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A Case-Control Association Study of 12 Candidate Genes and Attempted Suicide in French Adolescents

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Abstract:

Background: Suicide is the second leading cause of death for 10-19-year-olds. Evidence has shown that attempted suicide is a complex interplay of genes and environmental factors. In adult population, possible associations between genetic polymorphisms and suicidal behaviours have been investigated with several genes, most often with inconsistent findings and poor replicability of significant associations. This study aimed to identify gene variants conferring risk for adolescent suicide attempt. **Methods:** We selected the genes and variants after an analysis of the literature and a selection of the most significant associations identified. We performed analysis on 22 Single Nucleotide Polymorphisms (SNPs) in 12 genes (COMT, CRHR1, FKBP5, SLC6A4, HTR1B, HTR2A, TPH1, TPH2, BDNF, NTRK2, NOS1, and IL28RA) for association with suicide attempt, hopelessness and impulsivity in an independent sample, composed of 98 adolescent suicide attempters who required hospitalisation based on emergency assessments, and 150 healthy volunteers. Quality controls, deviations from Hardy-Weinberg disequilibrium and statistical tests of association (case/control) were calculated using PLINK. Asymptotic p-values were corrected with the Benjamini-Hochberg method. The level of significance was set to 0.05.

Results: We identified four polymorphisms of interest, rs10868235 (NTRK2), rs1659400 (NTRK2), rs2682826 (NOS1) and rs7305115 (TPH2), with significant associations for suicide attempts or for the quantitative hopelessness or impulsivity phenotypes. However, none of the associations withstand statistical correction tests.

Conclusion: Our results do not support the role of the 22 SNPs selected in suicide attempt or hopelessness and impulsivity in adolescent population. However, the relatively small sample size and the probable effect of gene–gene interaction or gene–environment interaction on suicidal behaviour could not be ruled out.

Key words: adolescent, attempt suicide, genetic association, polymorphisms, SNPs

Background:

Suicide is the second leading cause of death among young people aged 10-19 years in Europe [1] and the USA [2]. In addition, for every completed teenage suicide there are at least 40 suicide attempts and according to World Health Organization, this rate is increasing, particularly for girls [3, 4]. Suicidal behaviours (SB) cover a broad spectrum ranging from suicidal thoughts to suicide attempts and completed suicide. The etiology of SB in adolescence is complex, including biological, psychological, family, social, and cultural factors [5, 6, 7]. Several individual determinants have been associated with suicidal behaviour in adolescence, the foremost of which being mental disorders, including major depressive disorders [8-11].

There is sufficient epidemiological evidence from studies on families [12-14], adoption [15] or twins [16] to show that SB runs in families independently of the presence of a psychiatric disorder. Brent et al. (1996) [11] studied the relatives of 58 adolescent suicide probands and clearly showed that the rate of suicide attempts was increased in the first-degree relatives of suicide probands compared with the relatives of controls, even after adjusting for differences in rates of proband and familial Axis I and II disorders (odds ratio, 4.3; 95% confidence intervals, 1.1-16.6). Heritability figures indicate a multifactorial mode of inheritance, with genes explaining 21–55% of the variance in suicide attempts [13, 17].

SB has been related to dysfunction in many neurotransmitter systems (serotonergic, dopaminergic, noradrenergic or GABAergic) or neurotrophic systems but the exact neurobiological and genetic functions are far from clear [6, 18]. Possible associations between genetic polymorphisms and SB have been investigated with several genes (SLC6A4, TPH1, TPH2, HTR1A, HTR2A, HTR2C, HTR7, BDNF, COMT, MAOA, ADRA2A, CRHR1,

DRD2, NTRK2, SCN8A, NOS1), most often with inconsistent findings and poor replicability of significant associations [19-24].

