

Functional DNA methylation in a transcript specific 3'UTR region of TrkB associates with suicide

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Previous studies indicate that a subgroup of suicide completers has low cortical brain expression levels of TrkB-T1, a *TrkB* gene transcript that is highly expressed in astrocytes. Epigenetic modifications, including methylation changes in the *TrkB* promoter, partially explain TrkB-T1 low expression levels in brain tissue from suicide completers. The aim of this study was to investigate whether methylation changes in other regions of the *TrkB* gene could also contribute to the significant downregulation of the TrkB-T1 transcript observed in the brain of suicide completers. Methylation levels were assessed on BA8/9 from suicide completers expressing low TrkB-T1 transcript levels and controls, using custom-made Agilent arrays tiling the whole *TrkB* gene. After statistical correction for multiple testing, five probes located in the TrkB-T1 3'UTR region were found hypermethylated in the frontal cortex of suicide completers. These results were validated for four CpGs spanning a 150 bp sequence by cloning and Sanger sequencing bisulfite treated DNA. We found a significant correlation between the methylation level at these four CpGs and TrkB-T1 expression in BA8/9. Site-specific hypermethylation on this 3'UTR sequence induced decreased luciferase activity in reporter gene cell assays. Site-specific differential methylation in the TrkB-T1 3'UTR region associates with functional changes in TrkB-T1 expression and may play a significant role in the important decrease of cortical TrkB-T1 expression observed among suicide completers.

Introduction

Suicide is a major public health problem responsible for 10 to 20 deaths per 100 000 people.¹ Whereas suicidal behavior is multifactorial and results from the interaction of several different elements,² epigenetic factors seem to play an important role, for instance, by modifying stress response to environmental factors.^{3,4} Accordingly, recent studies have shown differential histone modifications and DNA methylation in brain regions of suicide completers as compared with controls.^{5,6} These epigenetic changes, especially DNA methylation, have been associated with environmental stressors⁷ and correlated with altered gene expression.⁸

Previous studies point to a change in the level of TrkB expression in suicide completers.^{9–11} The *TrkB* gene, located on chromosome 9, encodes three receptors of neurotrophins, characterized by specific 3'UTR sequences. *TrkB* gene products are involved in neurotransmission, calcium release, synaptic plasticity, and cell survival,¹² processes whose altered functions have been implicated in the neurobiology of suicide. The shortest TrkB transcript encodes a protein without a tyrosine kinase domain,

which is involved in the release of intracellular calcium in astrocytes.¹³ Downregulation of this truncated TrkB transcript, in coordination with a network of several other astrocytic genes,¹⁴ has been reported in several brain regions of suicide completers.⁹ Interestingly, convergent evidence from studies investigating gene expression patterns¹⁵ to studies focusing on cell morphology,¹⁶ has been increasingly suggesting that astrocyte dysregulation plays an important role in depression and suicide.

We have shown in the past that a subgroup of suicide completers has very low expression of the TrkB-T1 transcript in the frontal cortex. Studies of DNA methylation in the *TrkB* promoter sequence in cortical tissue from that brain region, and of microRNA binding in the 3'UTR sequence suggested that these marks could be partially responsible for the regulation of TrkB-T1 expression.^{9,10} However, DNA methylation in promoter sequence and the functional effect of microRNA binding described in the 3'UTR sequence, whether individually or combined, seem not to be sufficient to explain the large and specific decrease of TrkB-T1 transcript levels associated with suicide. Other mechanisms could be involved in that transcript specific regulation.

