Kinesin gene variability may affect tau phosphorylation in early Alzheimer's disease

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Received March 13, 2007; Accepted April 19, 2007

Abstract. Kinesin is a microtubule-associated motor protein that transports Alzheimer-associated amyloid precursor protein (APP) in neurons. In animal models, impaired kinesin-mediated APP transport seems to enhance formation of the neurotoxic 42 amino acid fragment of ß-amyloid (Aß42). In man, one study suggests that a polymorphism (rs8702, 56,836G>C) in the kinesin light chain 1 gene (*KNS2*) may affect the risk of Alzheimer's disease (AD). To further assess *KNS2* as a susceptibility gene for AD we analyzed 802 patients with sporadic AD and 286 controls, 134 longitudinally followed patients with mild cognitive impairment (MCI) and 39 cognitively stable controls for the rs8702 polymorphism. The rs8702 polymorphism did not influence risk of AD (p=0.46). However, rs8702 interacted with *APOE* ε4 carrier status in AD (p=0.006) and influenced cerebrospinal fluid levels of hyperphosphorylated tau in MCI patients who converted to AD during follow-up (p=0.018). These findings support earlier indications that genetic variability in the *KNS2* gene may play a role during early stages of AD pathogenesis.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder resulting in progressive cognitive impairment. The pathological hallmarks of AD are the presence of intracellular neurofibrillary tangles of hyperphosphorylated protein tau and extracellular deposits of amyloid ß-protein (Aß) in senile plaques in the cerebral cortex. Although these brain lesions are seen in aged non-demented individuals, the accumulation of Aß in the brain is believed by many to represent the earliest event in the pathogenesis of AD (1). Aß is generated from amyloid precursor protein (APP), a type I integral membrane protein with one transmembrane domain, by enzymatic digestion involving ß- and γ-secretase activities. The amyloid cascade hypothesis is supported by a wealth of genetic and biochemical data, especially for the familial forms of the disease (2). However, what starts Aß aggregation in sporadic AD remains an open question. Some data suggest that a primary alteration in the axonal compartment might be the initiating event (3). Hypothetically, a primary cause of sporadic AD might be dysfunctional axonal transport that leads to increased production of Aß and tau hyperphosphorylation, which further damage axonal transport in an autocatalytic and neurotoxic loop.

Kinesin is an ATP-dependent motor enzyme that travels along microtubules in a plus-ended direction and transports cargoes to the periphery of the cell (4). It is composed of two subunits. The first is the kinesin heavy chain protein encoded by the *KNS1* gene and contains the ATP- and microtubule-binding motifs that are essential for vesicle transport. The second is the kinesin light chain 1 (KLC1) protein encoded by the *KNS2* gene. This component associates with the heavy chain and with membrane vesicles that are transported along the axon from the neuronal cell body to nerve terminals (4). A number of observations suggest that impaired kinesin function may be specifically involved in the AD process. Firstly, KLC1 and APP exhibit high-affinity biochemical interaction (5-7). Secondly, in neurons, APP and its proteolytic machinery, ß- and γ-secretase, undergo kinesin-mediated fast anterograde axonal transport during which APP is proteolytically processed (6,8-10). Thirdly, reduced KLC1 dosage in mutant mice is associated with increased Aß generation in

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Key words: Alzheimer's disease, kinesin, polymorphism, biomarkers, apolipoprotein E, axonal transport

axonal swellings (11). Fourthly, overexpression of APP in *Drosophila* causes axonal transport defects that are markedly enhanced by reductions in the amount of kinesin (12). Finally, APP, Aß, kinesin, ß- and γ-secretase accumulate in axonal swellings during brain aging and after traumatic brain injury, and eventually form senile plaque-like aggregates that may represent early stages of AD (13-15).