Despite the fact that SB constitutes a major public health problem, genetic studies on it in adolescent populations remain rare [25]. We identified four adolescent population studies [26-29]. Zalsman et al. (2001) [28] attempted to clarify the role of the A218C polymorphism in intron 7 of the TPH gene. The family-based method was used in a cohort of 88 teenagers (hospitalised for a suicide attempt). The authors show that there was no significant allelic association of A218C polymorphism with suicide attempt or other phenotypic measures according to the Haplotype-Relative-Risk (HRR) method (chi-square = 0.094; P = 0.76) and the Transmission Desequilibrium Test (TDT) method (chi-square = 0.258; P = 0.61). In the same population, Zalsman et al. (2005) [29] tested T102C polymorphism (5-HTR2A gene) without demonstrating any significant association in allelic distribution between transmitted and non-transmitted alleles. The same team conducted a case-control association study in four groups of adolescents: (i) suicidal psychiatric inpatient adolescents (N=35), (ii) non-suicidal psychiatric inpatient adolescents (N=30), (iii) adolescents admitted to psychiatric emergency rooms due to a suicide attempt (N=51), and (iv) a community-based control group (N=95) [28]. The authors found that homozygosity for T (TT) of the HTR2A 102T/C polymorphism was associated with lower impulsivity (P=0.03) and aggression (P=0.01) compared to TC carriers and that a low activity MAOA was significantly associated with suicidality (P=0.04).

Studying a subsample of adolescent depression (n=155) sufferers who participated in the Treatment of SSRI-Resistant Depression in Adolescents (TORDIA) trial, Brent et al. (2010) [26] found that two polymorphisms in FKBP5 (rs1360780TT and rs3800373GG) were linked to suicide events (n=18), even when they controlled for related covariates and treatment effects.

In previous work, we identified key association studies in adult populations (case-control association studies, family-based association studies and GWAS) from the past 10 years targeting suicidal behaviour [19]. In support of the studies we identified as having significant results, we attempted to replicate these findings in an adolescent population. In the current study, we tested 22 SNPs in 12 genes (COMT, CRHR1, FKBP5, SLC6A4, HTR1B, HTR2A, TPH1, TPH2, BDNF, NTRK2, NOS1, IL28RA) for association with suicide attempt in an independent sample, composed of 98 adolescent suicide attempters who required hospitalization based on emergency assessments and 150 healthy volunteers.

Methods:

Sample:

The participants were recruited during a hospitalisation for attempted suicide at the University Hospital Charles Nicolle (Rouen, France), between September 2014 and May 2016. Suicide attempts were defined according to the Columbia Classification Algorithm of Suicide Assessment (C-CASA) [30]. This definition stresses the suicidal intentionality behind the self-aggressive acting out, contrary to non-suicidal self-injuries. Adolescents with no suicidal intention were excluded. Similarly, we excluded patients who had a concomitant medical or neurological illness and intellectual disability. To reduce ethnic variation and stratification effects, we included only subjects from CEU populations (Utah Residents with Northern and Western Ancestry). We screened 131 adolescent inpatients. The parents of nine of them refused to give their consent. Twenty-one were excluded because of their ethnic origin and three adolescents withdrew their consent during the study. Consequently, 98 adolescents were included.

The comparison group consisted of healthy volunteers registered on the database of the Clinical Investigation Centre (CIC) of the University Hospital of Rouen. To ensure the homogeneity of the comparison group, we included only volunteers aged 18-25 years. These controls were evaluated by psychiatrists as regards psychiatric diagnoses, personality disorders and suicidal behaviours. The controls were excluded if they had: (i) an Axis I or II diagnosis (ii) a personal history of suicidal thoughts or attempted suicide, or (iii) a family history of attempted or completed suicide (Mini). Similarly, we included only controls from CEU countries.

Instruments :

The clinical phenotype of the adolescent attempters was determined by a semi-structured diagnostic interview and six questionnaires.

- The Kiddie Schedule for Affective Disorders and Schizophrenia - Present and Lifetime (K-SADS-PL) version is a well established, semi-structured diagnostic interview. It serves to assess current and past episodes of Axis I psychopathology in children and adolescents according to the criteria of the DSM-IV-TR. The interview was translated into French by Mouren- Siméoni et al. (2002) [31].
- The Columbia–Suicide Severity Rating Scale was used to quantify severity of suicidal ideation and behavior [32]. This scale allows for assessment of suicidal behaviour and suicidal intentionality. It was completed by the clinician based on clinical interviews conducted with the adolescents.
- The Beck Depression Inventory, Second Edition [33] is a questionnaire that assesses severity of depression symptoms. This scale comprises 21 items rated on a 4-point scale. Scores can range from 0 to 63.

