CHAPTER 6

GENETIC VARIATION IN *VTCN1* (B7-H4) IS ASSOCIATED WITH COURSE OF DISEASE IN JUVENILE IDIOPATHIC ARTHRITIS

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ABSTRACT

Objective

The course of disease in Juvenile Idiopathic Arthritis (JIA) is unpredictable with episodes of activity and remission. In order to identify predictive factors, 93 SNPs, JIA subtype, age at onset and ANA status were studied in relation to disease course.

Methods

Genetic and clinical parameters were analysed in a cohort of 272 Caucasian patients with persistent oligoarthritis (n=129), extended oligoarthritis (n=57) and rheumatoid factor negative polyarthritis (n=86). Categories of disease course (remitting (n=65), intermediate (n=96) and unremitting (n=111)) were designed based on the cumulative time spent in active disease in the first two years.

Results

Univariate analysis revealed association of the course of disease with JIA subtype ($p=5.7*10^{-5}$) and three SNPs; *VTCN1* rs10923223 ($p=4.4*10^{-5}$), *VTCN1* rs12046117 (p=0.017) and *CDK6* rs42041 (p=0.038). In a subsequent multivariate ordinal logistic regression analysis, *VTCN1* rs10923223 (OR 0.41, 95%-CI 0.26-0.63) and JIA subtype (OR 3.8, 95%-CI 2.0-7.2; OR 2.5, 95%-CI 1.4-4.2, for extended oligoarthritis and RF-negative polyarthritis versus persistent oligoarthritis, respectively) were the strongest independent factors for course of disease.

Conclusion

This study provides evidence that *VTCN1*, encoding B7-H4, is associated with course of disease in selected subtypes of JIA. *VTCN1* might be useful in predicting the course of disease.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a heterogeneous group of disorders characterized by chronic inflammation of the joint(s). JIA is thought to be an autoimmune disease in which the immune response is deregulated.[1] Clinical course and outcome are of great importance to patients, parents and physician. In outcome studies using a variety of criteria for remission, an overall remission rate of 40% has been reported. The highest remission rate was consistently observed in persistent oligoarthritis compared to extended oligoarthritis and rheumatoid factor (RF)-negative polyarthritis.[2-4] Because the course of disease is unpredictable and fluctuating in all JIA subtypes with episodes of active and inactive disease, the cumulative time spent in a state of active disease is an accurate measure for the disease activity over time.[4-6] Since it is unethical to study patients who do not receive treatment, course of disease is inevitably defined for patients receiving treatment if and when necessary. The percentage of active disease in the first years is not only predictive for the course of disease in the following years,[2, 6] but a prolonged disease activity is also related to damage to the joints or functional impairment.[7] The aim of treatment is to minimize the time spent in active disease.

No clinical parameters or biomarkers are available at disease onset to predict the course of disease.[8] Genetic markers would be ideal as predictive factors, already present at disease onset and not influenced by disease activity or medication. Recently, a number of genetic susceptibility factors for JIA (e.g. *HLA*, *PTPN22*, *PTPN2* and *IL2RA*) have been described and replicated by means of genome-wide and candidate gene association studies.[9-11] Some of these genetic risk factors are also involved in other autoimmune diseases.

The aim of this study was to identify genetic factors associated with severity of JIA. The relationship between single nucleotide polymorphisms (SNPs) in candidate genes involved in immunoregulation and autoimmunity and the percentage of active disease in the first two years after diagnosis was investigated.

MATERIALS AND METHODS

Patient population

Two hundred and seventy-two Caucasian JIA patients with persistent oligoarticular, extended oligoarticular and RF-negative polyarticular JIA of whom both DNA and clinical data were available, were studied. Patients, recruited from eight centers throughout North-Western Europe, were diagnosed between 1991 and 2006 and had a follow-up

of at least two years. Informed consent was obtained from all patients and/or parents and the review boards of the participating centers approved this study (Table 1).

Clinical data

All hospital visits during the first two years after diagnosis have been retrospectively evaluated to determine the state of disease activity at each visit. Inactive disease was defined as the absence of clinically active arthritis, the lowest possible physician's global assessment and an ESR <20 mm/hr (following a modified definition of clinically inactive disease).[12] The cumulative time spent in active disease in the first two years was determined and formed the basis for the categories of disease course:[6] remitting (<= 35% active disease), intermediate (35-65% active disease) and unremitting (>= 65% active disease) (Table 1).