Recently, a study including 100 neuropathologically confirmed AD patients and 103 non-demented controls revealed a possible association with one of three examined single nucleotide polymorphisms (SNPs) in the KLC1-encoding *KNS2* gene. The C allele of the polymorphism rs8702 (56,836G>C), located in intron 13 of the *KNS2* gene, was significantly overrepresented among AD patients (16). To further investigate this association, we analyzed the clinical material of 802 patients with sporadic AD and 286 controls, 134 longitudinally followed patients with mild cognitive impairment (MCI) and 39 cognitively stable controls for the *KNS2* rs8702 polymorphism. Apart from risk, our analysis was complemented with an evaluation of possible effects of the *KNS2* marker on quantitative biomarker traits that may reflect elements of AD pathology. Furthermore, we tested the hypothesis that *KNS2* might interact with the major susceptibility gene for AD, the apolipoprotein E (*APOE*) gene, to influence risk of AD.

Materials and methods

Participants. The study included 3 Swedish population groups (sets A-C). Set A consisted of clinically diagnosed AD patients (n=719) and controls (n=187) and neuropathologically diagnosed AD patients (n=83) and controls (n=99). Set B consisted of clinically diagnosed MCI patients and controls (17) and set C of healthy control subjects from a Swedish stroke study (18) (Table I). The study was approved by the ethics committees at the Universities of Göteborg, Lund and Umeå, Sweden. The patients (or their closest relatives) and the control subjects gave informed consent to participate in the study, which was conducted in accordance with the provisions of the Helsinki Declaration.

Patients in set A were clinically diagnosed with probable late-onset AD according to the National Institute of Neurological Disorders and Stroke - Association Internationale pour la Recherche et l'Enseignement en Neurosciences) (NINCDS-ADRDA) criteria (19). AD diagnosis was preceded by detailed clinical investigation including medical history, physical, neurological and psychiatric examination, screening laboratory tests, ECG, EEG, chest x-ray and computed tomography of the brain. All clinical evaluations were made without knowledge of the results from the biochemical and genetic analyses and vice versa. Mini-Mental State Examination (MMSE) was performed according to Folstein and co-workers (20). No patient had a family history of autosomal dominant dementia. Control cases had no history of dementia and did not show any signs of psychiatric illness, malignant disease or systematic disorder. Potential control subjects with MMSE scores <28 were not included in the control group. AD patients in the post mortem material were diagnosed with probable late-onset AD according to the NINCDS-ADRDA criteria and the diagnosis was confirmed upon neuropathological examination of the brain (21). None showed infarcts or other brain changes that could have caused the dementia. Control cases did not have a history of dementia, psychiatric or malignant disease. All controls fulfilled the requirements of histopathological scores <4.0 (21).

Table I. Descriptive data for the populations studied.

	Population	No. of subjects	Sex (M/F)	Age (mean ± SD)
Set A	Swedish			
AD		802	293/509	76±7.0
Controls		286	117/169	72±9.2
Set B	Swedish			
Early AD		57	16/41	74±5.9
Controls		39	15/24	72±7.7
Stable MCI		56	30/26	64±9.0
Progressing to other dementias		21	13/8	73±9.3
Set C	Swedish			
Healthy controls		593	379/214	56±10.3

AD, Alzheimer's disease; MCI, mild cognitive impairment; SD, standard deviation.

Sets B and C have been described elsewhere (17,18). In brief, set B consisted of 134 patients with MCI and 39 controls. At baseline, patients underwent physical, neurological and psychiatric examination, careful clinical history and functional assessment. Moreover, computed tomography of the brain and cognitive tests were performed. The criteria of MCI were those defined by Petersen and collaborators (22), which include: (i) memory complaint, preferably corroborated by an informant, (ii) objective memory impairment adjusted for age and education, (iii) preservation of general cognitive functioning, (iv) no or minimal impairment of daily life activities, and (v) not fulfilling the DSM-IIIR (Diagnostic and Statistical Manual of Mental Disorders, 3rd edition, revised) criteria of dementia (23). The patients were followed clinically at least until they developed a certain type of dementia or until they had been cognitively stable for more than 4 years (average 5.2 years; range 4.0-6.8 years). The patients who received a diagnosis of AD during follow-up were required to meet the DSM-IIIR criteria of dementia (23) and the criteria of probable AD defined by NINCDS-ADRDA (19). The patients who received a diagnosis of vascular dementia (VaD) fulfilled the DSM-IIIR criteria of dementia (23) and the requirements of NINDS-AIREN (National Institute of Neurological Disorders and Stroke - Association Internationale pour la Recherche et l'Enseignement en Neurosciences) for probable VaD (24). For patients who developed dementia with Lewy bodies or frontotemporal dementia the consensus criteria by McKeith and collaborators (25) and Brun and colleagues (26) were used, respectively. Additionally, 39 healthy individuals, cognitively stable over 3 years, served as controls.