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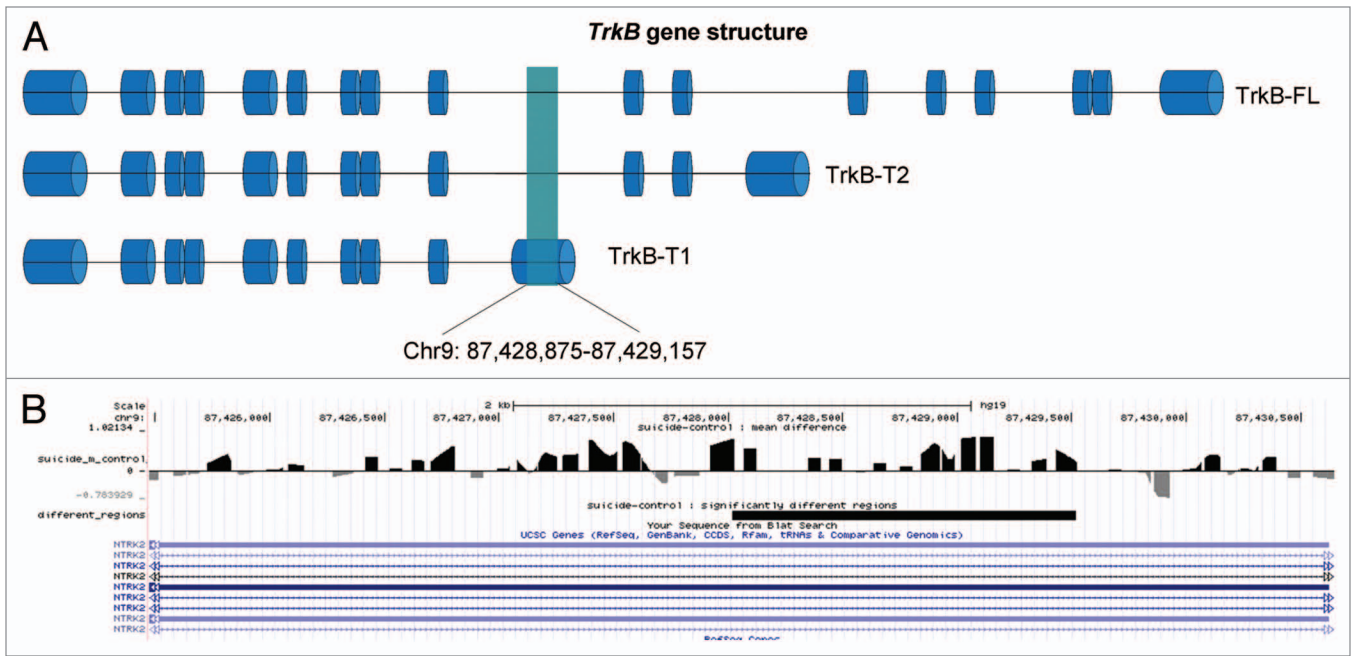


Figure 1. Differential DNA methylation in frontal cortex of suicide completers. **(A)** *TrkB* gene structure. **(B)** Differentially methylated region show through UCSC genome browser with the Track reporting the methylation level and differences between control and suicide.

In this study, we focused on the frontal cortex, where the difference in gene expression between controls and suicide completers is the most important. Furthermore, this brain area is involved in executive function, working memory and decision making,¹⁷ which are known to be impaired in patients with suicidal ideation.¹⁸

Most methylation studies conducted to date have focused on the investigation of DNA methylation differences in the promoter regions of genes of interest. However, growing evidence has indicated that gene body, as well as intergenic methylation associates with differential gene function regulation.¹⁹ Thus, this study aimed to investigate whether other *TrkB* gene regions may be differentially methylated and contribute to the significant downregulation of TrkB-T1 reported in the frontal cortex of suicide completers.

A custom-built microarray followed by a validation step allowed us to find a differentially methylated region in the *TrkB* gene between controls and suicide completers and determine if differential methylation could be correlated with TrkB-T1 mRNA expression. We assessed whether an A/G polymorphism (rs1624327), located in the DMR and able to generate a CpG site, could be associated with suicide or could drive TrkB-T1 expression through a CpG site formation. Finally, performing a patch methylation luciferase assay, we analyzed the functional effect of differential DNA methylation of the TrkB-T1 3'UTR sequence on TrkB-T1 expression.

