Error bars indicate SEM.

B, Levels of Aβ generated in the cell-free assay following alkali pre-treatment. Levels of Aβ40 and 42 were measured by western blotting with and without alkali pre-treatment and the Aβ42 to 40 ratios calculated.

C, Mass spectra of AICD generated in the cell-free assay at the indicated pH following

alkali pre-treatment.

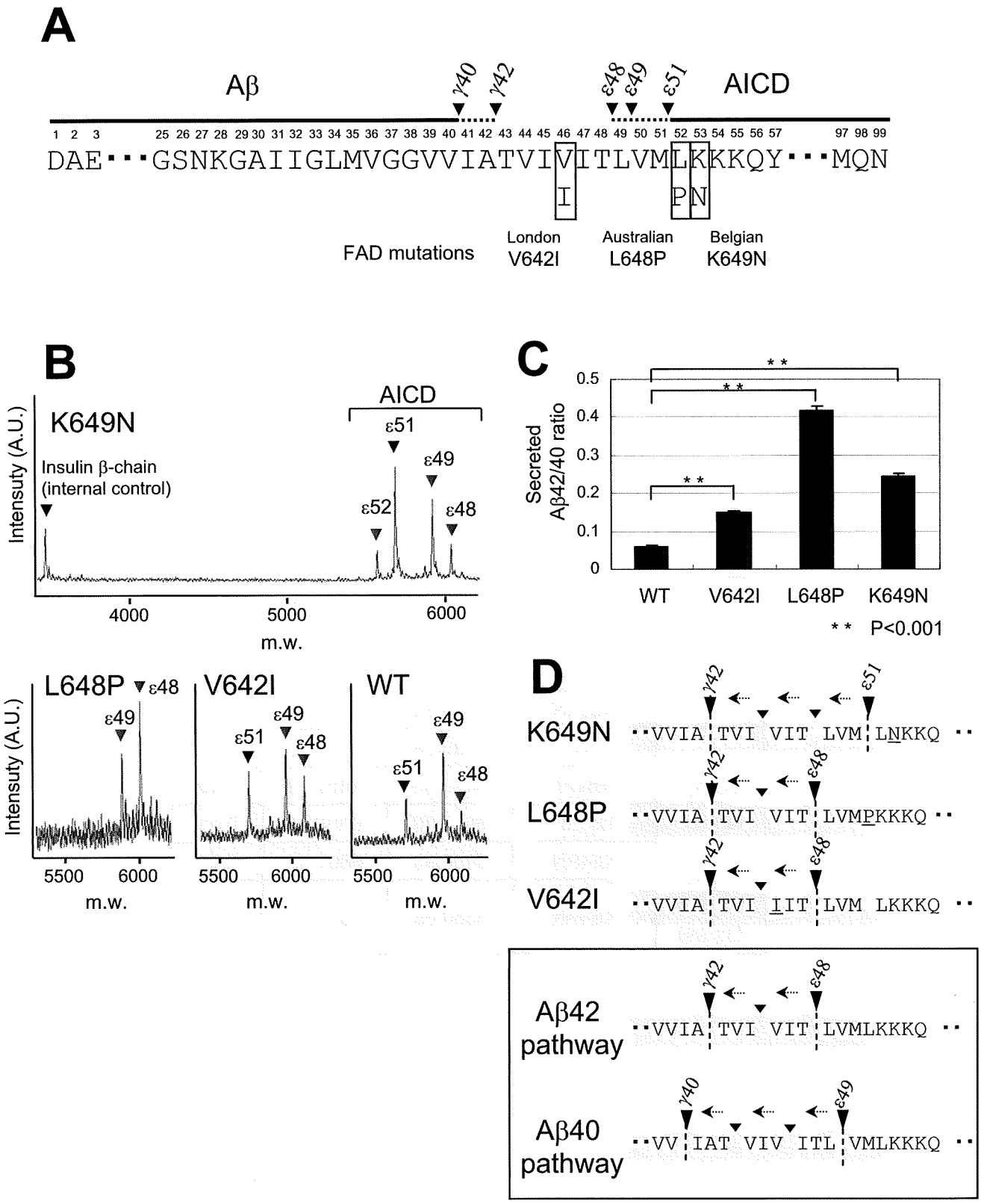
Figure 4. Effect of alkali pre-treatment on the precision of ε/γ-cleavages of βAPP K649N Belgian mutant.