Table II. MMSE, CSF data and *APOE* genotype frequencies for AD and control populations.

	AD	Controls	p-value
Cognition			
MMSE (mean ± SEM)	21.6±0.2	29.2±0.1	<0.001
Biomarkers			
Aß42 (mean ± SEM, ng/l)	457±6.6 (n=585)	712±14.8 (n=161)	<0.001
T-tau (mean ± SEM, ng/l)	668±12.8 (n=599)	351±12.2 (n=161)	<0.001
P-tau (mean ± SEM, ng/l)	81.4±1.4 (n=567)	62.1±1.6 (n=131)	<0.001
*APO*E genotype frequencies			
No ε4 [n (%)]	253 (31.5)	199 (69.6)	
One ε4 [n (%)]	414 (51.6)	78 (27.3)	
Two ε4 [n (%)]	135 (16.8)	9 (3.2)	<0.001

MMSE, Mini-Mental State Examination; CSF, cerebrospinal fluid; *APOE*, apolipoprotein E; SEM, standard error of the mean.

Table III. *KNS2* rs8702 genotype and allele frequencies in AD patients and controls.

	AD [n (%)]	Controls [n (%)]	p-value
KNS2 rs8702 genotype frequencies			
GG	413 (51.5)	147 (51.4)	
GC	324 (40.4)	122 (42.7)	
CC	65 (8.1)	17 (5.9)	0.46
KNS2 rs8702 allele frequencies			
G	1150 (71.7)	416 (72.7)	
C	454 (28.3)	156 (27.3)	0.64

KNS2, the kinesin light chain 1 gene; AD, Alzheimer's disease.

Set C consisted of healthy individuals randomly selected from participants in a population-based health survey (27) or the Swedish Population Register. Exclusion criteria were history or signs of vascular disease and dementing illness (18).

CSF sampling and biomarker analysis. Approximately 12 ml of CSF was collected by lumbar puncture through the L3/L4 or L4/L5 interspace in polypropylene tubes. Further details are described elsewhere (28). CSF samples were stored at -80˚C pending analysis. Determination of CSF total tau (T-tau), tau phosphorylated at threonine 181 (P-tau) and the 42 amino acid fragment of Aß (Aß42) was performed using immunoassays as previously described (17,29-31).

Genetic analyses. Gene symbols used in this study follow the recommendations of the HUGO Gene Nomenclature Committee (32). Genomic DNA for set A and B was obtained from 100 µl whole blood or from 10 mg brain tissue using a GenoPrep™ DNA Blood kit and DNA MagAttract kit (Qiagen, Germany) with GenoM™-48 Robotic Workstation (Geno Vision, Norway). Genomic DNA for set C was extracted from peripheral blood using a mini column method in single tube or 96-well microtiter well format (QIAamp® DNA Blood Mini kit and QIAamp® 96 DNA Blood kit; Qiagen). DNA was stored at -20˚C pending analysis.

APOE genotyping was performed by minisequencing as described previously in detail (33). Genotypes were obtained for the two SNPs, which are used to unambiguously define ε2, ε3, and ε4 alleles (rs7412 and rs429358).

Primers for Dynamic Allele-Specific Hybridization (DASH) and sequence PCRs enclosing *KNS2* rs8702 and DASH probes were designed using sequence information deposited in the UCSC genome browser (http://genome.ucsc.edu). DNA for DASH analysis was amplified using AmpliTaq Gold® (Applied Biosystems, Branchburg, NJ, USA) under optimal conditions: 3.0 mM $MgCl_2$, 0.16 pmol/µl forward primer (Biotin-TGAC GGTGACCTGTTGACGAAA), 0.64 pmol/µl reverse primer (GAGCACGTGCGGCACATTC) (Invitrogen, Life Technologies) and 52.5˚C hybridization temperature. Genotyping of the SNP was performed using the C probe CTTGCTCTA AGGCTTAG-rox (MWG Biotech, London, UK).

