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A functional polymorphism within plasminogen activator urokinase (*PLAU*) is associated with Alzheimer's disease

Matthias Riemenschneider[1,*], Lidija Konta[1], Patricia Friedrich[1], Sandra Schwarz[1], Kevin Taddei[5,13], Frauke Neff[2], Alessandro Padovani[6], Heike Kölsch[7], Simon M. Laws[1,13], Norman Klopp[8], Heike Bickeböller[9], Stefan Wagenpfeil[3], Jakob C. Mueller[1,3,14], Albert Rosenberger[9], Janine Diehl-Schmid[4], Silvana Archetti[6], Nicola Lautenschlager[10], Barbara Borroni[6], Ulrich Müller[11], Thomas Illig[8], Reinhard Heun[7], Rupert Egensperger[12], Jürgen Schlegel[2], Hans Förstl[4], Ralph N. Martins[5,13] the German Sib-Pair Study Group and Alexander Kurz[4]

[1]Neurochemistry and Neurogenetics Laboratory, Department of Psychiatry and Psychotherapy, [2]Institute of Pathology, [3]Department of Medical Statistics and Epidemiology , [4]Department of Psychiatry and Psychotherapy, Technische Universität München (TUM), Ismaningerstr. 22, 81675 Munich, Germany, [5]Sir James McCusker Alzheimer's Disease Research Unit, Hollywood Private Hospital, Perth, Western Australia, [6]Clinica Neurologica, Università degli Studi di Brescia, Brescia, Italy, [7]Department of Psychiatry and Psychotherapy, Friedrich Wilhelms Universität, Bonn, Germany, [8]Institute of Epidemiology, GSF, München-Neuherberg, Germany, [9]Department of Genetic Epidemiology, University of Göttingen, Germany, [10]School of Psychiatry and Clinical Neurosciences, University of Western Australia, Perth, Australia, [11]Institute of Human Genetics, University of Giessen, Germany, [12]Institute of Neuropathology, University Hospital Muenster, Germany, [13]Alzheimer's and Aging, School of Biomedical and Sports Science, Edith Cowan University, Joondalup, Australia and [14]Hertie-Institute for Clinical Brain Research, Tuebingen, Germany

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A number of susceptibility loci for Alzheimer's disease (AD) have been identified including a region on Chromosome 10q21–q22. Within this region the plasminogen activator urokinase gene (*PLAU*) was considered as a reasonable candidate from its functional implication in plasmin generation, a serine protease capable of degrading beta-Amyloid (Aβ) protein. We screened 56 single nucleotide polymorphisms (SNPs) around *PLAU* using 1751 individuals from four independent case–control samples (Munich, $N = 679$; Bonn $N = 282$; Brescia (Italy) $N = 219$; Perth (Australia) $N = 557$ and one discordant sib-pair sample (Munich $N = 622$). In brain tissue samples of neuropathologically confirmed cases with AD ($N = 33$) we analyzed plaque counts according to the risk allele. We identified that one functional exonic SNP (rs2227564) is associated with development of AD using the four independent case–control samples (Munich, $P = 0.02$; Bonn, $P = 0.005$; Brescia (Italy), $P = 0.001$; Perth (Australia), $P = 0.03$) and the discordant sib-pair sample ($P = 0.001$). In brain tissue, from neuropathologically confirmed cases with AD, we identified significantly higher plaque counts in carriers of the risk allele ($N = 6$; 60.3 ± 16.9) compared with non-carriers ($N = 9$; 26.3 ± 8.8; $P = 0.007$). This study provides compelling evidence of a genetic and functional involvement of a common *PLAU* variant into the pathogenesis of AD. Further functional investigations are warranted to elucidate the specific role of *PLAU*, respectively, *PLAU* variants in the metabolism of Aβ proteins.

*To whom correspondence should be addressed: Tel: +49 8941404214; Fax: +49 8941404888; Email: m.riemenschneider@lrz.tu-muenchen.de

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INTRODUCTION

Alzheimer's disease (AD) is a common, genetically complex neurodegenerative disorder that causes a progressive decline of cognitive abilities. The spectrum of factors that contribute risk to AD is wide and ranges from rare mutations causing an autosomal-dominant early-onset form of AD, to susceptibility genes and further to epigenetic and non-genetic risk factors, such as advanced age. Abundant evidence suggests that elevated amounts of the small, highly amyloidogenic protein beta-amyloid (Aβ), which is mainly composed of 40–42 amino acids (Aβ40, Aβ42) contributes to the neurodegenerative process in AD through its neurotoxic properties (1). While an increased production of Aβ peptides has been demonstrated for the rare autosomal dominant forms of AD due to mutations within the amyloid precursor protein gene (*APP*; OMIM*104760) or the presenilins (*PSEN1*, OMIM*104311; *PSEN2*, OMIM*600759), a decreased degradation of Aβ proteins may contribute to the sporadic forms of the disease. Ongoing research identified several proteases capable of degrading Aβ proteins. Among these the insulin degrading enzyme (*IDE*, OMIM*146680), plasmin and activators of the plasmin system are of particular interest, as they may represent reasonable candidate genes conferring risk to the development of AD.

Previous genome-wide linkage studies in patients with late-onset AD have identified several regions with considerable linkage, (2–7) which strongly suggests the existence of additional susceptibility genes beside apolipoprotein E (*APOE*), the only unequivocally confirmed risk gene of AD so far (8,9). One broad linkage region on Chromosome 10q [10q21–q24; multipoint LOD score (MLS) ≈4] has gained increasing attention due to the linkage to this region being repeatedly demonstrated in independent patient samples (10,11) and in addition, by a linkage study using elevated Aβ42 levels in plasma as a quantitative trait (12). The genetic basis of this linkage regions has yet to be determined; however, there is increasing evidence from functional and genetic studies to suggest that two proteases, the plasminogen activator urokinase (*PLAU*, OMIM*191840) located at the middle and insulin degrading enzyme (*IDE*) at the distal end of the Chromosome 10 linkage region are strong functional and positional candidate genes. *PLAU* encodes the urokinase-type plasmin activator (uPA), a serine protease, which activates plasmin from its inactive precursor plasminogen after binding to its receptor (*uPAR*, OMIM*173391). In the context of AD-related pathological mechanisms it has been shown that Aβ induces the expression of *PLAU* and its receptor (*PLAUR*) (13,14) and that uPA was found to inhibit Aβ-mediated neurotoxicity and fibrillogenesis via activation of plasmin, a serine protease capable of degrading aggregated and soluble forms of Aβ proteins (15,16). Besides its capability to degrade Aβ peptides directly, plasmin has also been shown to enhance the non-amyloidogenic processing of APP by α-secretase cleavage (17).