Results

Microarray analysis

Following meDIP precipitation, samples were hybridized in custom-designed arrays, tiling the full-length of the *TrkB* gene

in a total sample of 24 individuals, including 11 suicide completers having low TrkB-T1 expression levels and 13 controls not selected for TrkB-T1 levels. After correction for multiple testing, we found 5 consecutive probes differentially methylated (Fig. 1A; Table 1). These probes were located in a 500-bp range within the TrkB-T1 3'UTR (Chr9: 87.428.875–87.429.157). The five differentially methylated regions were hypermethylated in suicide completers in a log₂FC ranging from 0.8 to 1.0. The length of DNA characterized as differentially methylated and captured by the probes was estimated to be approximately 1.2 kb (Fig. 1B), representing 25% of TrkB-T1 3'UTR sequence.

The TrkB-T1 transcript was distinguished from the other TrkB transcripts by its 3' untranslated region. The differential methylation between control and suicide groups in the region Chr9: 87.428.875–87.429.157, which is specifically transcribed in TrkB-T1 mRNA, suggests that this genomic region plays a key role in the regulation of TrkB-T1 transcription/expression in the frontal cortex.

Validation of the DMR corresponding to TrkB-T1 3'UTR sequence

To validate the results observed with the array, we focused on the 1 kb region encompassing the five probes that were differentially methylated between groups. This region contains 13 CpGs (Fig. 2A). We initially cloned and then Sanger sequenced PCR products of bisulfite treated DNA. Twenty clones for each subject and for each of the 3 PCR fragments covering the 1 kb long, differentially methylated region were sequenced. We found significant overall hypermethylation in *TrkB* DNA sequence (chr9:87.428.550–87.429.575) suicide completers (0.800 ± 0.015) when compared with controls (0.720 ± 0.014) ($f = 14.848$; $df = 169$; $P = 1.67 \times 10^{-4}$) (Fig. 2B).

Table 1. Probes differentially methylated between control and suicide completers

Chromosome	Start	End	cpG level	Methylation level	P value	Q value	Differential	Gene name	Gene strand	tss distance	More methylated in
9	87428875	87428928	0.1331	0.7637	0.0018	0.0029	1.0213	NTRK2	1	-143326	Suicide
9	87429019	87429078	0.1313	0.7735	0.0077	0.0029	0.8204	NTRK2	1	-143473	Suicide
9	87429029	87429084	0.1300	0.7735	0.0038	0.0029	0.9259	NTRK2	1	-143481	Suicide
9	87429105	87429156	0.1292	0.7296	0.0017	0.0029	0.9471	NTRK2	1	-143555	Suicide
9	87429107	87429157	0.1292	0.7296	0.0061	0.0029	0.8241	NTRK2	1	-143557	Suicide

Criteria used to select the differential probes are the following: *P* values: < 0.01; *Q* values < 0.05; CpG level > 0.1, Methylation level > 0.6; Log2 Fold Change: < -0.8 and > 0.8.

When looking at site-specific CpG sites, we found a group of four CpGs (CpG6 to CpG9), spanning 150 bp, that were differentially methylated between suicide completers and controls (Fig. 2C; Table 2; Fig. S1). These data suggest that the significant difference in methylation found across this 1 kb region was driven by these four CpG.

To determine whether these CpG sites could be considered as a cluster with regard to the differential methylation observed between suicide completers and controls, we compared the $-\log P$ values (Fig. 3A) of these four CpGs to the values for the other CpGs. Using ANOVA, we found a significant differences in the $-\log P$ values between CpG regions ($f = 4.198$; $df = 2$; $P = 0.047$). The CpG6–9 region has a higher $-\log P$ value (1.764 ± 0.269) compared with the CpG1 to 5 region (0.665 ± 0.208) ($P = 0.026$) and to the CpG 10 to 14 region (0.389 ± 0.127) ($P = 0.026$).