The accuracy of the DASH method was verified by DNA sequencing of 23 samples representing all three genotypes. DNA for sequencing was amplified using Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) under the optimal conditions of 1.5 mM $MgCl_2$, 0.4 pmol/µl primers (forward: AGCTGTTCACTTTGGTAACAGG, reverse: TGCTACTGGGGCATATCCTAG; Invitrogen, Life Technologies) and 56.4˚C hybridization temperature. PCR products were purified using MicroSpin™ S-300 HR Columns (Amersham Biosciences, Buckinghamshire, UK). Sequencing reactions were run in sense and anti-sense direction using cycle sequencing with fluorescent dNTPs (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1; Applied Biosystems, UK). Separation by capillary electrophoresis and detection by laser-induced fluorescence was performed with an ABI PRISM 3100 genetic analyzer (Applied Biosystems, USA).

Table IV. Descriptive data and CSF biomarker levels in AD patients according to *KNS2* rs8702 genotype.

	KNS2 rs8702			
	GG	GC	CC	p-value
Descriptive data				
No. of subjects	413	324	65	
Gender (M/F)	136/277	133/191	24/41	0.076
Age (mean ± SD)	76±7.1	76±7.1	76±4.5	0.55
MMSE (mean ± SEM)	21.6±0.3	21.7±0.3	21.4±0.66	0.62
Biomarkers				
Aß42 (mean ± SEM, ng/l)	456±9.2 (n=300)	451±9.8 (n=240)	491±29.4 (n=45)	0.54
T-tau (mean ± SEM, ng/l)	680±18.6 (n=311)	658±19.2 (n=243)	634±42.8 (n=45)	0.51
P-tau (mean ± SEM, ng/l)	82.5±2.0 (n=288)	80.3±2.1 (n=236)	80.3±6.0 (n=43)	0.52

CSF, cerebrospinal fluid; AD, Alzheimer's disease; *KNS2*, the kinesin light chain 1 gene; MMSE, Mini-Mental State Examination; SD, standard deviation; SEM, standard error of the mean.

Table V. Gene-gene interaction between *KNS2* and *APOE*.

	No *APOE* ε4 [n (%)]	One *APOE* ε4 [n (%)]	Two *APOE* ε4 [n (%)]	p-value
AD patients				
KNS2 rs8702 genotype frequencies				
GG	114 (45)	223 (53.9)	76 (56.3)	
GC	112 (44.3)	155 (37.4)	57 (42.2)	
CC	27 (10.7)	36 (8.7)	2 (1.5)	0.006
KNS2 rs8702 allele frequencies				
G	340 (67.2)	601 (72.6)	209 (77.4)	
C	166 (32.8)	227 (27.4)	61 (22.6)	0.008
Non-demented controls				
KNS2 rs8702 genotype frequencies				
GG	224 (54.2)	92 (56.1)	6 (37.5)	
GC	154 (37.3)	61 (37.2)	6 (37.5)	
CC	35 (8.48)	11 (6.71)	4 (25.0)	0.15
KNS2 rs8702 allele frequencies				
G	602 (72.9)	245 (74.7)	18 (56.3)	
C	224 (27.1)	83 (25.3)	14 (43.8)	0.081

AD, Alzeimer's disease; *KNS2*, the kinesin light chain 1 gene; *APOE*, the apolipoprotein E gene.

Statistical analysis. Deviation from Hardy-Weinberg equilibrium for alleles at individual loci as well as differences in allele and genotype distributions between groups were assessed by the χ^2 statistics. As the distribution of most quantitative measures was significantly skewed, all statistical tests involving quantitative variables were conducted using nonparametric Kruskal-Wallis analysis of ranks. All statistical calculations were performed using SYSTAT 11.0 (SYSTAT Software GmbH, Erkrath, Germany). The power of the study for detecting the difference in rs8702 C allele frequency between AD cases and controls found by Dhaenens and co-workers (16) was >95% after correction for unequal sample size.

Results

We began by confirming the well-replicated finding of association between genetic variants of *APOE* and AD. AD patients had a significantly higher number of *APOE* ε4 alleles than controls ($p<0.001$, Table II). CSF Aß42 levels were lower in AD patients than control subjects ($p<0.001$), while T-tau and

Table VI. *KNS2* genotype and allele frequencies in *APOE* ε4-positive and -negative AD patients and controls.