Thus, several groups have investigated genetic variations in *PLAU* for association with AD (18–21) and AD-related endophenotypes, such as plasma Aβ42 levels (22), cerebrospinal fluid (CSF) Aβ42 levels and cognitive performance as assessed by the Mini Mental State Examination (MMSE) (23). However, the results of these genetic studies remain controversial, with some studies unable to find any association with AD (19–21) and others, which demonstrated significant association with AD (18,22), plasma Aβ42 concentrations (22), CSF Aβ42 concentrations and MMSE (23). The situation of *PLAU* appears to be even more puzzling, as Myers *et al*. (19) excluded *PLAU* as being the major locus underlying their linkage signal, whereas Ertekin-Taner (22) identified a substantial contribution of *PLAU* to their QTL-plasma Aβ42 signal and Finckh *et al*. (18) published a risk association to the opposite allele compared with Ertekin-Taner *et al*. (22).

In an attempt to clarify the discrepancy of association results, we applied a whole gene approach (24) and conducted a detailed analysis of the *PLAU* gene locus by genotyping 56 single nucleotide polymorphisms (SNPs) spanning a genomic region of ~300 kB around the *PLAU* gene using a large case–control sample from Germany. The most promising association signal of a coding polymorphism at Exon 6 was successfully replicated using three independent case–control samples and one discordant sib-pair sample. Another coding polymorphism was identified by sequencing, but excluded as a risk variant through genotyping. Finally, possible functional consequences on the cerebral plaque load were investigated using human brain samples with AD.

RESULTS

The initial genetic and statistical analyses to determine the LD structure, the single marker and haplotype association signals, as well as the sliding window approach were performed using the Munich cohort.

LD structure

Within the Munich sample, the LD structure based on 52 out of 53 SNPs (one SNP, rs2227579, was excluded because of deviation from Hardy–Weinberg equilibrium; Fig. 1A), shows that the *PLAU* region is partially separated by likely historical recombination from the neighboring gene, *CAMK2G* upstream (between markers hCV27069354 and rs2675675) and completely unlinked to the *VCL* downstream (between markers rs2688624 and rs4745730). Continuous blocks of relatively high r^2 values are observed at the 5′ flanking and promoter region of *PLAU* (from rs2675675 to rs2227552), and from *PLAU* Exon 11 (rs4065) to the 3′-UTR region (rs2688624). Accordingly, the markers at the *PLAU* gene form two blocks of high D' values (Block 3 and Block 4) separated by a partial breakpoint located at and including the marker rs2227564 (*PLAU* Exon 6; Fig. 1B). In addition, there were some scattered long-distance correlations between several markers within the genotyped region between rs2292307 and rs2688624, but these were uncorrelated with the markers at the *VCL* gene.

Association with AD

Analysis of *PLAU* SNP alleles in the screening sample from Munich revealed evidence of an allelic association with AD for several markers located at the intergenic region upstream

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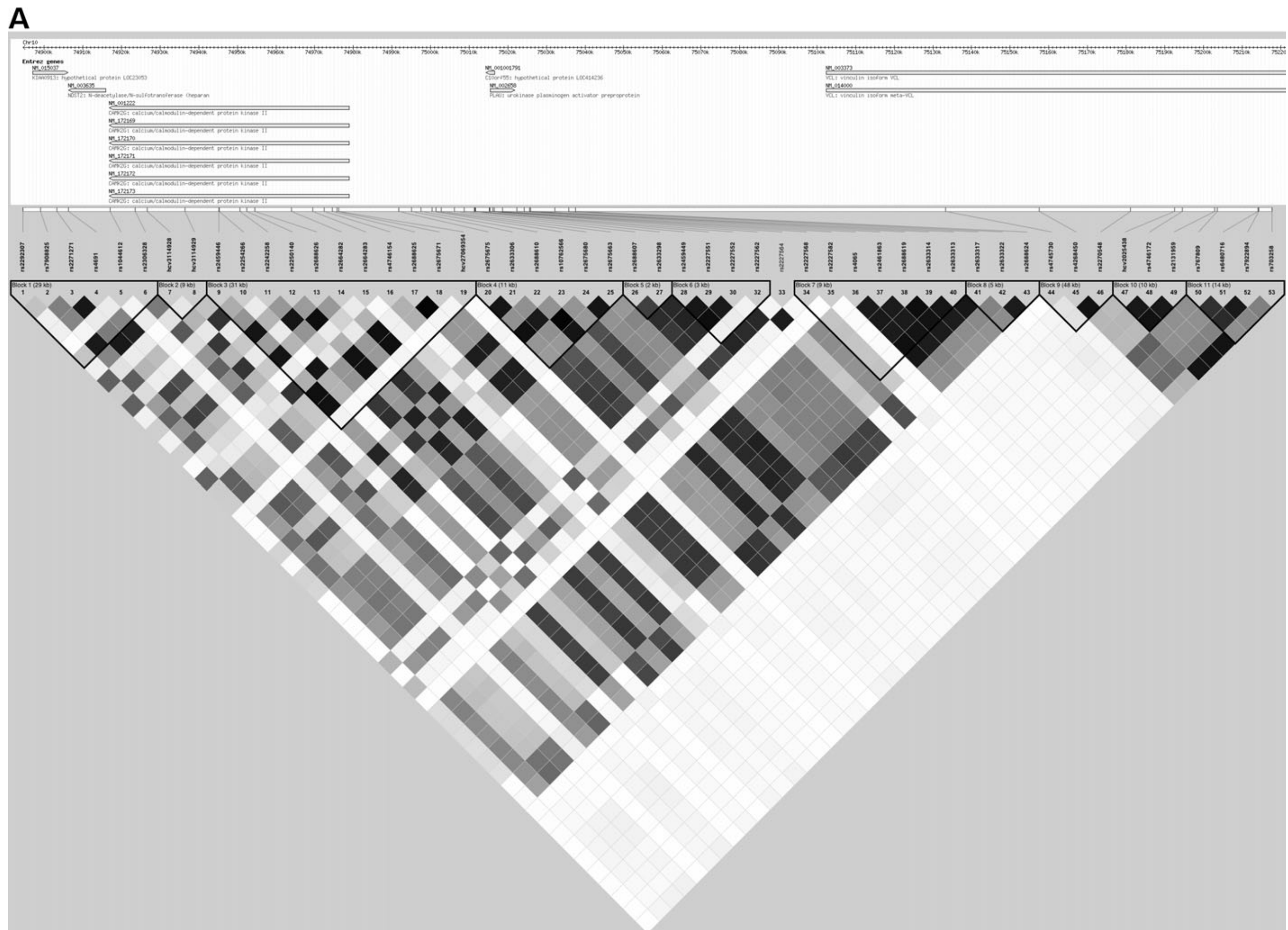

Figure 1. (**A**) Linkage disequilibrium structure of the extended *PLAU* gene region including the neighboring genes. (**B**) Linkage disequilibrium structure of the *PLAU* gene region. Linkage disequilibrium structure of the *PLAU* gene region. Pairwise r^2 values are color-coded: black, high r^2 values; white, low r^2 values. The haplotype blocks indicated by high D' values are superimposed. The gene structure of the genomic region is shown on top.

of *PLAU*, the LD breakpoint region within *PLAU* around Exon 6, and the 3′-UTR region. Specifically, three genetic markers within *PLAU* (rs2227562 Intron 5, rs2227564 Exon 6, rs2227568 Exon 8) were found to be associated with AD (Table 1). The risk conferred to AD was associated with the minor T allele for marker rs2227564, but was due to the major allele for both neighboring markers rs2227562 and rs2227568. The latter association pattern may be described by a common risk variant with a frequency of ∼86% or, alternatively, by a rare protective variant (frequency ∼14%). Both markers were also highly intercorrelated ($r^2 = 0.9$; $D' = 0.96$), whereas the marker rs2227564 was separated from this correlated cluster (pairwise comparison with rs2227562: $D' = 0.73$, $r^2 = 0.036$ and rs2227568: $D' = 0.63$, $r^2 = 0.025$).