Comparison of methylation levels in the 150 bp fragment containing the CpG sites 6 to 9 between suicides and controls showed a significant difference ($P = 4.3 \times 10^{-6}$) (Fig. 3B and D), which is characterized by a significant hypermethylation in suicide completers (0.817 ± 0.020) as compared with controls (0.679 ± 0.018) (Fig. 3D). The fold change in average methylation levels in the suicide group as compared with controls was: (1) CpG6: FC = 1.17; (2) CpG7: FC = 1.79; (3) CpG8: FC = 1.14; and (4) CpG9: FC = 1.16 (Fig. 3C). Interestingly, however, in both groups, the CpG7 had lower methylation levels than the other CpGs (Fig. 3B; Table S1).

We then determined if the methylation levels were associated with TrkB-T1 expression levels. Using a linear regression model, we found significant correlation between the methylation levels and TrkB-T1 expression ($P = 5.7 \times 10^{-4}$) (Fig. 4). These data suggested that TrkB-T1 decrease in BA8/9 of suicide completers is associated with methylation levels in CpG6 to 9 in our sequence of interest.

Relationship between TrkB-T1 expression levels and the polymorphism at the rs1624327 locus

One of the CpG sites in the differentially methylated region contains the SNP rs1624327, an A/G substitution (minor allele frequency $A = 0.247$) that is the tenth CpG of the sequence investigated in the validation studies (Fig. 2A). This A/G polymorphism significantly changes methylation levels, as homozygous AA would not have any methylation at this locus; heterozygous would have a maximum of 50%, and homozygous GG could present up to 100% of methylation. We found, in average, a 17% difference in methylation between the control (0.623 ± 0.092)

and the suicide groups (0.799 ± 0.100), with high coefficients of variation being, respectively, 53.2% and 41.7% (Fig. 3C). In fact, with the exception of one control subject, the methylation levels directly reflected the genotype (Fig. S2A). With average methylation levels at: (1) 0.94 ± 0.04 for the G/G patients, (2) 0.54 ± 0.05 for G/A patients, and (3) 0 for the A/A patients. We found significant correlation between genotype and methylation level ($R = 0.923$ $P = 1.4 \times 10^{-10}$). Consequently, we genotyped the rs1624327 locus in our initial sample of 24 patients (13 controls and 11 suicide completers), as well as in an extended sample of 61 patients (28 controls and 33 suicide completers with low TrkB-T1 expression) (Fig. S2B; Table S2). The genotypic distributions are presented in Tables 3A and C. We found no significant difference in the allelic distribution between controls and suicides, whether in the initial cohort ($\chi^2 = 0.16$ and $P = 0.69$; Table 3B) or in the extended cohort ($\chi^2 = 0.09$ $P = 0.77$; Table 3D). Although the methylation level at the rs1624327 is driven by the presence of a G allele at this locus, the absence of significant association between the allele at the rs1624327 and suicide suggests that the allele at the rs1624327 is not involved in the regulation of TrkB-T1 expression, as the results of bisulfite sequencing were suggestive, in spite of high variances in each group.

Functional validation of differential methylation of TrkB-T1 3'UTR sequence

To assess the functional effect of site specific differential methylation in TrkB-T1 3'UTR sequence, a 150 bp fragment containing the CpG6, 7, 8, and 9 was cloned in a pCpGfree vector, 3' to the luciferase coding sequence, in sense and antisense orientation (Fig. 5A). These constructs (methylated or unmethylated) were transfected into HEK293 and luciferase activity was compared among cells containing native vector, unmethylated or methylated vector.