Genetic factors

112 SNPs in 65 genetic regions were genotyped, because of their association with JIA, other autoimmune diseases and/or their role in immunoregulation (Supplementary Table S1).

Statistical analysis

The three categories representing course of disease were used as outcome parameter. Univariate analysis was performed with the clinical parameters JIA subtype, age at onset and ANA status using Chi-square and Kruskal-Wallis tests, and with the genetic parameters comparing the genotype distribution of each SNP between the categories of disease course with a linear by linear trend test. The linkage disequilibrium of associated SNPs ($p<0.05$) was studied using parental data (Haploview).[13]

Parameters with a p-value <0.05 in the univariate analysis were analyzed by multivariate ordinal logistic regression. Odds ratios and 95% confidence intervals (CI) are presented. A p-value <0.001 was considered significant after Bonferroni correction for multiple testing (57 successfully typed genes/loci). ROC curves were plotted for two models with different parameters predicting course at disease onset (Supplementary Table S2 and Figure S1). A case-control analysis was performed with the SNPs that were associated with course of disease ($p<0.05$), to evaluate their role in susceptibility to JIA (Supplementary Table S3). Data were analyzed using IBM SPSS Statistics 20.

Table 1. Characteristics of the JIA cohort

	n	(%)
Total cohort[a]	272	
Country[b]		
The Netherlands	138	(50.7)
Belgium	45	(16.5)
Germany	49	(18.0)
Switzerland	40	(14.7)
Subtype		
Persistent oligoarthritis	129	(47.4)
Extended oligoarthritis	57	(21.0)
RF negative polyarthritis	86	(31.6)
Gender		
Female	190	(69.9)
Male	82	(30.1)
ANA		
Positive	153	(56.3)
Negative	86	(31.6)
Inconclusive/unknown	33	(12.1)
Course of disease[c]		
Remitting course	65	(23.9)
Intermediate course	96	(35.3)
Unremitting course	111	(40.8)
Medication[d]		
Intra-articular steroids (n=166)	63	(38.0)
Sulfasalazine (n=218)	73	(33.5)
Methotrexate (n=238)	97	(40.8)
Etanercept (n=233)	4	(1.7)
	median	**range**
Percentage of active disease[e]		
Total cohort	54.9	(2-100)
Remitting course	22.1	(2-35)
Intermediate course	49.8	(36-65)
Unremitting course	91.1	(65-100)
Age at onset	3.98	(0.6-16.2)

a) Written informed consent from patients and parents was obtained after a personalised informative letter from the local investigator and the coordinating centre, in their native language. The majority of patients participated by taking a buccal swab at home and sending this to the coordinating centre. They authorised storage and analysis of their DNA for this and further JIA related research.

b) Paediatric rheumatologists in the participating centres (The Netherlands n=5, Belgium n=1, Germany n=1, and Switzerland n=1) are all members of the Paediatric Rheumatology European Society (PRES).

c) Remitting clinical course: percentage of active disease in the first two years <= 35%; intermediate clinical course: percentage of active disease in the first two years >35 and <65%; unremitting clinical course: percentage of active disease in first two years >=65%.

d) Treatment was started in the first two years after diagnosis. Notable is that not of all patients the detailed data on use of medication are known (n).

e) Percentage of active disease in the first two years after diagnosis.

RESULTS

93 of 112 SNPs (83%) located in 57 genes/loci were successfully genotyped in 272 patients (Supplementary Table S1). This cohort consisted of 65 patients with a remitting course of disease, 96 patients with an intermediate course, and 111 patients with an unremitting course (Table 1).

In univariate analysis JIA subtype, but not ANA status or age at onset, was significantly associated with the course of disease; the persistent oligoarthritis patients had more intermediate and remitting disease course, whereas the majority of extended oligoarthritis and RF-negative polyarthritis patients followed an unremitting

Table 2. Univariate analysis of clinical and genetic parameters with the categories representing disease course as outcome parameter[a].