A more detailed analysis of the association signals within the Munich screening sample revealed differences in the strength of the association according to the disease onset. To further explore this association we separately investigated patients with an early-onset of the disease (EOAD; onset $\leq$65 years; $N = 140$) and those with late-onset AD (LOAD $>$65 years; $N = 282$). In the EOAD group, we observed stronger allelic association ($P = 0.001$) and a risk allele (T-allele) dose-dependent increase of the odds ratio (OR) from 1.7 (1.1–2.6) in heterozygous subjects up to 3.0 (1.1–8.0) in homozygous subjects (Table 2). No significant association was obtained in patients with LOAD (Table 1). Concerning both neighboring SNPs, the opposite effect was observed. Both markers show a trend or significant allelic association with LOAD [rs2227562: $P = 0.04$; OR = 1.5 (1.01–2.2) and rs2227568: $P = 0.05$; OR = 1.47 (0.99–2.1)], whereas no association was present in patients with EOAD (Table 1). Because of the number of SNPs genotyped at this region the genotypic association signals would no longer be significant when adjusting for the multiple comparisons performed.

Two LD blocks which include the associated markers rs2227562 (Block 3; Fig. 1B) and rs2227568 (Block 4; Fig. 1B) were identified at the *PLAU* region. Within Block 3, three haplotypes are estimated. The most common haplotype shows a non-significant trend for association with AD ($\chi^2 = 3.72$; $P = 0.054$; Table 3) and another haplotype with a frequency of 13.4% in cases and 18.1% in controls indicate a significant association ($\chi^2 = 5.7$; $P = 0.017$; Table 3). Within

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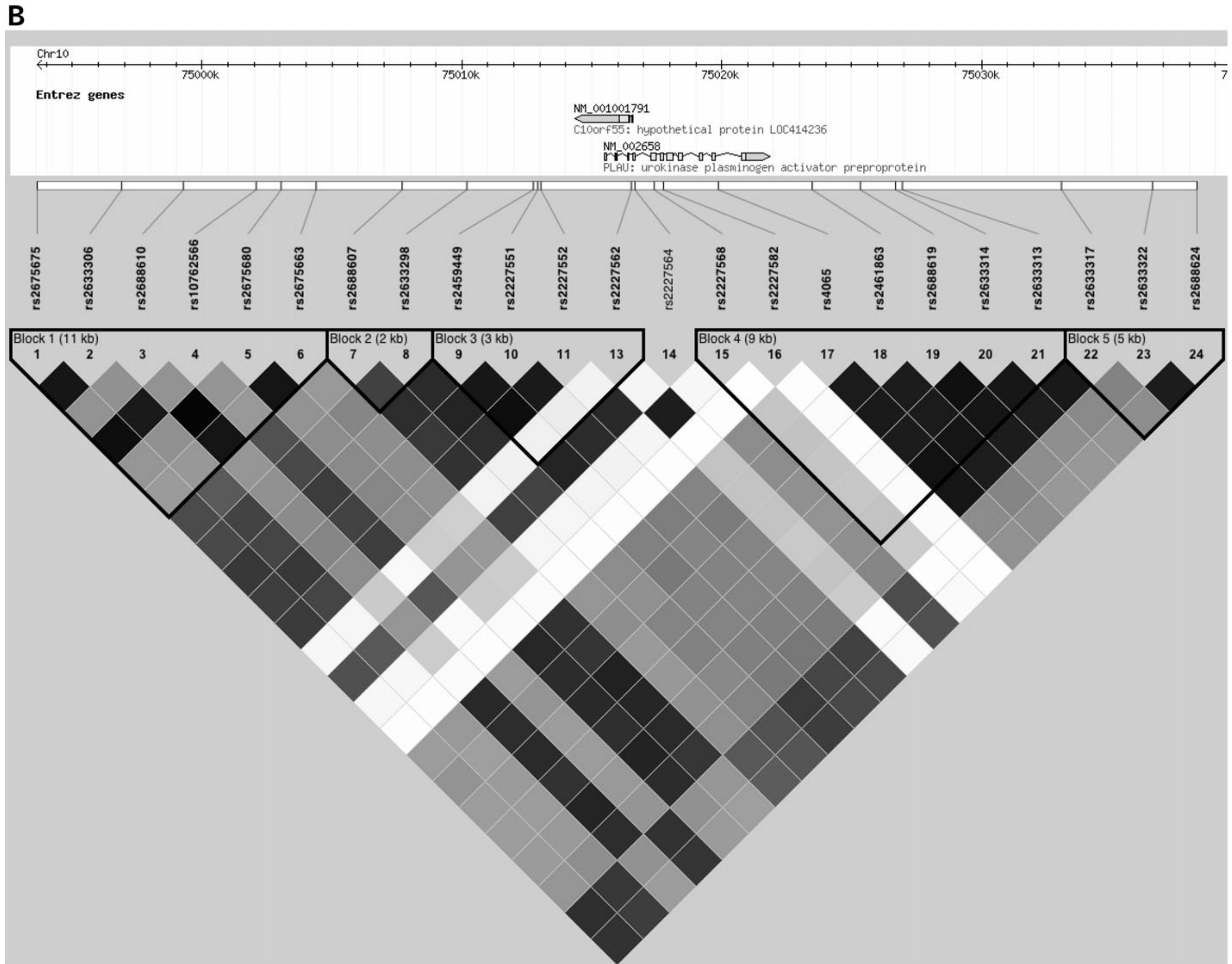

Figure 1. Continued.

Block 4, four haplotpyes are estimated and one haplotype showing a frequency of 12% in cases and 16.8% in controls was significantly associated ($\chi^2 = 6.1$; $P = 0.014$; Table 3).

Using a sliding window approach for three marker associations, we identified two regions showing evidence for a table-wide significant association. The proximal signal was obtained at the intergenic region within window 1 (Haplotype 1.3; adjusted $P = 0.024$) and a trend for window 2 (Haplotype 2.3; adjusted $P = 0.051$; Supplementary Table, S1). The strongest, second signal was observed at the breakpoint for window 11 (marker combinations rs2227552, rs2227562, rs2227564) and window 12 (marker combination rs2227562, rs2227564, rs2227568; Supplementary Table, S1). Two haplotypes with frequencies of 12.5% in cases and 17.9% in controls (Haplotype 11.3; adjusted $P = 0.057$) and 0.7% in cases and 3% in controls (Haplotype 11.4; adjusted $P = 0.009$) showed a trend or table-wide significant association (1000 permutations). In addition, the haplotypic combination 12.3 of window 12 with frequencies of 11.3% in cases and 16.7% in controls was also significantly associated (adjusted $P = 0.048$).