A, Mass spectra of AICD generated in the cell-free assay with and without alkali pre-treatment.

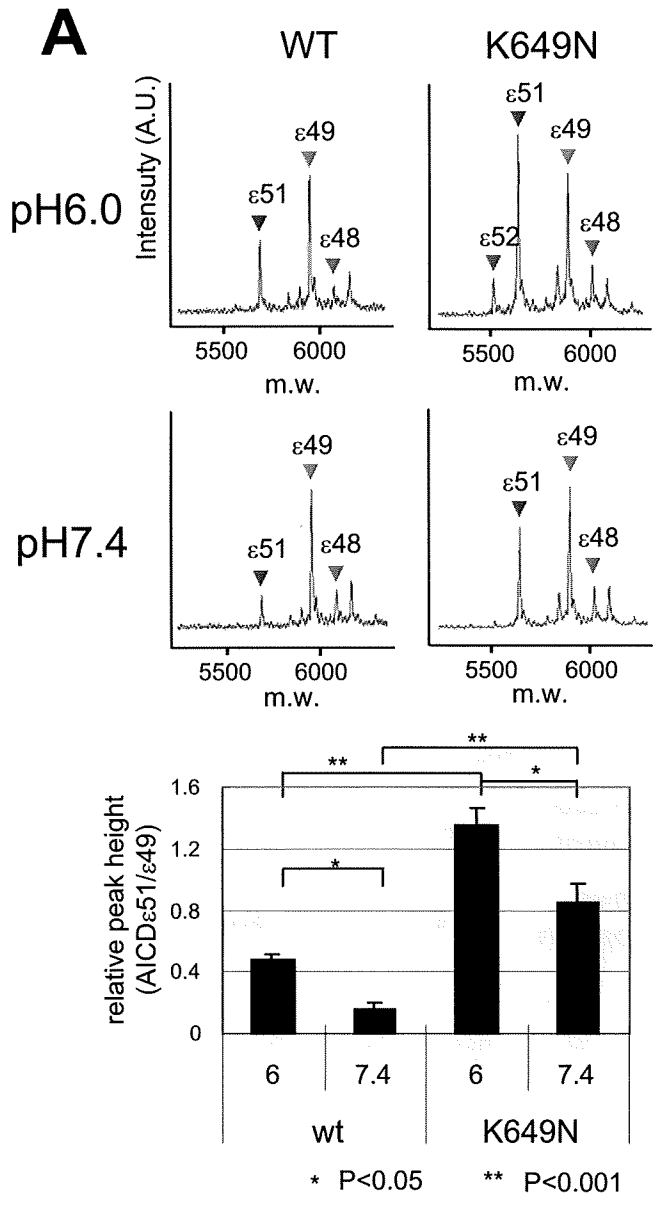
B, Levels of Aβ generated in the cell-free assay following alkali pre-treatment.

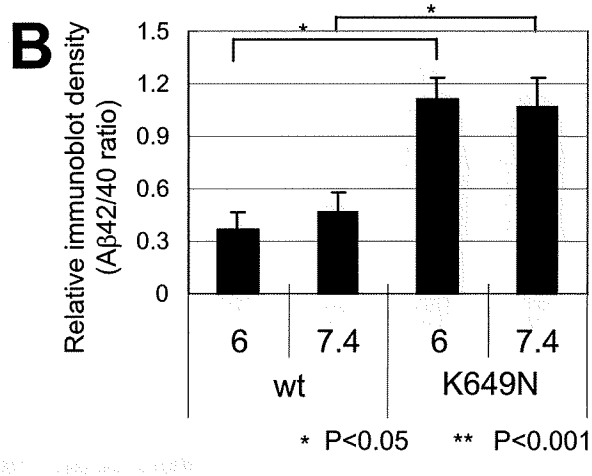
Table 1. Molecular species of AICD generated in the cell-free assay.