- The Beck Hopelessness Scale [34] consists of 20 true or false items that serve to assess negative attitudes about the future. Its score range is 0 to 20.
- The Eysenck Questionnaire to score impulsity [35]. This self-administered questionnaire of 24 items completed by youngsters aged 8-17 years complies with the standards defined in two studies carried out in the general Canadian and British populations.

Procedure:

Within 48 hours of admission, each patient was diagnosed by a psychiatrist or a trained psychiatric intern using criteria based on the K-SADS-PL. All the questionnaires and saliva DNA sampling were implemented during the hospitalisation.

SNP selection and genotyping:

We targeted SNPs that had shown significant associations or significant trends in previous studies, highlighting the results of meta-analyses. We also selected the SNPs with a significant genome-wide association, from the GWAS.

DNA was extracted from the saliva sampling kits using standard methods (DNA Genotek) [36]. All subjects included in the study underwent genotyping. The genotyping process by AS-PCR was developed from the methodology described by Nazarenko (1997) [37] and Myakishev (2001) [38]. For each SNP, 3 primers were designed using primer3 (http://frodo.wi.mit.edu/primer3/): Allele Specific 1 (AS1), Allele Specific 2 (AS2), and common reverse. A universal primer tail1 sequence was added at the 5' end of the sequence of AS1 and also with the universal tail2 for AS2. All primers were ordered from IDT. SNP genotyping was performed on the Biomark (Fluidigm) in a microfluidic multiplex. The

genotypes were obtained by clustering, using Fluidigm SNP Genotyping Analysis version 4.1.3 according to Fluidigm's recommendations [39].

Statistical analysis:

Quality controls (genotype frequencies, missing genotypes…), deviations from Hardy-Weinberg disequilibrium and statistical tests were calculated using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). It is a free and open-source whole genome association analysis toolset, designed to focus on genotype and phenotype data. Statistical tests of association (case/control) testing the relationship between genotype and suicide attempt were performed in PLINK. Asymptotic p-values were corrected with the Benjamini-Hochberg method. The level of significance was set to 0.05. The results were verified by logistic regression analysis. The genotypes were also tested with R software (v3.1) using Pearson Chi-Square test or Fishier exact test. R (https://cran.r-project.org/) is a freely available language and environment for statistical computing. It is not specifically designed for biological data.

Results

Descriptive and clinical analyses:

The suicidal inpatient group aged 13 - 17 years (mean = 15.4, + /- 1.53), included 81 (82,66 %) girls and 17 (17.34 %) boys. All the adolescents were of CEU origin. The CEU origin was considered to be proven if all four grandparents were from Northern or Western Europe. The diagnostic evaluation produced the following results: major depressive disorder (32%), adjustment disorders with depressed mood (25%), anxiety disorders (22%), oppositional defiant and conduct disorders (16%), substance related disorders (9%). 22.5% of

the adolescents had already made at least one previous suicide attempt. The healthy control group from the community was recruited by the Clinical Investigation Centre (CIC) of the University Hospital of Rouen. Their age range was 21 - 27 years (mean= 25.1, + /- 3.04) and the group included 118 (78.66%) girls and 32 (21.33%) boys.

We used the Fischer or Chi 2 tests to evaluate the relationship between the selected clinical variables and the suicidal profile of the individuals in the study. Several variables were highly significant ($p < 0.0001$): Beck hopelessness total score, Beck depression total score, impulsivity total score, major depressive episode, other depressive disorders, and addictive behaviours).

Genetic analyses:

Analyses with PLINK:

The distribution of 22 polymorphisms in the case group and the group of suicidal adolescents did not differ from Hardy–Weinberg equilibrium. After removal of three individuals with missing data per individual and of the rs25531 marker (Supplement 1), the frequencies of minority alleles are calculated for each marker of interest, depending on the genotypes of the individuals in this study (**Table 1**).