Sequencing

To identify additional SNPs which are in LD with the functional rs2227564 polymorphism, we sequenced the complete Exon 6 of the PLAU gene including both exon/intron boundaries using an independent sample from Munich consisting of 80 patients with AD and 80 controls. By sequencing we identified one additional functional, but rare variation within Exon 6 (1811 C/T His → Tyr), 23 bp upstream of the rs2227564 (1788 C/T; Leu → Pro) polymorphism (Fig. 2). Subsequent genotyping of this novel polymorphism in the Munich sample did not reveal any evidence of an association with AD or patients with EOAD (data not shown).

Replication

To replicate our initial genetic results we investigated the rs2227564 polymorphism in three independent case−control series from Bonn (Germany), Brescia (Italy), Perth (Australia) and in addition applied a family-based approach using a discordant sib-pair sample from Germany.

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Table 1. SNP description, allele distribution and association with AD

SNP	Position (hg17)	Position in *PLAU* gene	Alleles (major/minor)	Major allele frequency (cases/controls)	AD affection allelic *P*-values (risk allele) OR and (95%CI)		
					AD complete $N = 422$	EOAD ($N = 140$) (onset ≤65 years)	LOAD ($N = 282$) (onset > 65 years)
rs2675675	75319054	Intergenic	A/G	0.766/0.749	0.490	0.947	0.366
rs2633306	75322453	Intergenic	C/A	0.749/0.745	0.879	0.67	0.728
rs2688610	75324937	Intergenic	A/G	0.591/0.520	0.012 (A); 1.34 (1.07–1.67)	0.492	0.013 (A); 1.51 (1.1–2.1)
rs10762566	75327866	Intergenic	G/A	0.765/0.752	0.574	0.937	0.503
rs2675680	75328870	Intergenic	C/T	0.589/0.529	0.032 (C); 1.27 (1.02–1.59)	0.968	0.01 (C); 1.5 (1.1–2.0)
rs2675663	75330276	C10orf 55	G/T	0.574/0.520	0.042 (G); 1.26 (1.01–1.57)	0.905	0.009 (G); 1.5 (1.1–2.0)
rs2688607	75333742	Promoter	G/A	0.764/0.753	0.6402	0.808	0.596
rs2633298	75336345	Promoter	G/C	0.723/0.729	0.8159	0.474	0.998
rs2459449	75339019	Promoter	C/T	0.736/0.722	0.5708	0.901	0.394
rs2227551	75339196	Promoter	T/G	0.735/0.732	0.9021	0.759	0.739
rs2227552	75339325	Promoter	T/C	0.729/0.711	0.5034	0.698	0.481
rs2227579	75341295	Exon 2	C/T	n.a.	n.a.	n.a.	n.a.
rs2227562	75342967	Intron 5	G/A	0.864/0.816	0.019 (G); 1.47 (1.09–1.98)	0.072	0.042 (G); 1.5 (1.01–2.2)
rs2227564	75343107	Exon 6	C/T	0.718/0.782	0.020 (T); 1.37 (1.05–1.78)	0.001 (T); 1.91 (1.3–2.8)	0.517
rs2227568	75343885	Exon 8	C/T	0.876/0.826	0.033 (C); 1.37 (1.03–1.84)	0.173	0.05 (C); 1.47 (10.99–2.1)
rs2227582	75344258	Intron 8	T/C	0.988/0.990	0.7139	0.914	0.678
rs4065	75346470	Exon 11	T/C	0.599/0.562	0.1883	0.912	0.115
rs2461863	75350242	3′-UTR	T/C	0.620/0.556	0.018 (T); 1.31 (1.04–1.65)	0.647	0.007(T) 1.6 (1.2–2.24)
rs2688619	75352182	3′-UTR	G/A	0.593/0.531	0.034 (G); 1.28 (1.02–1.61)	0.813	0.008 (G) 1.54 (1.1–2.14)
rs2633314	75353596	3′-UTR	T/C	0.595/0.545	0.068	0.403	0.09
rs2633313	75353871	3′-UTR	T/C	0.603/0.549	0.049 (T); 1.26 (1.01–1.58)	0.659	0.029 (T); 1.42 (1.03–1.95)
rs2633317	75360274	Intergenic	G/A	0.613/0.559	0.053	0.375	0.028 (G); 1.41 (1.04–1.9)
rs2633322	75363938	Intergenic	C/T	0.757/0.742	0.526	0.156	0.323
rs2688624	75365730	Intergenic	G/T	0.784/0.738	0.061	0.739	0.011 (G); 1.59 (1.1–2.23)

The locations of the SNPs on Chromosome 10 and *PLAU* according to the UCSC map (hg17) are shown. The distribution of major allele frequencies in cases and controls from Munich, the association results (logistic regression including the covariates age at onset/exam and sex) for the complete sample, patients with early onset AD (EOAD) and late onset AD (LOAD) with *P*-values and OR with 95%CI for significant SNPs are shown. n.a., not available.

Regarding the case–control collectives we observed a significant allelic association for all series and further obtained a T-allele dose-dependent increase of the OR in the sample from Bonn and a similar trend in the series from Brescia, which just failed to reach significance in homozygous carriers of the T allele, probably because of the limited sample size (Table 2). In the Australian series, this effect was, likewise to the Munich sample, only present in patients with a younger onset of the disease. Because of a relatively small number of patients with EOAD, we divided this sample into two equal-sized subgroups defined by the cohort median age of 80 years (Table 2).

As a final step of our replication procedure, we applied a family-based strategy and analyzed the *PLAU* rs2227564 polymorphism in a discordant sib-pair sample from Germany. For the statistical analysis we applied the S-TDT (25) and obtained a highly significant over-representation of the risk-allele in the affected group ($P = 0.001$; Table 2).

In addition, no significant interaction between the rs2227564 polymorphism and the *APOE*ϵ4 carrier status was observed in the larger series from Munich and Perth ($P > 0.05$).

Functional analyses

To corroborate our genetic data, we extended our analysis to histopathologically confirmed brain samples of subjects with AD ($N = 33$) and investigated possible effects of the *PLAU* rs2227564 polymorphism on cerebral plaque counts, as one characteristic feature of AD associated with Aβ metabolism. The brain samples used for this analysis are presented in Table 4. In a first approach we restricted our analysis to patients lacking the *APOE*ϵ4 allele because of its known effect on plaque pathology. We found significantly higher plaque counts in temporal cortices of patients carrying the *PLAU* rs2227564 T allele (60.3 ± 16.9; $N = 6$) compared with patients without the rs2227564 T allele (26.3 ± 8.8; $N = 9$; $P = 0.007$) (Table 4, Figs 3 and 4). In the complete histopathological sample ($N = 33$), we then determined the influence of the *APOE*ϵ4 and *PLAU* rs2227564 T allele on cerebral plaque count by linear regression analysis, using age and sex as covariates. As expected, we found a significant effect for presence of the *APOE* ϵ4 allele ($P = 0.0069$), but also a weaker independent effect for the *PLAU* rs2227564 T allele ($P = 0.015$). Age and sex, however, did not contribute to the number of plaques.