Subtype			Remitting	Intermediate	Unremitting	p
Persistent oligoarthritis			45 (0.35)	48 (0.37)	36 (0.28)	
Extended oligoarthritis			6 (0.11)	17 (0.30)	34 (0.60)	
RF-negative polyarthritis			14 (0.16)	31 (0.36)	41 (0.48)	$5.7*10^{-5}$ [b]
ANA						
Positive			21 (0.24)	28 (0.33)	37 (0.43)	
Negative			33 (0.22)	59 (0.39)	61 (0.40)	0.65[b]
Age at onset (median, range)			4.0 (1.0-15.0)	3.2 (0.6- 14.9)	4.3 (1.0- 16.2)	0.49[c]
Gene	**SNP**	**Genotype**	**Remitting**	**Intermediate**	**Unremitting**	**p[d]**
CDK6	rs42041	00	31 (49.2)	55 (59.8)	69 (65.1)	
		01	27 (42.9)	32 (34.8)	33 (31.1)	
		11	5 (7.9)	5 (5.4)	4 (3.8)	0.038
		MAF (G)	0.29	0.23	0.19	
VTCN1	rs10923223	00	33 (51.6)	63 (67.0)	88 (79.3)	
		01	26 (40.6)	29 (30.9)	22 (19.8)	
		11	5 (7.8)	2 (2.1)	1 (0.9)	$4.4*10^{-5}$
		MAF (C)	0.28	0.18	0.11	
VTCN1	rs12046117	00	40 (62.5)	64 (69.6)	88 (79.3)	
		01	23 (35.9)	27 (29.3)	22 (19.8)	
		11	1 (1.6)	1 (1.1)	1 (0.9)	0.017
		MAF (T)	0.20	0.16	0.11	
LCK	rs695161	00	22 (33.8)	27 (28.4)	28 (25.7)	
		01	33 (50.8)	53 (55.8)	51 (46.8)	
		11	10 (15.4)	15 (15.8)	30 (27.5)	0.051
		MAF (C)	0.41	0.45	0.51	

a) Only the SNPs of interest with a p-value <0.1 are shown.
b) p-value of Pearson Chi-square test
c) p-value of Kruskal-Wallis test
d) p-value of linear by linear association (trend test) with one degree of freedom

disease course ($p=5.7*10^{-5}$) (Table 2). Comparing the genotype distribution in the three categories reflecting course of disease revealed three SNPs that were associated ($p<0.05$). Two of these SNPs were located in the *VTCN1* gene, rs10923223 ($p=4.4*10^{-5}$) and rs12046117 ($p=0.017$) and a third association was found with *CDK6* rs42041 ($p=0.038$) (Table 2). The minor alleles of the three SNPs were associated with a remitting course. None of the other investigated SNPs were associated, except a trend towards association with *LCK* rs695161 ($p=0.051$). Because two of the associated SNPs (rs10923223 and rs12046117) were located in the *VTCN1* gene, the linkage disequilibrium was studied. The two SNPs were highly correlated ($D'=0.97$; $r^2=0.77$). Therefore we have included only *VTCN1* rs10923223 in further analyses.
In multivariate ordinal regression analysis, JIA subtype (extended oligoarthritis and RF-negative polyarthritis, with persistent oligoarthritis as reference; extended oligoarthritis OR 3.8, 95%-CI 2.0-7.2, $p=6.3*10^{-5}$; RF-negative polyarthritis OR 2.5, 95%-CI 1.4-4.2, $p=9.0*10^{-4}$) and the genetic factors *VTCN1* rs109232203 (OR 0.41, 95%-CI 0.26-0.63, $p=5.8*10^{-5}$) and *CDK6* rs42041 (OR 0.66, 95%-CI 0.44-0.97, $p=0.036$) were independently associated with course of disease (Table 3). The addition of *VTCN1* rs10923223 and *CDK6* rs42041 to a model predicting course of disease at diagnosis in which only subtype at onset is included, increases the AUC of an ROC curve from 0.57 to 0.67 (Supplementary Table S2 and Figure S1).

Table 3. Multivariate ordinal logistic regression of associated clinical (subtype) and genetic parameters (*VTCN1*, *CDK6*).

