
Analysis of Genetic Variations of Protein Tyrosine Kinase *fyn* and their Association with Alcohol Dependence in Two Independent Cohorts

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Background: *Decreased sensitivity to and increased tolerance for the effects of alcohol is a phenotype, which was shown to be associated with an increased risk for alcoholism in humans and was observed in protein tyrosine kinase (PTK) *fyn* knockout mice.*

Methods: *We performed an association study of genetic variations of PTK *fyn* in 430 alcohol-dependent patients and 365 unrelated control subjects from two independent samples.*

Results: *In a combined analysis, we found an association of alcohol dependence with the single nucleotide polymorphism (SNP) T137346C in the 5' untranslated region (UTR) of the gene. A relevant association could be excluded for the remaining two informative SNPs. Selection by phenotype showed that a high number of withdrawal symptoms, high amount of alcohol intake, and high maximum number of drinks compared with unrelated control subjects was associated with the SNP in the 5'-UTR region but not with the remaining SNPs.*

Conclusions: *Our results indicate a possible association of alcohol dependence with a genotype of the SNP T137346C of the PTK *fyn*, with C being the risk allele. Biol Psychiatry 2003;54:1422–1426 © 2003 Society of Biological Psychiatry*

Key Words: Alcohol dependence, protein tyrosine kinase *fyn*, early onset, withdrawal, alcohol intake, association, linkage disequilibrium

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Introduction

Alterations in neurotransmitter systems have been associated with alcoholism phenotypes with a high genetic load, which include withdrawal characteristics (Tsai and Coyle 1998) and decreased sensitivity and increased tolerance to the effects of alcohol (Schuckit and Smith 1996). Glutamatergic signaling has been shown to be a crucial component of the pathophysiology of alcohol dependence (Spanagel and Bienkowski 2002). Knockout mice with a deletion of the intracellular src-like kinase gene, *fyn*, show increased alcohol sensitivity and lack of tolerance to the effects of ethanol (Miyakawa et al 1997) due to a reduction of *fyn*-dependent phosphorylation of the N-methyl-D-aspartate (NMDA) receptor subunits 2A (NR2A) (Tezuka et al 1999) and 2B (NR2B). The subunits NR2A and NR2B are crucial in mediating glutamatergic effects of ethanol (Fink and Gothert 1996; Masood et al 1994).

Based on these findings, we hypothesized that PTK *fyn* is involved in the pathophysiology of alcohol dependence in humans. The present study was intended to generate hypotheses regarding the role of genetic variations of *fyn*. To this end, we analyzed whether six single nucleotide polymorphisms (SNPs) in the 5' untranslated region (UTR) and various exons of the *fyn* gene are associated with alcohol dependence.

Methods and Materials

Subjects and Psychiatric Assessment

A total of 430 alcohol dependent patients recruited by the Departments of Psychiatry of the Universities of Mainz ($n = 247$) and Munich ($n = 183$) and 365 unrelated control subjects from the Mainz ($n = 100$) and Munich areas ($n = 265$) were included in the study. Table 1 shows the characteristics of our samples. All patients were consecutively admitted for inpatient alcohol withdrawal therapy and fulfilled the DSM-IV criteria for

Table 1. Description of Patient and Control Subject Samples

	n	Age (years) ^b Mean (SD)	Gender ^a	
			Male n (%)	Female n (%)
All Patients	430	42.8 (9.7)	293 (76.9)	88 (23.1)
Mainz	247	44.5 (10.1)	191 (78.3)	53 (21.7)
Munich	183	40.2 (8.5)	102 (74.5)	35 (25.5)
All Control Subjects	365	44.5 (14.8)	187 (51.2)	178 (48.8)
Mainz	100	41.9 (11.3)	72 (72.0)	28 (28.0)
Munich	265	45.3 (15.7)	115 (43.4)	150 (56.6)

^aGender differences between all patients and all control subjects: $|z| = 1.76$, $p < .001$.