DISCUSSION

PLAU represents a reasonable functional and positional candidate gene as it is located under a linkage region of AD on Chromosome 10q and functionally is involved in the degradation of Aβ via plasmin activation.

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Table 2. Common *PLAU* haplotypes, frequencies and associations with AD

Haplotype ID	Sequence	Frequency cases/controls	AD affection *P*-values (uncorrected)
Block 1			
1.1	ACAGCG	0.572/0.516	0.0441
1.2	GAGATT	0.236/0.244	0.7227
1.3	ACGGTT	0.162/0.217	0.0124
1.4	ACAGCT	0.014/0.008	0.2884
Block 2			
2.1	GG	0.711/0.717	0.8356
2.2	AC	0.230/0.236	0.7922
2.3	GC	0.052/0.037	0.1975
Block 3			
3.1	CTTG	0.590, 0.537	0.0538
3.2	TGCG	0.255, 0.268	0.5938
3.3	CTTA	0.134, 0.181	0.0171
Block 4			
4.1	CTTTGTT	0.572/0.526	0.0953
4.2	CTCCACC	0.260/0.270	0.6679
4.3	TTCCACC	0.120/0.168	0.0139
4.4	CCTTGTT	0.012/0.010	0.7208
Block 5			
5.1	GCG	0.604/0.558	0.0920
5.2	ATT	0.229/0.249	0.3883
5.3	ACG	0.147/0.180	0.1009
5.4	ATG	0.017/0.009	0.2083

All haplotypes with a frequency >1% within the *PLAU* gene region (23 SNPs) in the Munich sample were tested for association with AD affection status using χ^2 statistics.

In an attempt to elucidate the genetic contribution of *PLAU* to the development of AD and to further understand the conflicting results of several association studies on *PLAU*, we analyzed a large case–control sample on a high-density SNP map using a whole gene approach (24) and analyzed the LD structure of the genomic region. Using a sliding window approach, we fine-mapped the associated region. The strongest single marker association conferring risk to the disease, particular EOAD was observed for the functional rs2227564 polymorphism, which is located at an LD breakpoint within *PLAU* Exon 6. Interestingly, the strength of this association increased in patients with a younger onset of the disease, particularly in patients with EOAD where we observed a T-allele dose-dependent increase of the ORs, whereas other, weaker association signals appeared in patients with LOAD. Sequencing of *PLAU* Exon 6 identified a new functional polymorphism, which did not contribute to the observed association. As the rs2227564 polymorphism showed the strongest association signal and because of its functional nature we replicated this SNP in three independent case–control series from Bonn, Brescia and Perth and confirmed our initial results. All series showed an allelic association and a T-allele dose-dependent increase of the ORs. In the Australian series the association appeared, likewise to the Munich sample, to be stronger in patients with a younger onset of the disease. To reduce the possibility of a spurious association finding due to hidden stratification we included a discordant sib-pair sample from Germany, which also showed a significant over-representation of the risk allele in the affected group. Because of the hypothesized functional involvement of *PLAU* in Aβ catabolism, we finally examined possible effects of the *PLAU* rs2227564 T-allele on cerebral plaque counts using post-mortem brain material of subjects with neuropathological confirmed AD. Brain samples with the rs2227564 T-allele showed significantly higher plaque counts compared with non-carriers, and this effect was independent from the presence of the *APOE*ϵ4 allele.

Taken together our results provide considerable evidence of a genetic and functional involvement of *PLAU* polymorphisms, respectively, of a yet untyped causal variant in LD, into the pathogenesis of AD.

However, six articles investigating a possible association of *PLAU* polymorphisms with AD and related endophenotypes, such as plasma Aβ42 concentrations and cognitive performance (MMSE) were published so far. In summary, three of these articles by Myers *et al*. (19), Papassotiropoulos *et al*. (20) and Bagnoli *et al*. (21) failed to identify a significant association for the rs2227564 polymorphism with AD. While Ertekin-Taner *et al*. (22), reported a similar association to our study, Finckh *et al*. (18) observed a risk association to the opposite allele.

On the first view, these discrepant results do not substantially support a major role of *PLAU* as a new susceptibility gene of Alzheimer's disease, but rather may reflect the typical situation often observed in genetically complex diseases. Typical reasons for the discrepancies are well known and include factors such as statistical over-interpretation of the first study, small sample sizes, genetic and phenotypic heterogeneity of the analyzed trait, population differences in allele frequencies and correlation structures among genetic markers, drawbacks in study design and differences regarding ethnic background and population sub-structures (e.g. different age structures in patients).

Regarding the correlation structures, there is evidence that the LD structure of *PLAU* differs substantially among population samples. The finding of a LD breakpoint at *PLAU* Exon 6 in the German population is supported by a previous study which examined block boundary shifts in a number of genes, including *PLAU* in several European populations (26). This breakpoint has not been observed in two larger studies from the US which genotyped five (22), respectively, seven SNPs (19) within *PLAU*. Even between European and German samples, the probability for LD block boundaries around markers rs2633306/rs2688610 and rs2227564 (markers 14 and 22 in figure 2 of Mueller *et al*.) (26) are highly variable. Such differences in LD-block structures including boundary shifts are expected among different populations and may help to limit association signals to smaller regions (26). Moreover, the low r^2 values at the associated region of *PLAU* rs2227562 (Intron 5), rs2227564 (Exon 6) and rs2227568 (Exon 8) and some scattered high r^2 values among non-neighboring markers, may indicate a long and independent mutational history of the individual markers. Recent mutational events may vary in different populations, thus leading to different correlation structures between populations, which complicate replications and comparison of genetic association studies. However, all polymorphisms in correlation with our tested coding SNP have to be considered as equally likely causal candidates. There is a large block (~300 kb) of scattered correlated SNPs between two indicated recombination hotspots (see HapMap). This block

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Table 3. *PLAU* rs2227564 allele and genotype frequencies in case–control and discordant sib-pair samples