Subtype	B	OR	95%CI	p
Persistent oligoarthritis vs. extended oligoarthritis	1.33	3.8	2.0-7.2	$6.3*10^{-5}$
Persistent oligoarthritis vs. RF-negative polyarthritis	0.90	2.5	1.4-4.2	$9.0*10^{-4}$
VTCN1 rs10923223	-0.90	0.41	0.26-0.63	$5.8*10^{-5}$
CDK6 rs42041	-0.42	0.66	0.44-0.97	0.036

DISCUSSION

In this study we describe that genetic variations in *VTCN1* (rs10923223 and rs12046117) and in *CDK6* (rs42041) are associated with the course of disease in JIA. Our data show a protective effect of the minor allele of all three SNPs on the course of disease. This effect is independent of the association that exists between the JIA subtype and the course of disease. Because CDK6 is involved in cell-cycling, the association of disease

course with *CDK6* rs42041 is not unlikely. However, this association is not significant after correction for multiple testing.

Interestingly, the association of *VTCN1* with the course of disease is in line with the described function of the encoded protein. *VTCN1,* V-set domain containing T cell activation inhibitor 1, encodes B7-H4, a member of the B7 co-signaling molecule family expressed on antigen presenting cells.[14,15] Ligation of B7-H4 has an inhibitory effect on T-cell proliferation and production of cytokines.[14,15] Because of its inhibiting effects on immune responses B7-H4 has been associated with prognosis in cancer and autoimmunity.[16,17]

In B7-H4 deficient mice an exacerbation of collagen induced arthritis was observed.[18] An agonistic soluble B7-H4Ig suppressed the progression of collagen induced arthritis and improved the progression of experimental autoimmune encephalomyelitis.[18,19] Targeting of this inhibitory B7-H4 pathway might be of therapeutic interest, similar to the CTLA4 fusion protein (Abatacept) in rheumatoid arthritis.

In our cohort no significant association of *VTCN1* with the susceptibility to JIA was observed (Supplementary Table S3), but instead an association of the minor allele of rs10923223 with a less severe course of disease (Table 2). It is conceivable that the minor allele is associated with a gain of function of *VTCN1*, leading to a stronger inhibitory signal to activated T cells. This hypothesis implies that *VTCN1* does not contribute to initiation but rather to the course of the disease. At present it is very difficult to predict the course of disease at the moment of diagnosis. The addition of *VTCN1* and *CDK6* to the predictive model for disease course increased the AUC (Supplementary Table S2 and Figure S1). Although the total predictive power is still limited it suggests that genetic information may contribute to prognosis in the case of JIA.

It is remarkable that the well-established JIA loci *PTPN22*, 4q27 *(IL2-IL21)*, 5q11 *(ANKRD55)* and *TNFA* were not associated with course of disease, although they were associated with susceptibility in our cohort as well *(Reinards et al. manuscript in preparation).*[9-11, 20] This suggests that genes involved in the development of disease are not necessarily involved in the progression of disease. Replication of our data remains necessary since patient numbers are relatively small when comparing categories of disease course. However, if association of *VTCN1* with course of disease will be confirmed, these data support the development of therapeutic tools based on interfering with this receptor ligand interaction in JIA and maybe other autoimmune diseases.

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SUPPLEMENTARY DATA

ROC curve

To give more insight into the predictive value for disease course of the parameters that can be used at disease onset (when persistent oligoarthritis cannot be distinguished from extended oligoarthritis yet), a ROC curve was plotted. The predictive value of an ordinal logistic regression model with subtype at onset (oligoarthritis versus polyarthritis at presentation) as only parameter was plotted (Figure S1a), followed by a model of the subtype at onset together with the genetic parameters with an independent effect in the multivariate analysis (Figure S1b).

Control population

A case-control analysis was performed with the SNPs that were associated with course of disease ($p<0.05$), to evaluate their role in developing JIA.

The control population, used to determine allele frequencies of *VTCN1* rs10923223 and rs12046117 in healthy individuals, consisted of blood bank donors that were randomly selected by the Immunogenetics and Transplantation Immunology Section at Leiden University Medical Center, The Netherlands (n=758), or that were recruited via participating patients and their families (but unrelated) (n=111). Because there was not sufficient DNA for all of these controls to type all SNP's, 744 additional individuals were included in case of *CDK6* rs42041. These individuals were blood bank donors (n=372) or requested genetic counselling at the Laboratory for Diagnostic Genome Analyses, Leiden University Medical Center, regarding a monogenic disease in their families, but tested negative for this single genetic defect (n=372).

All controls were unrelated, of Caucasian descent, above the age of 18 years, and gave informed consent for anonymized (further) use of their (left over) DNA for scientific purposes, and for anonymized use of their DNA in a biobank.