^bAge difference between all patients and all control subjects: $t = 1.76$, $p = .076$.

alcohol dependence. Written informed consent was obtained from all individuals when they were in a state of full legal capacity. The study was approved by the ethics committees of the Landesärztekammer Rheinland-Pfalz and the University of Munich. Symptoms related to alcohol dependence were assessed in the Munich group with the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA; Buchholz et al 1994), whereas in the Mainz group the Michigan Alcoholism Screening Test (Selzer 1971) and the WHO-Composite International Diagnostic Interview (CIDI) (Wittchen 1994) were used. Standardized and clinical interviews were performed by staff members, who received intensive training from licensed trainers, and rated independently. Withdrawal symptoms were assessed in 291 patients as numbers of DSM-IV criteria for withdrawal according to CIDI (Mainz; $n = 162$) and SSAGA (Munich; $n = 129$). Patients were asked for subjective withdrawal symptoms during their current and past treatments. These data were corroborated with inpatient files to control for discrepancies

between staff observation and patient self-report. The number of fulfilled withdrawal symptoms was used for stratification into subgroups with high (four or more symptoms; $n = 181$) or low number of withdrawal symptoms (three or fewer; $n = 110$).

Selected phenotypes in the Munich patient group were assessed with the SSAGA. The amount of alcohol intake was defined as average alcohol intake 1 week before admission. It was assessed in 128 patients and was used for stratification into subgroups with high (≥ 300 g/day; $n = 65$) or low alcohol intake (< 300 g/day; $n = 63$). The maximum number of drinks was defined as maximum number of drinks within 24 hours (g alcohol/24 hours). It was assessed in 160 patients and was used for stratification into subgroups with high (≥ 600 g/24 hours; $n = 81$) or low alcohol intake (< 600 g/day; $n = 79$).

Munich control subjects were randomly selected from the city registration office. Mainz control subjects were selected from hospital personnel and by board advertisement. In both control groups a detailed medical and psychiatric history was performed and all Axis 1 psychiatric diagnoses were excluded by use of the Structured Clinical Interview for DSM-IV or CIDI questionnaires and clinical assessment by experienced psychiatrists.

Genotype Analysis

Genomic deoxyribonucleic acid (DNA) was isolated from venous blood by a standard salting-out method. The PTK fyn genotypes assessed were derived from the HGBASE databank (Brookes et al 2000) or described by Ishiguro et al (2000) (Table 2). Polymerase chain reaction (PCR) was performed with primers and PCR condition described in Table 3. The resulting product was purified with a DNA purification kit (Invisorb PCR-HTS-96-Kit, Invitex, Berlin, Germany). Genotyping was performed with RFLP analysis (Table 3). An ABI 377 sequencer

Table 2. Single Nucleotide Polymorphism Characteristics

Genomic Nucleotide No. ^a	Nucleotide Exchange	Amino Acid Exchange	Allele Frequency	Alleles Analyzed	Previous Nomenclature
78244	A/C		C = .127	398	T894G ^b
91653	A/G		G = .117	398	T37C ^b
91761	A/T	Ile/Phe	.00	72	HGV 2333 ^c
91811	G/T	Ala/Asp	.00	156	HGV 2303 ^c
117301	G/T	Gln/Lys	.00	72	HGV 11277 ^c
137346	T/C		C = .244	398	A93G ^b

^aThe genomic nucleotide numbers are based on locus HS66H14, accession No. Z97989.

^bNomenclature according to Ishiguro et al 2000.

^cNomenclature according to Brookes et al 2000.

Table 3. Genotype Analysis

Polymorphism	Forward Primer 5'-3' (2.5 ng/ μ L)	Reverse Primer 5'-3' (2.5 ng/ μ L)	Annealing Temperature/Cycles	PCR Fragment Size	Restriction Enzyme
A78244C	GGG GTG GTT CCT GAA AAG AG	CCA ATC AGG ACA GGT GTT TG	Hot start 52°C 40 cycles	312 bp	Apo I 50°C
A91653G	AGT GCC CTC TGC CTG ATG AAT AAC C	TGA AGT TTT CCC AAA TGG TGT CAA A	60°C 35 cycles	244 bp	Dpn II 37°C
T137346C	TTG TGC TCC GTG ATG ATG CTG TCA	GTT GCT TCT TTA TCC TTA CAT TGC	58°C 35 cycles	280 bp	Hph I 37°C

PCR, polymerase chain reaction; bp, base pairs.