Sample	*N*	Age at onset/exam	Allele frequency [%]			Genotypes (%), *P*-values and OR (95%CI)				
			C	T	*P*-values	CC	CT	*P*-values	TT	*P*-values
Munich										
AD	422	69.1 ± 9.4	0.72	0.28	0.022	0.51	0.42	0.247	0.06	0.069
EOAD	140	59.5 ± 5.6	0.70	0.30	0.001	0.47	0.46	0.023 OR = 1.7 (1.1–2.6)	0.07	0.029 OR = 3.0 (1.1–8.0)
Controls	257	68.8 ± 13.1	0.78	0.22		0.59	0.38		0.03	
Bonn										
AD	109	74.8 ± 6.4	0.76	0.24	0.005	0.59	0.34	0.02 OR = 1.9 (1.1–3.3)	0.07	0.026 OR = 3.7 (1.1–11.9)
Controls	173	75.1 ± 8.6	0.85	0.15		0.73	0.24		0.03	
Brescia										
AD	120	67.3 ± 8.5	0.79	0.21	0.004	0.64	0.30	0.002 OR= 3.2 (1.5–6.9)	0.06	0.059 OR= 8.7 (0.9–82)
Controls	99	64.6 ± 7.2	0.92	0.08		0.86	0.13		0.01	
Perth										
AD	219	74.3 ± 10.7	0.72	0.28	0.009	0.53	0.39	0.01	0.08	0.08
AD >80 years	104	84.2 ± 3.7	0.78	0.22	n.s.	0.63	0.32	n.s.	0.05	n.s.
AD ≤80 years	115	65.7 ± 9.5	0.67	0.33	0.001	0.45	0.42	0.001 OR= 2.4 (1.4–4.2)	0.13	0.01 OR= 2.9 (1.3–7.1)
Controls	338	77.1 ± 10.6	0.81	0.19		0.66	0.29		0.05	
Discordant sib-pairs										
Affected	251	67.1 ± 8.8	0.72	0.28	0.001[a]	0.49	0.44		0.08	
Unaffected	371	64.8 ± 11.5	0.81	0.19		0.68	0.28		0.04	

Description of the case–control cohorts from Munich, Bonn, Brescia and Perth and the discordant sib-pair sample with age of onset for patients and age at exam for controls. Allele frequencies, allelic association results (logistic regression including the covariates age at onset/exam and sex), genotype frequencies and genotypic association results (logistic regression including the covariates age and sex) with *P*-values and OR with 95%CI are given for the four independent case–control series. n.s., not significant.
[a]Statistical analysis of the discordant sib-pair sample was performed using the S-TDT as described in the methods section.

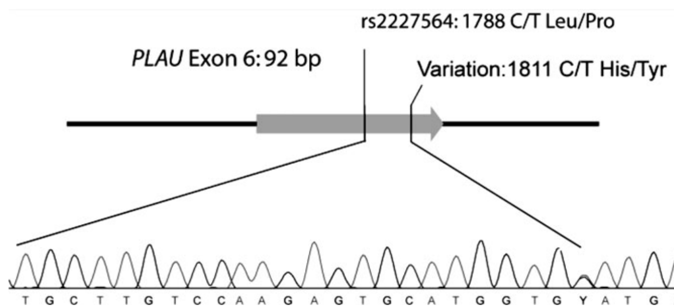

Figure 2. Sequencing chromatogram of the *PLAU* Exon 6 region. Identification of SNPs at the PLAU Exon 6 region. One additional coding SNP was identified by sequencing of genomic DNA in 80 patients with AD and 80 controls (320 alleles).

encompasses the following 10 genes: *FLJ32658*, *SEC24C*, *FUT11*, *CHCHD1*, *LOC196752*, *KIAA0913*, *NDST2*, *CAMK2G*, *C10orf55* and *PLAU*. On the basis of our results we assume, however, that our association signal maps to the *PLAU* region, which is supported by relatively high, although variable, probabilities for LD block boundaries between the neighboring genes *CAMK2G* and *PLAU* in most European samples [around marker 8/9 (rs2675671/rs2633303) and marker 14 (rs2688611) in figure 2 of Mueller *et al.*] (26), which in turn corresponds to higher estimated recombination rates in the HapMap data. In addition, the 3′ neighboring gene *VCL* is clearly unlinked to *PLAU*.

Sample size considerations may apply to the studies of Papassotiropoulos *et al.* (20) and Finckh *et al.* (18) who used small sample sizes, which were less than 100 individuals in some of their analyzed samples (e.g. 43 cases and 55 controls from Basel, Switzerland and 94 patients and 30 controls from Brescia, Italy) (18), or 181 cases and 99 controls from Greece (20). Another weak point of both studies is the applied pooling strategy, thereby combining several underpowered case–control series of different ethnic backgrounds and considerable differences of the genotype distributions (upto 10%). In contrast to all other studies, Finckh *et al.* (18) identified the major allele to confer risk to AD. This is of particular interest as we observed a similar association pattern for both neighboring SNPs (rs2227562 and rs2227568). Although there appears to be large variability of the rs2227564 minor allele frequencies among different populations with a gradient from Northern Europe (30%) to the Southern part (13%) (26), which is accompanied by an increase of the recombination signal in the southern parts, the reason for this observation remains to be elucidated.

An often neglected and underestimated factor that certainly contributes to the large variability of genetic association studies concerns sample differences in the age structures. We also observed only a weak association in our larger series from Munich and Perth, but subsequent analysis of the patient substructure revealed that the risk conferred by rs2227564 appears to be stronger in patients with a younger onset of AD, whereas no effect was present in elderly patients. This age-dependency seems reasonable as it may be hypothesized that the genetic contribution, in general and that of a specific genetic risk factor may vary according to differences

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Table 4. Description of brain samples used for analysis of cerebral plaque counts, description of brain samples and plaque counts of the temporal cerebral cortex

Groups	*N*	Sex (male/female)	Age at death (mean ± SD)	Duration (mean ± SD)	Plaque counts (mean ± SD)
rs2227564 T-allele absent *APOE*ϵ4 allele absent	9	3:6	79.3 ± 9.5	7.4 ± 2.9	26.3 ± 8.8
rs2227564 T-allele present *APOE*ϵ4 allele absent	6	2:4	80.2 ± 7.6	7.5 ± 3.2	60.3 ± 16.9
rs2227564 T-allele absent *APOE*ϵ4 allele present	13	3:10	78.1 ± 7.2	7.7 ± 3.5	59.8 ± 38.8
rs2227564 T-allele present *APOE*ϵ4 allele present	5	3:2	78.5 ± 9.2	7.1 ± 3.6	79.2 ± 26.8

Numbers, sex distribution, age at death (years, mean ± SD), duration of the disease (years, mean ± SD) and cortical plaque counts (mean ± SD) according to the presence or absence of the *PLAU* rs 2227564 T- and the *APOE*ϵ4 allele are shown. Plaques were counted by examination of three slides per case and eight consecutive representative fields from severely affected tissue areas.