Supplementary Table S1
Genetic variants selected for association analysis with course of disease in JIA

Chromosome	Position*	Gene/region	SNP	Minor allele
1	2553624	*TNFRSF14-MMEL1*	rs3890745	G
1	12091210	*TNFRSF8-MIIP*	rs946461	T
1	12252955	*TNFRSF1B*	rs1061622	G
1	32729702	*LCK*	rs1004420	T
1	32743866	*LCK*	rs695161	C
1	114377568	*PTPN22*	rs2476601	A
1	117685992	*VTCN1*	rs6673837	A
1	117690758	*VTCN1*	rs2358817	T
1	117711911	*VTCN1*	rs2358820	A
1	117730048	*VTCN1*	rs10923217	C
1	117730623	*VTCN1*	rs6669320	A
1	117746573	*VTCN1*	rs10923223	C
1	117751365	*VTCN1*	rs12046117	T
1	198700442	*PTPRC*	rs10919563	A
1	206946897	*IL10*	rs1800896	C
1	207015957	*IL19*	rs2243191	T
1	207038686	*IL20*	rs1400986	T
2	100832155	*AFF3*	rs1160542	G
2	100835734	*AFF3*	rs10865035	A
2	103070568	*IL18RAP*	rs917997	T
2	113537223	*IL1A*	rs17561	A
2	113542960	*IL1A*	rs1800587	A
2	113590390	*IL1B*	rs1143634	A
2	113594867	*IL1B*	rs16944	A
2	162856148	*DPP4*	rs2268894	C
2	191835596	*STAT1*	rs3771300	C
2	191843445	*STAT1*	rs13010343	A
2	191845725	*STAT1*	rs1547550	C
2	191855521	*STAT1*	rs7562024	T
2	204610396	*CD28*	rs1980422	C
2	204732714	*CTLA4*	rs231775	G
3	46414947	*CCR5*	rs333	del
3	58556841	*FAM107A*	rs13315591	C
4	26108197	4p15	rs874040	G
4	123132492	*KIAA1109*	rs4505848	G
4	123348345	*ADAD1*	rs11732095	G
4	123514528	*IL2-IL21*	rs4492018	A
4	123548068	*IL21*	rs1398553	T

Supplementary Table S1 (continued)
Genetic variants selected for association analysis with course of disease in JIA

Chromosome	Position*	Gene/region	SNP	Minor allele
5	55438580	*ANKRD55*	rs6859219	A
5	96124330	*ERAP1*	rs30187	T
5	135287029	*LECT2*	rs31517	A
6	31540141	*LTA*	rs2239704	A
6	31540313	*LTA*	rs909253	G
6	31540784	*LTA*	rs1041981	A
6	31542482	*TNFA*	rs1799724	T
6	31542963	*TNFA*	rs1800750	A
6	31543031	*TNFA*	rs1800629	A
6	31543101	*TNFA*	rs361525	A
6	31543827	*TNFA*	rs1800610	A
6	31544189	*TNFA*	rs3093662	G
6	57012930	*ZNF451*	rs3734738	A
6	106568034	*PRDM1*	rs548234	C
6	138006504	6q23	rs6920220	A
6	159482521	*TAGAP*	rs394581	C
6	167534290	*CCR6*	rs3093023	A
7	75442759	*CCL24*	rs2302005	T
7	75442855	*CCL24*	rs2302004	C
7	92246744	*CDK6*	rs42041	G
7	128594183	*TNPO3*	rs10488631	C
9	34743681	*CCL21*	rs951005	C
9	139775146	*TRAF2*	rs7048473	C
9	139787453	*TRAF2*	rs2811761	G
9	139815053	*TRAF2*	rs10781522	G
9	139821068	*TRAF2*	rs3750512	C
10	6053163	*IL2RA*	rs12722605	T
10	6099045	*IL2RA*	rs2104286	G
10	6114660	*IL2RA*	rs41295061	A
10	6393260	*PRKCQ*	rs4750316	C
11	36525293	*TRAF6*	rs540386	T
11	71709272	*IL18BP*	rs3814721	C
11	71710478	*IL18BP*	rs2298455	C
11	71714078	*IL18BP*	rs1541304	T
11	112035458	*IL18*	rs1946518	T
11	117869670	*IL10RA*	rs2229113	A
12	6450945	*TNFRSF1A*	rs767455	C
12	6451590	*TNFRSF1A*	rs4149570	A