Twenty-four hours after transfection into HEK293 cells, we found significant differences in luciferase activity among conditions ($f = 23.965$; $df = 20$; $P = 1.37 \times 10^{-6}$) (Fig. 5B). Luciferase activity in cells transfected with unmethylated 150 bp TrkB-T1 sequences cloned in sense and antisense orientations were significantly lower (15.9% for sense orientation [$P = 0.033$] and 19.4% for antisense orientation [$P = 0.002$]) than luciferase activity in cells transfected with empty vector. A 29.1% decrease of luciferase activity was found in cells transfected with the methylated 150 bp in sense orientation compared with the luciferase activity from cells transfected with the unmethylated constructs (P

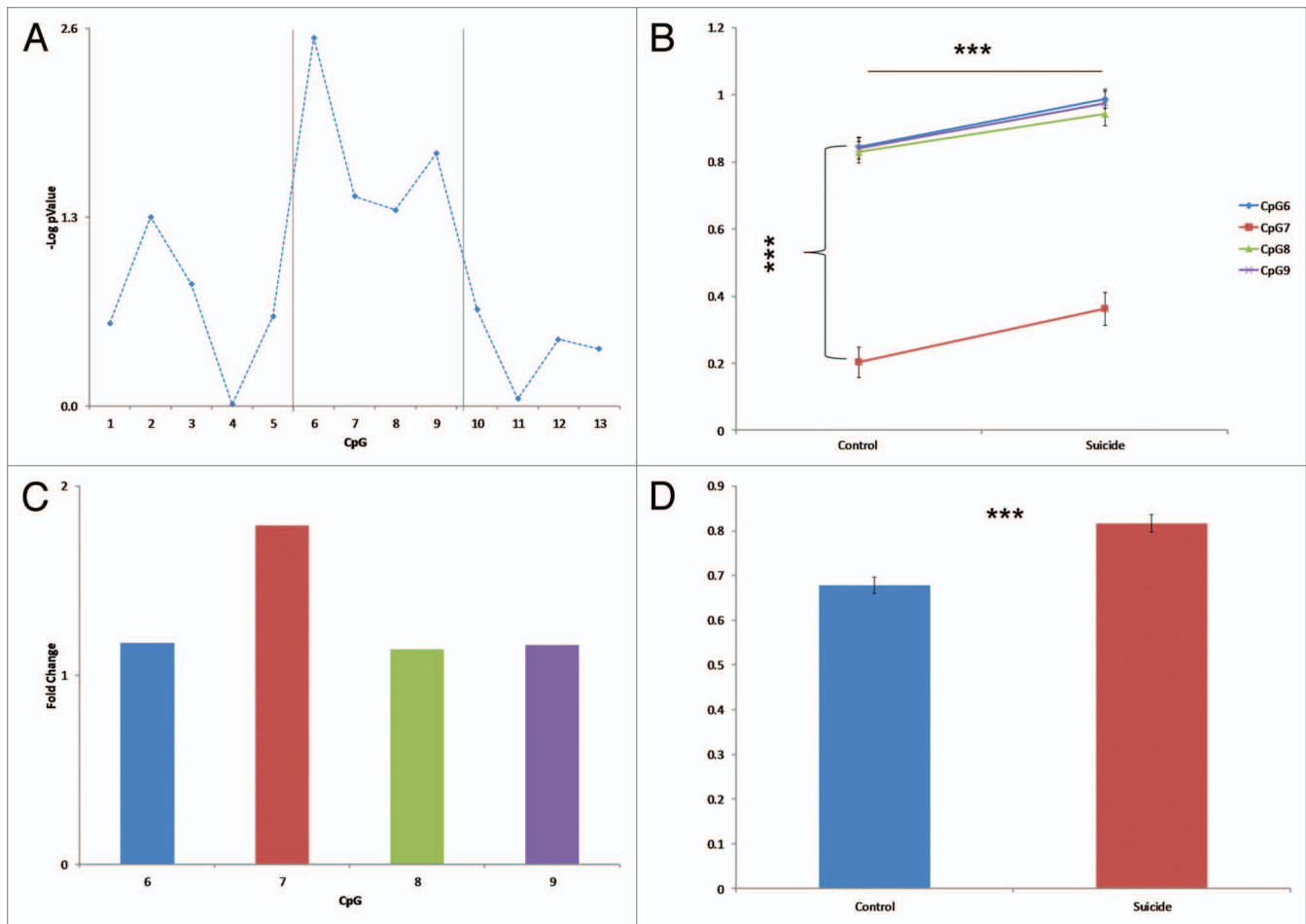


Figure 3. (A) Plot of $-\log P$ values for the 13 CpGs group in the TrkB-T1 3'UTR DNA sequence (B) Representation of the paired comparison of methylation levels on individuals CpG6, 7, 8, and 9 between control and suicide. (C) Histogram of the individual fold changes in methylation on CpG6, 7, 8, and 9 in suicide group compared with control. (D) Histogram of the average level of methylation in the CpG6, 7, 8, and 9 in suicide and control group.

3'UTR DNA sequence, described here, suggests that methylation in this DNA region may be among the main causes of TrkB-T1 decrease in suicide completers.

Methylation in the TrkB gene

A previous study by our group found that histone modification and site-specific differential methylation in the *TrkB* promoter contribute to regulate TrkB-T1 expression.