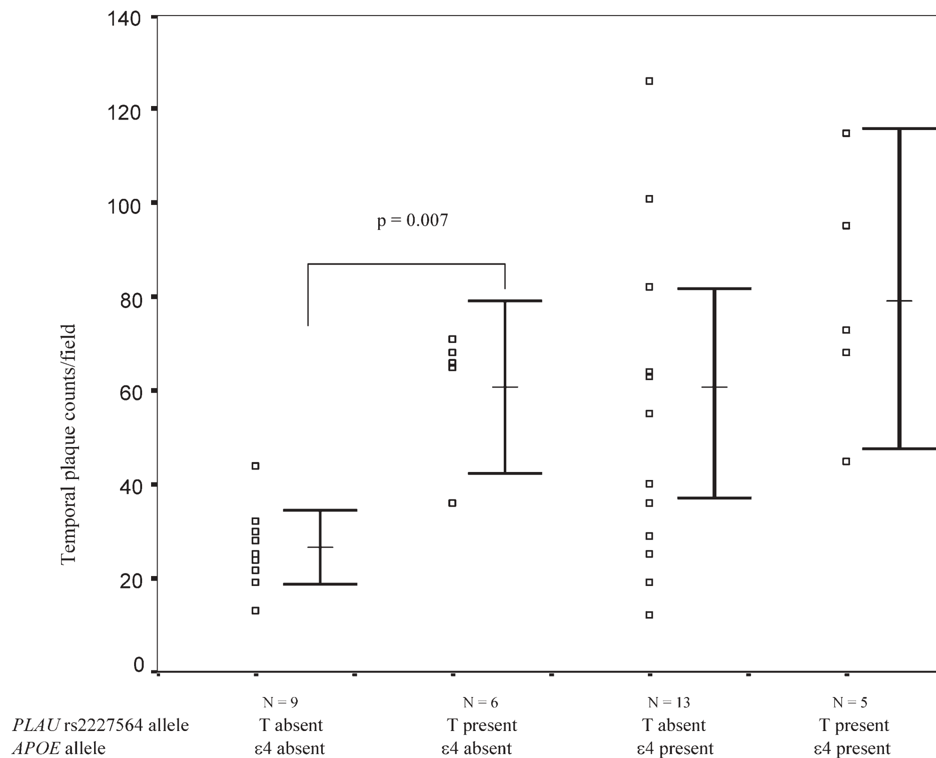

Figure 3. Plaque counts according to presence or absence of the *PLAU* s2227564 T and the *APOE*ϵ4 alleles. Scatter of temporal plaque counts with mean and 95%CI. Grouping with number of samples according to presence or absence of the *PLAU* rs2227564 T allele and the *APOE*ϵ4 allele. *P*-value for group differences using the two-sided Mann–Whitney *U* test as indicated.

regarding the onset of the disease. This fact has been repeatedly demonstrated for *APOE*, the strongest genetic risk factor of AD so far (27). Therefore, studies using relatively old patient samples, such as Myers *et al*. (19) (e.g. Mayo series mean age 82.7 years) may have missed to detect the possible association with EOAD.

In contrast, Ertekin-Taner *et al*. (22) demonstrated genetic results similar to this study in their larger series (MCR) from the US. They observed not only a significant OR of 1.4 (1.018–1.9) for the functional Exon 6 rs2227564 polymorphism, but also significant results for some neighboring markers at Intron 7, Intron 9 and Exon 11.

Moreover, they present functional data of significantly elevated plasma Aβ42 levels in carriers of the *PLAU* risk genotypes, which are believed to reflect a quantitative trait of AD. This result corresponds to our findings of higher cerebral plaque counts in brain samples with the risk allele. In addition, they suggest that the elevation of Aβ42 levels may be due to a loss of function of *PLAU* as aged mice with a knock-out of the *PLAU* gene also showed substantially elevated Aβ42 levels. Further support of a functional involvement of *PLAU* in the mediation of lethal effects due to *APP* over-expression comes from transgenic mouse studies (28). Analysis of age at death as a quantitative trait in transgenic animals vulnerable to *APP* over-expression identified a region on mouse Chromosome 14, which counteracts the lethal effects of APP. This region harbors several sequences with human homologues, including two genes, *PLAU* and neuregulin 3 (*NRG3*) on Chromosome 10 (28). As preliminary genotyping data from our group do not support *NRG3* as a possible susceptibility gene of AD (data not shown), it remains to be seen whether *PLAU* alone is responsible for the linkage signal.

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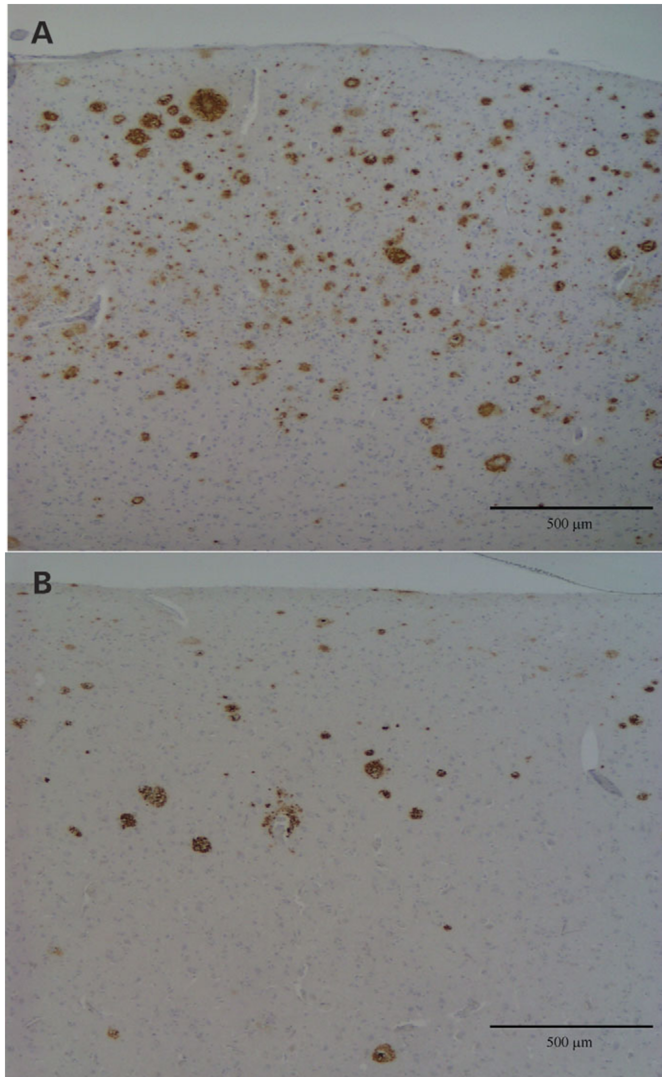

Figure 4. Immunohistochemistry of plaques according to the *PLAU* rs2227564 alleles. Comparison of Aβ load of the temporal cortex in two cases lacking the *APOE*ϵ4 allele with (**A**) and without (**B**) the *PLAU* rs2227564 T allele. Immunohistochemistry with monoclonal Aβ antibody (DAKO; 6F/3D; 1:100), peroxidase/DAB. Scale bar: 500 μm.

Likewise to our study, also Papassotiropoulos *et al*. (20) investigated possible functional effects of the rs2227564 polymorphism in human brain material but failed to find any effects. However, the use of brain samples from subjects without dementia and any neuropathological abnormalities may be the reason for not identifying any effects on the cerebral plaque load. While Myers *et al*. (19) excluded variations in *PLAU* to contribute to their linkage region at Chromosome 10q (2,10), Ertekin-Taner *et al*. (22) demonstrated for the rs2227564 polymorphism, a contribution of 22% to their plasma Aβ42 phenotype (22).

In summary, this study provides compelling evidence of a genetic and functional involvement of a common *PLAU* variant into the pathogenesis of AD. Further functional investigations are required to elucidate the role of *PLAU* and *PLAU* polymorphisms in the pathogenesis of AD and the processing of Aβ proteins.