Insert table 1

The association tests enabled us to identify four markers with a p-value of less than 0.05. These are the polymorphisms rs10868235 (NTRK2), rs1659400 (NTRK2), rs2682826 (NOS1) and rs7305115 (TPH2). However, after correction, none of the markers remains significant (**Table 2**). The variant rs7305115 (TPH2) shows a significant trend $p = 0.065$ after Benjamini-Hochberg correction. In the logistic regression analyses, we again identified the same variants rs10868235 (NTRK2), rs1659400 (NTRK2), rs2682826 (NOS1) and rs7305115

(TPH2) with an asymptomatic p-value of less than 0.05. But after correction, none of them passes the significance threshold (**Table 3**).

Insert Table 2

We also performed an association and linear regression analysis for two quantitative hopelessness and impulsivity phenotypes (Supplement 2). Two variants, rs2682826 (NOS1) and rs7305115 (TPH2), were significantly associated with hopelessness, respectively (β = -1.025, SE 0.4684, Stat -2.188, p = 0.029) and (β = -1.163, SE = 0.4508, Stat =-2.579, p = 0.015). Concerning impulsivity, only the variant rs7305115 (TPH2) is significant (β = -0.998, SE = 0.478, Stat = -2.087, p = 0.037). However, none of the associations are significant after applying Bonferroni or Benjamini-Hochberg statistical corrections.

Insert Table 3

Analyses using the R software (without correction) :

We found a significant relationship for two polymorphisms, rs10868235 (NTRK2) (chi-square = 7.8003, df = 2, p = 0.020) and rs7305115 (TPH2) (chi-square = 7.3346, df = 2, p = 0.025). The marker rs1659400 (NTRK2) shows a significant trend (chi-square = 4.844, df = 2, p = 0.088).

In a second stage, we conducted a logistic regression to identify an association between the explanatory variables (SNPs of interest) and the dependent variable (suicide risk). We found a significant association with two markers rs1659400 (NTRK2) (p = 0.041) and rs7305115 (TPH2) (p = 0.042). Regarding the rs1659400 (NTRK2) marker, the TT

genotype is present at a higher level than expected in the control population (71% vs. 29%, p = 0.0277). GG carriers for the rs7305115 variant (TPH2) are present in greater numbers than expected in the population of suicidal adolescents (43% vs. 57% p = 0.017).

Discussion

We conducted an association study with 22 variants which had been previously identified as being significantly associated with suicidal behavior. These variants are located in or close to 12 candidate genes (COMT, CRHR1, FKBP5, SLC6A4, HTR1B, HTR2A, TPH1, TPH2, BDNF, NTRK2, NOS1, IL28RA) suspected of having a role in the pathophysiology of suicidal behavior. Our results failed to demonstrate any significant association between the markers of interest and suicide attempts in adolescence after correction for multiple testing. Similarly, we failed to identify any significant associations with the two quantitative traits, hopelessness and impulsivity. We identified four variants, rs10868235 (NTRK2), rs1659400 (NTRK2), rs2682826 (NOS1) and rs7305115 (TPH2), which were associated with suicidal behavior in different statistical tests, but without being able to withstand statistical corrections. The variant rs7305115 (TPH2) shows a significant trend after Bonferroni correction (p = 0.08843).

It is interesting to note that the rs7305115 polymorphism has been the subject of several publications with significant associations. The human TPH2 gene is located on chromosome 12q15; it comprises 11 exons and covers a region of approximately 93.5 kilobases [40]. TPH2 is preferentially expressed in the brain, and an elevated expression of TPH2 mRNA has been found in the dorsal and median raphe nuclei of suicidal depressed patients [41] and in the prefrontal cortex of suicide victims [42]. TPH2 is one of the most

promising candidate genes for psychiatric disorders [43]. However, the question of a possible SNP TPH2 association with suicide-related behavior is complex and controversial. Several studies have shown a possible association between polymorphisms TPH2 and major depression with suicidal behavior [44-45]. On the other hand, other studies have failed to find any associations between TPH2 polymorphisms and suicidal behavior [46-47]. In two samples of Chinese depressed patients, Zhang et al. (2010) [48] and Ke et al. (2006) [49] showed that the TPH2 rs7305115 AA was still a significant protective predictor of SB (OR=0.33 and OR=0.35). The findings suggest that the carriers of the A → G mutation of the TPH2 rs7305115 SNP might run a greater risk of attempted suicide than the carriers of the AA homozygous genotype in MDD patients. More particularly, the results suggest that the association between the SNP of the TPH2 gene and tendency to suicidal behaviour in major depression might be distinct from the heritability of mood disorders. Be that as it may, the absence in both studies of potentially functional SNPs indicates a pressing need for investigation of the polymorphisms present in both the TPH2 regulatory and adjacent regions.