^{5,9} While the current study did not find evidence of differential methylation in the promoter region, our current results are not discordant from our previous findings. The microarray methodology used in this study integrates methylation levels from several CpG sites,²⁰ whereas the findings reported previously on the *TrkB* promoter were observed using bisulfite sequencing and, thus, had single base resolution but interrogated a very short sequence. In the array study, each probe covered a 600 bp sequence,^{20,21} and this method is particularly powerful to detect signals coming from a cluster of differentially methylated CpGs, as was the case for the 3'UTR findings reported here. Together, our results suggest that TrkB-T1 transcription is regulated by multiple factors: DNA methylation and histone modification in the promoter region and DNA methylation and microRNA binding in 3'UTR sequence.

Table 2. Descriptive statistics of the differential methylation levels in CpG6, 7, 8, and 9 in the region differentially methylated between control and suicide

	Mean	Std. Error	Mean	Std. Error		
CpG	Control		Suicide		F	Sig
6	0.851	0.026	0.981	0.029	11.082	0.003
7	0.207	0.045	0.355	0.049	4.960	0.036
8	0.834	0.032	0.937	0.035	4.505	0.045
9	0.846	0.032	0.967	0.035	6.454	0.018

Function of methylation in 3'UTR sequences

Although DNA methylation is a relatively stable epigenetic modification,²² it has primarily been investigated in the promoter regions because of its repressive function on gene expression. Consequently, the functional effect of DNA methylation on other regulatory regions, such as in 3'UTR regions, as well as in other non-coding and coding regions remain still poorly understood, including their functional consequence on gene function. A growing number of studies, however, have recently investigated