MATERIAL AND METHODS

Subjects and material

This study was approved by the review boards of each medical faculty and refers to a total of 2359 Caucasian subjects compromising four independent case–control samples from Munich, Germany (AD, $N = 422$; controls $= 257$); Bonn, Germany (AD $= 109$; controls $= 173$); Brescia, Italy (AD $= 120$; controls $= 99$); Perth, Australia (AD $= 219$; controls $= 338$) and a discordant sib-pair sample from Germany (affected $= 251$; unaffected $= 371$). All individuals were recruited from specialists at University Memory Clinics and each control group was matched for geographical location, ethnicity, sex and age and consisted of cognitively healthy individuals. The clinical diagnosis of probable AD was established according to National Institute of Neurological and Communicative disorders and Stroke-Alzheimer's Disease and Related Disorder Association (NINCDS-ADRDA) criteria (29). After informed consent had been obtained, blood samples of each individual were taken by venous puncture. Cognitive performance was assessed using standard neuropsychological tests, such as the Cambridge Cognitive Examination (30) or the Consortium to Establish a Registry for Alzheimer's disease (31) which includes the Mini Mental State Examination (MMSE) (32), which was used as a measure of global cognitive performance in the control groups. Control subjects with an MMSE score below 28 were excluded from further analyses. All patients and controls underwent a thorough psychiatric, neurological and neuropsychological evaluation. The diagnostic work-up also included an informant interview, a chemistry survey and structural brain imaging.

Frozen and paraffin-embedded brain tissue from 33 randomly selected patients (11 male, 22 female; mean age at death 78.5 ± 7.9 years; disease duration 7.5 ± 3.4 years) with clinically and neuropathologically confirmed late-onset AD was obtained from the Departments of Neuropathology at the University of Munich and the Technical University Munich.

The neuropathological diagnosis of AD was performed according to established criteria (33).

Genotyping and sequencing

We genotyped 56 SNPs derived from Public or the Celera human genome databases covering ~299 kb of the *PLAU* gene region on Chromosome 10 including the upstream located genes calcium/calmodulin-dependent protein kinase II-gamma (*CAMK2G*; OMIM # 602123), *N*-deacetylase/*N*-sulfotransferase 2 (*NDST2*; OMIM # 603268), KIAA0913 and downstream Vinculin (*VCL*; OMIM # 193065). A total of 52 SNPs with a median spacing of 6.2′kb showed sufficient genotyping quality (mean call rate above 95%), Hardy–Weinberg equilibrium and a minor allele frequency of more than 0.05 in the controls (Fig. 1). At the *PLAU* region, we genotyped 24 markers with a mean intermarker distance of 2 kb. The markers and locations are described in detail in Table 1. *APOE* genotypes were determined by the restriction enzyme approach (34). Genotyping was performed at the Department of Psychiatry, TU Munich and the Genome Analysis Center, GSF, Munich by a primer extension of multiplex polymerase

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chain reaction products and detection of the allele-specific extension products by matrix-associated laser desorption/ ionization time of flight (MALDI-TOF) mass spectrometry (Sequenom, San Diego, USA).

We sequenced genomic DNA (*PLAU* Exon 6 including the Exon/Intron boundaries at both sides) from 80 patients with AD and 80 cognitively healthy controls using Applied Biosystems BigDye Chemistry according to the recommendations of the manufacturer.

DNA from paraffin-embedded brain tissues was extracted as described (35), whereas standard methodologies were used for frozen brain samples. *APOE* and *PLAU* genotypes of brain material were determined using two independent methodologies, the MALDI-TOF and the restriction enzyme approach (34,36).

Analysis of cerebral plaque counts and immunohistochemistry

The quantitative analysis of plaques and Aβ immunohistochemistry was performed using sections from the medial temporal gyrus according to the methodology described before (37,38). In particular, the number of plaques were counted by an experienced neuropathologist who was blinded for the genotyping results by examination of three slides per case and eight consecutive representative fields from severely affected tissue areas.

Statistics

The pairwise linkage disequilibrium measures, D' and r^2, were calculated using the software package Haploview (39). Association between AD affection state and the alleles/ genotypes of SNP markers was tested by logistic regression analysis including age of onset/examination and sex as covariates. Corrections for multiple comparisons were considered using a global permutation test (1000 permutations). Multiplicative interaction terms were tested between SNP effects and *APOE*ϵ4 status (present or absent), age and gender by appropriate logistic regression models. Haplotype assignments for each individual were estimated by the EM algorithm and subsequently used for association tests, both implemented in Haploview (39). A sliding window approach was performed to test for all two-, and three-locus haplotype associations. The discordant sib-pair sample was analyzed using the sib transmission/disequilibrium test (S-TDT) (25).

Differences in plaque counts were analyzed according to the presence or absence of the *PLAU* risk alleles by the two-sided Mann–Whitney *U* test. An overall analysis of genetic effects on cerebral plaque pathology was estimated by linear regression analysis using the *APOE*ϵ4 allele, *PLAU* risk allele, duration of the disease and gender as covariates.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None of the authors has had involvements that might raise the question of bias in the work reported or in the conclusions, implications or opinions stated.

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APPENDIX

The German Sib-Pair Study Group: Many data and biomaterials were collected by two recruitment sides of the German Sib-Pair Study Group at the TU-Munich (M. Krömmer, B. Cramer, A. Klimbacher), the GSF, Munich (P. Belcredi) and several centers that participated at this study. The principal investigators and co-investigators were: Bezirkskrankenhaus Gabersee; Professor Dr G. Laux and Dr Eberl; Department of Psychiatry, University of Regensburg, Professor Dr H.E. Klein and Dr Bernd Ibach; Neurologische Klinik Bad Aibling, Professor Dr Eberhard Koenig and Dr Barbara Romero; Bezirkskrankenhaus Bayreuth, Professor Dr Manfred Wolfersdorf and Dr Michael Schüler; Department of Psychiatry, University of Freiburg, Professor Dr Mathias Berger, PD Dr Schmidtke; E. Jost MSc; Bezirkskrankenhaus Augsburg Professor Dr M. Schmauss and Ch. Steber; Krankenhaus München-Neuperlach, Abt. für Akutgeriatrie, Professor Dr R. Heinrich and Dr Britta Wiegele; Bezirkskrankenhaus Werneck PD Dr H.-P. Volz and Dr M. Jähnel; Bezirkskrankenhaus Taufkirchen, Professor Dr M. Dose, Dr Marquard and Dr Bremer; Psychiatrische Klinik Agatharied, Dr N. Braunisch and Dr H. Nickl; Bezirksklinikum Mainkofen, Dr L. Blaha and Dr S. Herpich; Bezirkskrankenhaus Haar, Dr H.W. Dietl; Bezirkskrankenhaus Günzburg, Professor Dr R. Schüttler and PD Dr R. Hess; Bezirkskrankenhaus Landshut, Professor Dr M. Philipp and Dr A. Wermuth.

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