Table 4. Genotypes and Allele Frequencies in Patients and Control Subjects for SNP T137346

	<i>n</i>	Genotype <i>n</i> (%)			Hardy-Weinberg	Allele Frequencies <i>n</i> (%)	
		TT	TC	CC		T	C
Patients	430	231 (53.72)	169 (39.30)	30 (6.98)	<i>p</i> = 1.00	631 (73.37)	229 (26.63)
Mainz	247	131 (53.04)	97 (39.27)	19 (7.69)		359 (72.67)	135 (27.33)
Munich	183	100 (54.64)	72 (39.34)	11 (6.01)		272 (74.32)	94 (25.68)
Control Subjects	365	228 (62.47)	117 (32.05)	20 (5.48)	<i>p</i> = .35	573 (78.49)	157 (21.51)
Mainz	100	61 (61.00)	31 (31.00)	8 (8.00)		153 (76.50)	47 (23.50)
Munich	265	167 (63.02)	86 (32.45)	12 (4.53)		420 (79.25)	110 (20.75)

SNP, single nucleotide polymorphism.

(Applied Biosystems, Foster City, California) was used to verify the results of the RFLP analysis. Control restriction fragment length polymorphism (RFLP) experiments were performed in 15% of the samples obtained by random choice. Results of repeated genotyping were identical.

Statistical Analysis

Hardy-Weinberg equilibrium and allelic associations were determined in each group with the χ^2 test. The *p* values of genotypes reported refer to the trend test for 2×3 contingency tables, as originally proposed by Armitage (1955). The Armitage test is known to be more sensitive against trend alternatives typically occurring in applications to genetic epidemiology than the classic χ^2 procedure (see Devlin and Roeder 1999). For each odds ratio (OR), exact 95% one-sided confidence intervals were calculated. All significance statements made hold in the comparison-wise sense. Controlling the multiple significance levels was considered dispensable in view of the fact that prior knowledge about the role of genetic variations of *fyn* in alcohol dependence has been too sparse for formulating a comprehensive hypothesis. Linkage disequilibrium between the three polymorphisms was computed by Arlequin 2000 software (available at: <http://anthropologie.unige.ch/arlequin/>).

Results

We analyzed six SNPs from the 5'-UTR and exons of the PTK *fyn* gene (Table 2). Only the SNP in the 5'UTR region T137346C and the silent SNPs A78244C, A91653G, previously described by Ishiguro et al 2000, were informative. Genotype frequencies were within

Hardy-Weinberg equilibrium (Tables 4 and 5). No significant linkage disequilibrium could be detected between T137346C and A91653G (Mainz patients: $\chi^2 = .1243$, *p* = .725) and between T137346C and A78244C ($\chi^2 = .0058$, *p* = .939) in any of the groups analyzed. A91653G and A78244C were in linkage disequilibrium ($\chi^2 = 208.7527$, *p* < .00001). Logistic regression analysis showed no interaction between gender and disease status in all genotypes analyzed (data not shown).

In an analysis of the combined samples, we found an association of alcohol dependence with the genotype of T137346C ($|z| = 2.35$, *p* = .020), with C being the risk allele ($\chi^2 = 5.63$, *p* = .018, OR = 1.33) (Table 6). The remaining informative SNPs were not associated with alcohol dependence (Table 7).

In exploratory analyses, Munich and Mainz patients were compared with their respective control groups. We found a numeric increase of the CC-genotype and the C-allele of T137346C in either patient group, which was not statistically significant (Table 4).

We performed exploratory analyses of phenotypes presumed to be influenced by glutamatergic neurotransmission. Patients with a "high number of withdrawal symptoms" (four or more) compared with control subjects were associated with T137346C ($|z| = 2.00$, *p* = .027) in the combined group and in the Munich group ($|z| = 2.38$, *p* = .013) but not in the Mainz group (Table 8). The amount of alcohol intake and the maximum number of drinks was

Table 5. Genotypes and Allele Frequencies in Patients and Control Subjects for SNP A91653G and SNP A78244C

	Genotype <i>n</i> (%)			Hardy-Weinberg	Allele Frequencies <i>n</i> (%)	
	AA	AG	GG		A	G
SNP A91653G						
Control Subjects	285 (78.1)	74 (20.3)	6 (1.6)	<i>p</i> = .61	644 (88.2)	86 (11.8)
Patients	334 (77.8)	89 (20.7)	7 (1.6)	<i>p</i> = .66	737 (87.7)	103 (12.3)
SNP A78244C						
Control Subjects	277 (75.9)	83 (22.7)	5 (1.4)	<i>p</i> = .82	637 (87.3)	93 (12.7)
Patients	324 (75.3)	98 (22.8)	8 (1.9)	<i>p</i> = .83	746 (86.7)	114 (13.3)

SNP, single nucleotide polymorphism.