AICD species		m/z		
		Calculated [M+H]	observed [M+H]	
			mean	SD
AICDε51 (52-99)	wt	5677.79	5678.38	0.64
	V642I	5677.79	5678.30	0.70
	K649N	5663.74	5663.96	0.23
AICDε49 (50-99)	wt	5907.9	5908.35	0.29
	V642I	5907.9	5908.49	0.21
	L648P	5891.87	5892.48	0.20
	K649N	5893.84	5894.10	0.27
AICDε48 (49-99)	wt	6020.98	6021.36	0.40
	V642I	6020.98	6021.59	0.42
	L648P	6004.96	6005.59	0.33
	K649N	6006.93	6007.51	0.17
AICDε52 (53-99)	K649N	5550.65	5551.01	0.27

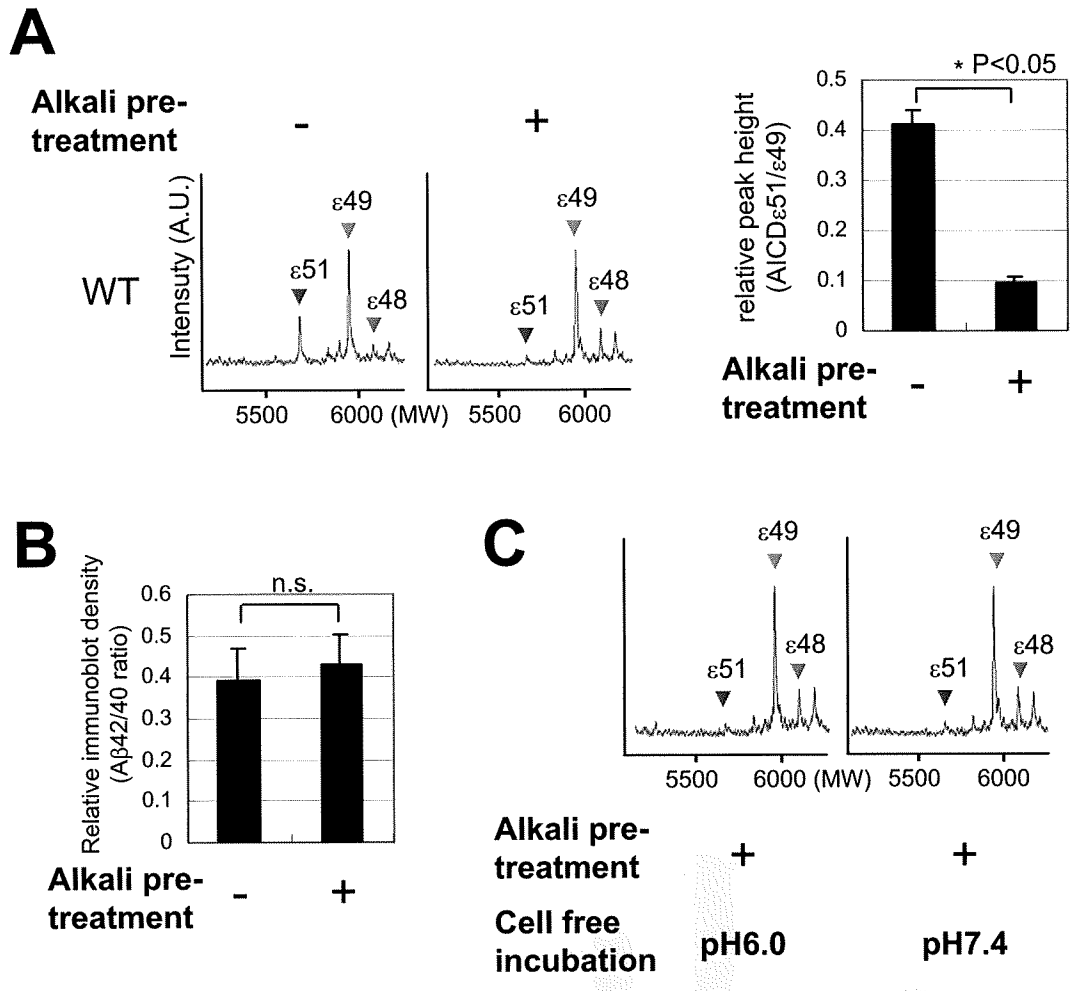


Mori et al., Figure 1

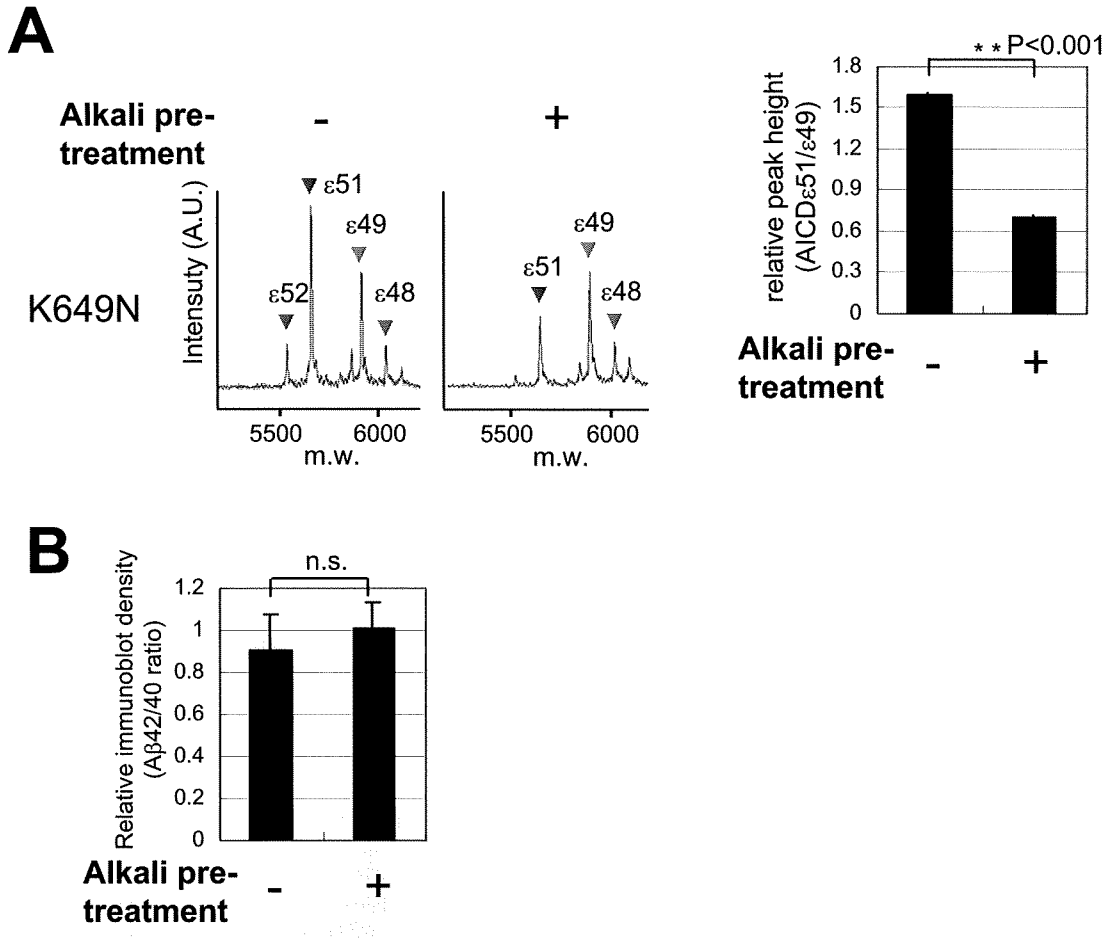




Mori et al., Figure 2



Mori et al., Figure 3



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The International Consortium on Lithium Genetics (ConLiGen): An Initiative by the NIMH and IGSLI to Study the Genetic Basis of Response to Lithium Treatment

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