	AD	Controls	p-value
APOE ε4-positive individuals			
KNS2 rs8702 genotype frequencies			
GG	299 (54.5)	49 (56.3)	
GC	212 (38.6)	33 (37.9)	
CC	38 (6.9)	5 (5.7)	0.90
KNS2 rs8702 allele frequencies			
G	810 (73.7)	131 (75.3)	
C	288 (26.2)	43 (24.7)	0.67
APOE ε4-negative individuals			
KNS2 rs8702 genotype frequencies			
GG	114 (45.1)	98 (49.2)	
GC	112 (44.3)	89 (44.7)	
CC	27 (10.7)	12 (6.0)[a]	0.20
KNS2 rs8702 allele frequencies			
G	340 (67.2)	285 (71.6)	
C	166 (32.8)	113 (28.4)	0.15

AD, Alzeimer's disease; *KNS2*, the kinesin light chain 1 gene; *APOE*, the apolipoprotein E gene. [a]P=0.081 when comparing the CC genotype frequency in ε4-negative AD cases and controls.

P-tau concentrations were significantly elevated (p<0.001, Table II).

KNS2 genotype and allele frequencies were in Hardy-Weinberg equilibrium and were not significantly different between AD cases and controls (Table III). There was no association between *KNS2* genotype and biological markers for AD (Table IV). However, when analyzing *KNS2* genotype frequencies in AD patients with different numbers of *APOE* ε4 alleles, an interaction between *KNS2* and *APOE* was revealed (Table V). Inheritance of one or two ε4 alleles was associated with a gene dose-dependent decrease in the *KNS2* rs8702 C allele frequency. Next, we questioned whether this was an AD-specific phenomenon. The control group in set A had too few ε4 homozygotes to address this question. Instead, we analyzed an unrelated healthy control population from a Swedish stroke study (set C, Table I). The data showed that the interaction was not present in this non-AD cohort (Table V). When stratifying set A into *APOE* ε4-positive and -negative individuals (Table VI), a tendency was seen that the rs8702 CC genotype might contribute to risk of AD in ε4-negative individuals (p=0.081, Table VI).

Finally, we questioned whether the *KNS2* rs8702 polymorphism might influence molecular changes in the early disease process, i.e. in AD in its MCI stage. The rs8702 C allele was significantly and gene dose-dependently associated with elevated concentrations of P-tau, specifically in MCI patients who progressed to AD (p=0.018, Fig. 1). T-tau levels were affected in a similar manner (p=0.050, Fig. 1), while Aß42 levels were comparable in the three *KNS2* genotype groups. The rs8702 C allele did not show association with increased P-tau or T-tau levels in MCI patients who were cognitively stable or in MCI patients who progressed to other forms of dementia or in the cognitively stable controls (data not shown).

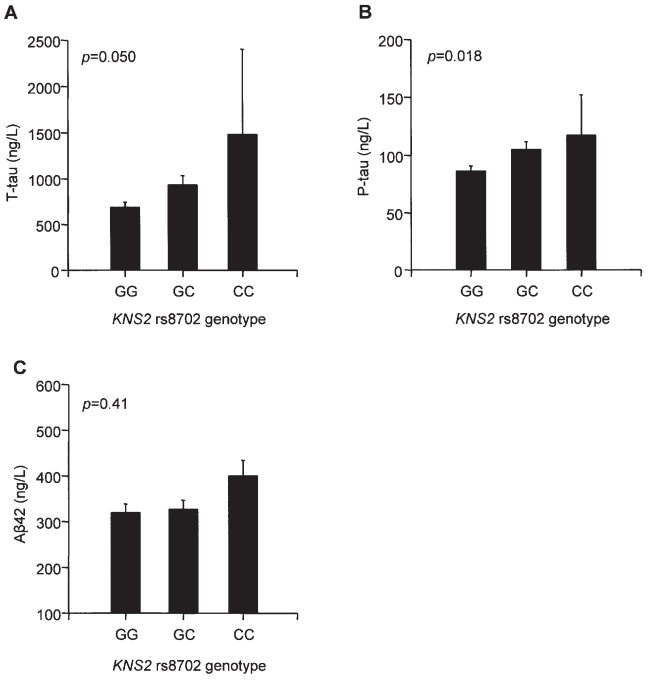

Figure 1. T-tau (A), P-tau (B) and Aß42 (C) levels according to the *KNS2* rs8702 genotypes GG (n=32), GC (n=23) and CC (n=2) in MCI patients who progressed to AD during follow-up. Values are means and error bars indicate standard errors of the mean (SEM).