NTRK2 (TRKB) encodes the receptor for BDNF. Aberrant neurotrophic signalling has been implicated in suicide risk by various studies [50]. BDNF and NTRK2 mRNA and protein expression are reported downregulated in the prefrontal cortex and hippocampus of suicide victims compared to controls [50]. Previously, a number of genetic variants within the NTRK2 gene have been associated with SA among depressed patients [51]. Kohli et al. (2010) [51] show association of 5 tagging SNPs located within the NTRK2 locus with a lifetime history of SA within depressed patients in 2 independent German samples. This association appears to be carried by several independent risk loci within this gene, and carriers of the risk genotypes of the 3 most significant markers had a 4.5-fold higher risk for SA than depressed patients carrying the nonrisk genotypes. Moreover, we report supportive

evidence for these associations in African American patients with MDD. Murphy et al. (2011) [52] observed a significant association with NTRK2 intronic genetic variant, rs1659404, and SA in females. Rs1659400 is in strong LD with several other SNPs within the NTRK1 gene, some of which have been implicated in alcohol dependence and depression [53-54].

Our study has many limitations. Firstly, the sample size is small and the number of variants is large compared to other studies with similar sample sizes. However, to our knowledge, it is the largest sample studied in an adolescent population. The genetic data is hence limited in this population and the results are difficult to compare with results in the adult population. Neurodevelopmental changes and the specific features of a pediatric population should be taken into account and are currently poorly identified. Studies that used MRI and fMRI showed both alterations related to age and differences related to gender in grey and white matter over the period of adolescence [55]. These results could go some way to explaining why a large number of psychiatric disorders, including SB, manifest themselves during this period of life and might explain the gender-related differences seen in adolescent SB, namely that females have a greater tendency to attempt suicide than males, who tend to achieve completed suicide more frequently. Zalsman (2010) [25] suggested that the fact of restricting the investigation of SB in adolescents simply to the interaction between gene and environment might prevent researchers from detecting other complex interaction factors, which involve timing. It seems legitimate to speculate that it is only when particular genotypes are exposed to specific environment-related risks during a critical period of brain development that suicidality would be the outcome.

In conclusion, our results do not support the role of the 22 polymorphisms selected in suicide attempt or hopelessness and impulsivity in adolescent population. However, the

relatively small sample size and the probable effect of gene–gene interaction or gene–environment interaction on suicidal behaviour could not be ruled out.

Abbreviations

COMT: Catechol-O-methyl transferase, **CRHR1:** Corticotropin-releasing hormone receptor 1, **FKBP5:** FK506 binding protein 5, **SLC6A4**: Solute Carrier Family 6 (Neurotransmitter Transporter, Serotonin), Member 4, **HTR1B:** 5-Hydroxytryptamine Receptor 1B, **HTR2A:** 5-Hydroxytryptamine Receptor 2A, **TPH1:** Tryptophan Hydroxylase 1, **TPH2:** Tryptophan Hydroxylase 2, **BDNF:** Brain Derived Neurotrophic Factor, **NTRK2:** Neurotrophic Receptor Tyrosine Kinase 2, **NOS1:** Nitric Oxide Synthase 1, **HTR2C:** 5-Hydroxytryptamine Receptor 2C, **HTR7:** 5-Hydroxytryptamine Receptor 7, **MAOA:** Monoamine Oxidase A, **ADRA2A:** Adrenoceptor Alpha 2A, **DRD2:** Dopamine Receptor D2, **SCN8A:** Sodium Voltage-Gated Channel Alpha Subunit 8, **IL28RA:** Interleukin 22 Receptor Subunit Alpha 1, **DSM IV-TR:** diagnostic and statistical manual of mental disorders, **MDD:** Major depressive disorder.