Table 6. Comparison in Genotypes and Allele Frequencies for SNP T137346

	Armitage-Trend Test (Two-Sided) for Testing Genotype Distribution		OR	CI (OR)	χ^2 Test (Two-Sided) for Testing Differences in Allele Frequencies	<i>p</i>
	<i>z</i>	<i>p</i>				
All	<i>z</i> = 2.35	<i>p</i> = .020	1.33	1.05-1.67	χ^2 = 5.63	.018
Munich	<i>z</i> = 1.73	<i>p</i> = .089	1.32	.96-1.81	χ^2 = 2.99	.084
Mainz	<i>z</i> = 1.01	<i>p</i> = .353	1.22	.84-1.79	χ^2 = 1.08	.299

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

Table 7. Comparison of Genotypes and Allele Frequencies of SNP A91653G and SNP A78244C

	Armitage-Trend Test (Two-Sided) for Testing Genotype Distribution		χ^2 Test (Two-Sided) for Testing Differences in Allele Frequencies	
	<i>z</i>	<i>p</i>	<i>z</i>	<i>p</i>
SNP A91653G All	<i>z</i> = .12	.9388	.01 (risk allele C)	.904
SNP A78244C All	<i>z</i> = .31	.7651	1.05 (risk allele G)	.761

SNP, single nucleotide polymorphism.

only assessed in the Munich group. We found an association of patients with "high amount of alcohol drinking" (≥ 300 g/day) ($|z| = 2.84$, $p = .004$; Table 8) and of "high maximum number of drinks" (≥ 600 g/24 hours) ($|z| = 2.54$, $p = .008$) with a T137346C genotype. No association of any of the selected phenotypes with A91653G or A78244C was observed (data not shown). Although there was a highly significant interaction between "high alcohol intake" and "high maximum number of drinks" (χ^2 test: $\Phi = .663$, $p < .0001$), analysis of independence revealed no interaction between the remaining phenotype combinations.

Discussion

Based on the combined analysis of two independent cohorts, our results indicate an association of alcohol

dependence with a genotype of the SNP T137346C of PTK fyn, with C being the risk allele. An association could be excluded for the remaining informative PTK fyn SNPs A91653G and A78244C. The differences in allele frequencies and association patterns between our cohort and a Japanese group (Ishiguro et al 2000) are most likely due to ethnic differences. The OR of 1.33 is in line with the polygenic nature of alcohol dependence; however, to rule out a false-positive finding, an independent replication is warranted. Pairwise linkage disequilibrium revealed no linkage disequilibrium for T137346C, whereas A91653G and A78244C were in linkage disequilibrium, which supports the association data and suggests recombination effects between T137346C and the remaining SNPs.

Selection by phenotype revealed an association of T137346C genotype but not A91653G and A78244C with "high number of withdrawal symptoms," "high amount of drinking," (Munich group) and "high maximum number of drinks" (Munich group). These phenotypes showed no interaction with each other, indicating a different composition of each individual phenotypic subgroup.

A "high number of withdrawal symptoms" correlates with enhanced glutamatergic neurotransmission and NMDA receptor activation (Fadda and Rossetti 1998). Activation of the the NMDA receptor depends on phosphorylation through PTK fyn. In this context, the localization of T137346C in a c-myc transcription factor binding site, which might potentially influence the transcriptional activity of the PTK fyn gene, is of interest.

Table 8. Genotype Frequencies of SNP T137346C in Selected Phenotypes

	Genotypes of Patients <i>n</i> (%)				Genotypes of Control Subjects <i>n</i> (%)				Armitage-Test (One-Sided) Patients vs. Control Subjects
	TT	TC	CC	Total	TT	TC	CC	Total	
High Number of Withdrawal Symptoms									
All	231 (53.7)	169 (39.3)	30 (7.0)	430	228 (62.5)	117 (32.1)	20 (5.5)	365	2.00 ($p = .027$)
Munich	97 (53.6)	70 (38.7)	14 (7.7)	181	228 (62.5)	117 (32.1)	20 (5.5)	365	2.38 ($p = .013$)
Mainz	36 (48.0)	33 (44.0)	6 (8.0)	75	167 (63.0)	86 (32.5)	12 (4.5)	265	.34 ($p = .410$)
High Alcohol Intake (>300 g/day)									
Munich	61 (57.5)	37 (34.9)	8 (7.5)	106	61 (61.0)	31 (31.0)	8 (8.0)	100	2.84 ($p = .004$)
High Maximum Number of Drinks (>600 g/24 hours)									
Munich	27 (41.5)	34 (52.3)	4 (6.2)	65	167 (63.0)	86 (32.5)	12 (4.5)	265	2.54 ($p = .008$)

SNP, single nucleotide polymorphism.