Discussion

AD has been the subject of numerous gene association studies (34). While many positive findings for candidate genes have been reported, none exhibits an effect as large and consistent as that of the *APOE* gene (35,36). Here, we attempted to replicate the recent finding in a small but autopsy-confirmed case-control cohort that the *KNS2* rs8702 C allele may be associated with increased risk of AD (16).

The *KNS2* rs8702 C allele frequency in our control and study populations (27% and 28%, respectively) was similar to that found by Dhaenens *et al* when grouping their patients and controls (29%) (16). Our results showed no influence of the *KNS2* rs8702 polymorphism on risk of AD, neither in the whole population, nor in the neuropathologically confirmed subset that had a size similar to the groups in the study by Dhaenens *et al* (16). However, an association between the rs8702 C allele and tau hyperphosphorylation in early AD was revealed, which suggests an involvement of kinesin gene variability in the AD process once it has started.

Kinesin function is regulated by alternative splicing and phosphorylation of C-terminal epitopes. There are at least 19 variants of human *KNS2* gene transcripts that are generated by alternative splicing of downstream exons (37). The alternative exons are all located 3' of exon 12 and the different splice forms produce both alternative C-termini and alternative 3' untranslated regions. Intron 13, in which rs8702 is located, may contain important sequences regulating this process. Interestingly, both tau and KLC1 are known *in vivo* substrates for glycogen synthase kinase-3ß (GSK3ß) (38,39). The major phosphorylation epitopes in KLC1 are located in the C-terminal domains and hyperphosphorylation leads to KLC1 detachment from microtubules and impaired axonal transport (38). Similarly, tau hyperphosphorylation leads to its detachment from the microtubules and may either be a consequence of or result in microtubule destabilization and axonal dysfunction. Alternative splicing that skips GSK3ß consensus motif-containing KLC1 exons would possibly result in different KLC1 isoforms with different susceptibility to the GSK3ß activation seen in AD brains (40). This might also explain the association between the *KNS2* rs8702 polymorphism and hyperphosphorylation of tau. However, this hypothesis needs to be evaluated in future studies.

The gene-gene interaction between *APOE* located on chromosome 19 and *KNS2* located on chromosome 14 was an unexpected finding. The combined *KNS2* rs8702 CC and *APOE* ε4/ε4 genotype was significantly underrepresented in the AD cohort and there was a dose-dependent decrease in the C allele frequency with increasing numbers of ε4 alleles. This interaction was not present in unrelated control material. Possibly, rs8702 C carriers are less susceptible to the AD-promoting effect of the APOE ε4 allele. However, we consider this unlikely in the light of the results that the C allele is associated with tau hyperphosphorylation and that the rs8702 CC genotype might impose an increased risk of AD in ε4-negative individuals. The functional basis for the *APOE-KNS2* gene interaction is at this stage difficult to discuss and the finding warrants independent confirmation.

Altogether, our results do not support *KNS2* as a major susceptibility gene for AD. Nonetheless, genetic variation in the *KNS2* gene may influence AD-specific tau hyperphosphorylation early in the disease process. Thus, genetic variability in *KNS2* may affect the course of the disease, once its process has started.

Acknowledgements

We thank Mona Seibt Palmér for skillful technical assistance. This study was supported by grants from the Swedish Research Council, the Swedish Council for Working Life and Social Research, the Göteborg Medical Society, the Swedish Society for Medical Research, the Royal Swedish Academy of Sciences, the Fredrik and Ingrid Thuring Foundation, the Gamla Tjänarinnor Foundation, the Åke Wiberg Foundation, the Gun and Bertil Stohne Foundation, the Magn. Bergvall Foundation, the Ragnar and Einar Lundström Foundation and Pfizer Inc. The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

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