Declarations :

Authors' contributions

The study was jointly designed by BM and PG. BM organized the collection of data, performed part of the statistical analysis and prepared the first draft. DC has made substantial contributions to the conception, design, analysis and interpretation of data. CL and ML partially participated in the study design and made substantial contributions to the writing of the manuscript, in data analysis and interpretation of the findings. Overall, all authors were involved in drafting the manuscript or revising it critically for important intellectual content and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Since all the participants signed a consent form to participate solely in the current study, the present data will be used exclusively for its purpose by the primary authors/researchers according to ethics.

Ethics approval and consent to participate

The study was approved by the Nord-Ouest I Group Ethics and Medical Research Committee (Rouen University Hospital, Rouen, France) and was conducted in accordance with the principles of the Declaration of Helsinki. The genetic nature of the study was discussed with the adolescents and their parents beforehand. They received oral and written explanations of the objectives of the study. They received no remuneration. A written informed consent was obtained from the adolescents and their parents or their legal guardian before they could participate in the study.

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Table 1 : Candidate Genes , Polymorphisms and Minor Allele Frequency

Chromosome	Gene	SNP	Minor Allele	Major Allele	Minor Allele Frequency
1	IL28RA	rs10903034	C	T	0.447
3	Intergene	rs1466846	A	G	0.2816
5	HTR1B	rs6295	C	G	0.4796
6	FKBP5	rs3800373	G	T	0.2837
6	FKBP5	rs1360780	T	C	0.3
6	HTR1B	rs6296	C	G	0.2245
9	NTRK2	rs1147198	C	A	0.2041
9	NTRK2	rs1659400	T	C	0.4835
9	NTRK2	rs1867283	A	G	0.4876
9	NTRK2	rs10868235	C	T	0.4918
11	TPH1	rs1800532	A	C	0.3551
11	TPH1	rs10488683	G	A	0.4447
11	BDNF	rs6265	A	G	0.2347
12	TPH2	rs1386494	A	G	0.1701
12	TPH2	rs7305115	A	G	0.402
12	NOS1	rs2682826	T	C	0.3286
12	NOS1	rs1353939	A	G	0.2541
12	NOS1	rs693534	A	G	0.3755
13	HTR2A	rs6313	T	C	0.4184
13	HTR2A	rs6311	T	C	0.4163
17	CRHR1	rs4792887	T	C	0.1042
22	COMT	rs4680	A	G	0.4816

Table 2 : Association of Candidate Markers With Attempt Suicide (case-control association)

Ch	SNP	Minor Allele	Frequency in cases	Frequency in controls	Major Allele	Basic Allelic test Chi-Square	Asymptomatic p-value	Estimated Odds ratio	Standard Error	Lower Bound of 95% Confidence Interval (OR)	Upper Bound of 95% Confidence Interval (OR)	Bonferroni	Benjamini & Hochberg
9	**rs10868235**	C	0.551	0.4524	T	4.578	**0.03238**	**1.486**	0.1853	1.033	2.136	0.7124	0.1781
9	**rs1659400**	T	0.4175	0.5276	C	5.638	**0.01757**	**0.6419**	0.1872	0.4448	0.9263	0.3866	0.1781
12	**rs2682826**	T	0.2704	0.3673	C	5.009	**0.02521**	**0.6383**	0.2012	0.4303	0.9469	0.5547	0.1781
12	**rs7305115**	A	0.3214	0.4558	G	8.83	**0.002963**	**0.5656**	0.1926	0.3877	0.825	0.06519	0.06519

Table 3 : Association of Candidate Markers With Attempt Suicide (logistic regression analysis)

Ch	SNP	Tested Allele	Number of non-missing individuals	Odds Ratio	Standard Error	Lower Bound of 95% Confidence Interval	Upper Bound of 95% Confidence Interval	Coefficient t-statistic	Asymptomatic p-value	Bonferroni	Benjamini & Hochberg
9	**rs10868235**	C	245	1.494	0.1885	1.033	2.162	2.13	**0.03317**	0.7297	0.1824
9	**rs1659400**	T	242	0.6306	0.1933	0.4318	0.9211	-2.385	**0.01708**	0.3757	0.1824
12	**rs2682826**	T	245	0.6485	0.1995	0.4386	0.9589	-2.17	**0.02997**	0.6594	0.1824
12	**rs7305115**	A	245	0.5729	0.1937	0.392	0.8374	-2.877	**0.004019**	0.08843	0.08843