Because the amount of alcohol intake is influenced by alcohol tolerance, our findings of an association of “high amount of alcohol intake” and “high maximum number of drinks” with T137346C in the Munich group point toward a contribution of this *fyn*-genotype to alcohol tolerance.

The PTK *fyn* gene is encoded on chromosome 6q21 (Boyle et al 1992) at 115.5 cM (Z97989). It is within chromosomal regions at 104 cM and at 166 cM, which are likely to contain genes predisposing for alcoholism (Cantor and Lanning 1999).

This study supports neurobiological research suggesting the involvement of PTK *fyn* in the pathophysiology of alcohol dependence and provides evidence for an association of alcohol dependence and alcohol phenotypes carrying a high genetic load with a PTK *fyn* SNP encoded in the promoter region of the gene.

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References

- Armitage P (1955): Tests for linear trends in proportions and frequencies. *Biometrics* 11:375–386.
- Boyle JM, Hey Y, Myers K, Stern PL, Grzeschik FH, Ikehara Y, et al (1992): Regional localization of a trophoblast antigen-related sequence and 16 other sequences to human chromosomes 6q using somatic cell hybrids. *Genomics* 12:693–698.
- Brookes AJ, Lehvaslaiho H, Siegfried M, Boehm JG, Yuan YP, Sarkar CM, et al (2000): HGBASE: A database of SNPs and other variations in and around human genes. *Nucleic Acids Res* 28:356–360.
- Bucholz KK, Cadoret R, Cloninger CR, Dinwiddie SH, Hesselbrock VM, Nurnberger JI Jr, et al (1994): A new, semi-structured psychiatric interview for use in genetic linkage studies: A report on the reliability of the SSAGA. *J Stud Alcohol* 55:149–158.
- Cantor RM, Lanning CD (1999): Comparison of evidence supporting a chromosome 6 alcoholism gene. *Genet Epidemiol* 17(suppl 1):S91–S96.
- Devlin B, Roeder K (1999): Genomic control for association studies. *Biometrics* 55:997–1004.
- Fadda F, Rossetti ZL (1998): Chronic ethanol consumption: From neuroadaptation to neurodegeneration. *Prog Neurobiol* 56:385–431.
- Fink K, Gothert M (1996): Both ethanol and ifenprodil inhibit NMDA-evoked release of various neurotransmitters at different, yet proportional potency: Potential relation to NMDA receptor subunit composition. *Naunyn Schmiedebergs Arch Pharmacol* 354:312–319.
- Ishiguro H, Saito T, Shibuya H, Toru M, Arinami T (2000): Mutation and association analysis of the *Fyn* kinase gene with alcoholism and schizophrenia. *Am J Med Genet* 96:716–720.
- Masood K, Wu C, Brauneis U, Weight FF (1994): Differential ethanol sensitivity of recombinant N-methyl-D-aspartate receptor subunits. *Mol Pharmacol* 45:324–329.
- Miyakawa T, Yagi T, Kitazawa H, Yasuda M, Kawai N, Tsuboi K, et al (1997): *Fyn*-kinase as a determinant of ethanol sensitivity: Relation to NMDA-receptor function. *Science* 278:698–701.
- Schuckit MA, Smith TL (1996): An 8-year follow-up of 450 sons of alcoholic and control subjects. *Arch Gen Psychiatry* 53:202–210.
- Selzer ML (1971): Michigan Alcoholism Screening Test: The quest for a new diagnostic instrument. *Am J Psychiatry* 127:1653–1658.
- Spanagel R, Bienkowski P (2002): Alcohol dependence and addiction. In: Lodge D, Danysz W, Parsons CG, editors. *Ionotropic Glutamate Receptors as Therapeutic Targets*. Johnson City, TN: F.P. Graham Publishing Co., 375–403.
- Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T (1999): PSD-95 promotes *Fyn*-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. *Proc Natl Acad Sci U S A* 96:435–440.
- Tsai G, Coyle JT (1998): The role of glutamatergic neurotransmission in the pathophysiology of alcoholism. *Annu Rev Med* 49:173–184.
- Wittchen HU (1994): Reliability and validity studies of the WHO Composite International Diagnostic Interview (CIDI): A critical review. *J Psychiatr Res* 28:57–84.