Supplementary Table S1 (continued)
Genetic variants selected for association analysis with course of disease in JIA

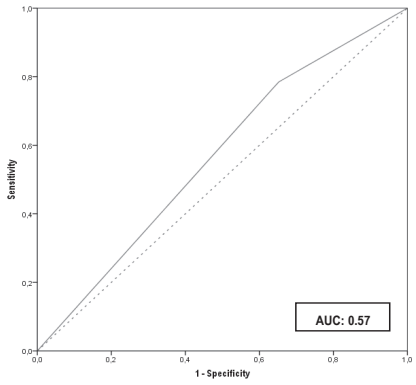
Chromosome	Position*	Gene/region	SNP	Minor allele
12	57968715	*KIF5A*	rs1678542	C
16	11179873	*CLEC16A*	rs12708716	G
16	11249329	*CLEC16A*	rs6498169	G
16	27448401	*IL21R*	rs3093341	G
16	67189486	*TRADD*	rs11574518	T
17	32594568	*CCL2-CCL7*	rs8079244	C
17	40447401	*STAT5A*	rs7217728	C
17	40461003	*STAT5A*	rs2293154	A
18	67531642	*CD226*	rs763361	T
19	44515514	*ZNF230*	rs12753	A
20	43280231	*ADA*	rs6031698	A
20	44746982	*CD40*	rs1883832	T
21	34640788	*IL10RB*	rs2834167	G
22	24236392	*MIF*	rs755622	C
22	37544245	*IL2RB*	rs3218258	T
22	37544810	*IL2RB*	rs3218253	T
22	37551607	*IL2RB*	rs743777	G

*) Base-pair position is based on NCBI dbSNP build 136

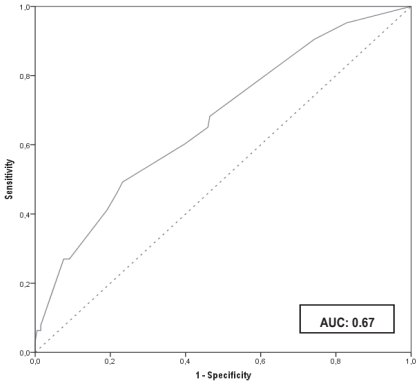
112 SNPs in 65 loci have been genotyped by the iPLEX MassARRAY platform according to the manufacturer's recommendations (Sequenom, San Diego, California, USA), of which these 93 SNPs in 57 loci passed quality control criteria. Only SNPs exceeding a 90% call rate were used for further analysis. SNP call rates per individual exceeded 90%.

Supplementary Figure S1
ROC curves of predictive models for course of disease

a. Only subtype at onset included as predictor



b. Subtype at onset and genetic parameters included as predictors



AUC: area under the (solid) curve
The dashed line represents the reference.

Supplementary Table S2
Clinical and genetic parameters as predictors for course of disease plotted in a ROC curve

Figure	Parameters included	AUC*
S1a	Subtype at onset#	0.57
S1b	Subtype at onset#, *VTCN1* rs10923223, and *CDK6* rs42041	0.67

*) Area under the curve (AUC) reflecting the ability to distinguish between remitting, intermediate and unremitting disease
#) Subtype at onset comparing oligoarthritis (persistent and extended oligoarthritis) to (RF-negative) polyarthritis

Supplementary Table S3
Genotype frequencies of the SNPs of interest in JIA cases compared to healthy controls

Gene	**SNP**		**n**	**MAF***	**Genotype**			**p#**
					00 (%)	01 (%)	11 (%)	
VTCN1	rs10923223	Cases	269	0.17	184 (68)	77 (29)	8 (3)	
		Controls	856	0.14	622 (73)	221 (26)	13 (2)	0.10
VTCN1	rs12046117	Cases	266	0.15	191 (72)	72 (27)	3 (1)	
		Controls	848	0.13	640 (76)	197 (23)	11 (1)	0.30
CDK6	rs42041	Cases	261	0.23	155 (59)	92 (35)	14 (5)	
		Controls	1274	0.26	713 (56)	465 (37)	96 (8)	0.19

*) MAF: minor allele frequency
#) p-value of linear-by-linear association (trend test with one degree of freedom)