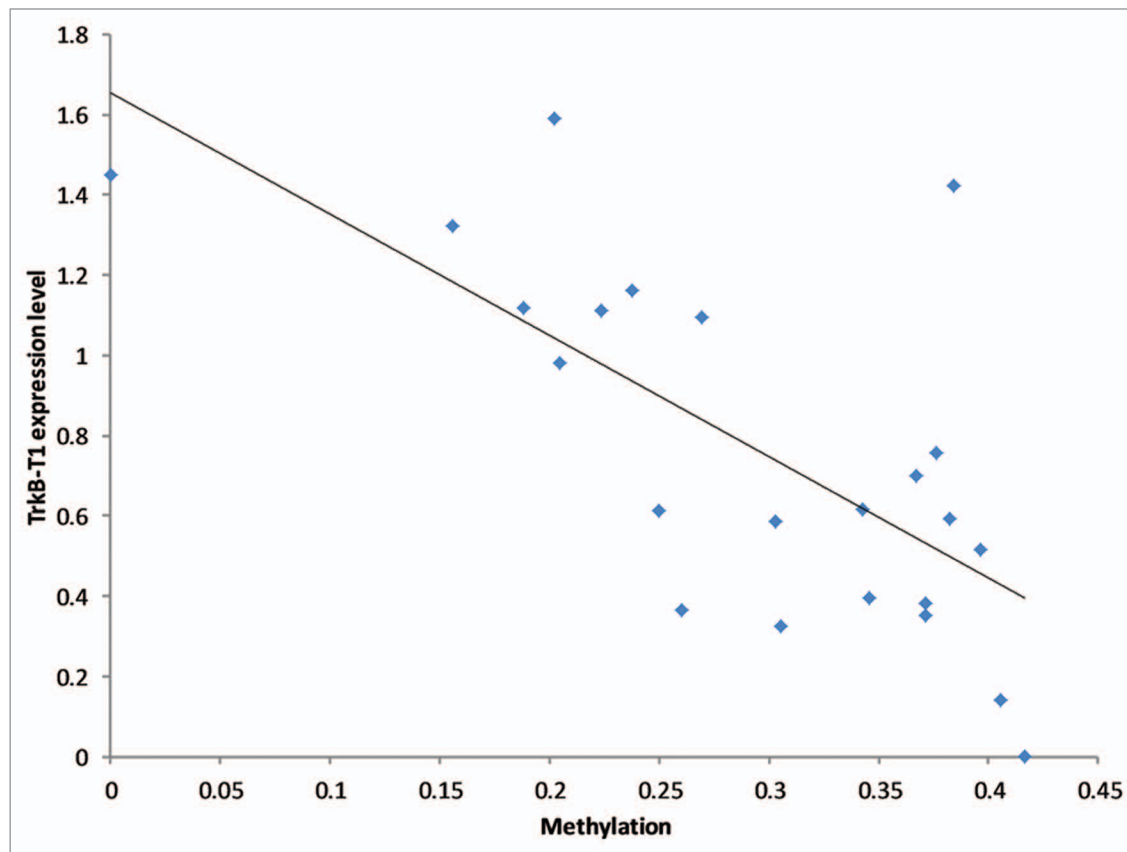


Figure 4. Linear regression analysis of the TrkB-T1 expression depending on DNA methylation levels on CpG6 to 9, corrected for age and PMI.

the role of DNA methylation in the 3'UTR on the regulation of gene expression.²³⁻²⁶

One of the first studies showing differential methylation in the 3'UTR region reported that hypermethylation of the cell cycle inhibitor gene *p15INK4b* interferes with its expression in primary lymphomas.²³ Hypomethylation of *CCDN1* 3'UTR DNA region, concomitant with an increased expression of the gene, was observed in many neuroblastoma cases.²⁴ Similarly, methylation of *Lhx8* DNA 3'UTR region was inversely correlated with the protein expression during different phases of oogenesis in the mouse.²⁶ Finally, hypermethylation in 3'UTR sequence of the *PDX1* and *OTX1* genes were positively correlated with gene expression in colorectal cancers.²⁵ Whereas promoter methylation has extensively been investigated and correlated with gene expression, our study, together with those cited above, have suggested that methylation in the 3'UTR could regulate the levels of RNA and/or proteins. These findings are in line with the idea that methylation changes in regions other than the promoter can lead to transcript specific regulation. In consequence, TrkB-T1 regulation may result from combined mechanisms involving different epigenetic mechanisms, including promoter DNA methylation, histone modifications, and microRNA binding. In addition, the current study suggests a potential role for methylation of the 3'UTR, specifically targeting regulation of the TrkB-T1 transcript.

Transcript-specific regulation and methylation in the 3'UTR position

Several studies, including ours, have shown that methylation of the 3'UTR has regulatory effects on gene expression. However, the mechanisms by which methylation in the 3'UTR change gene expression are not well understood. One possibility is through impacting regulation by the CTCF factor, which is part of a regulatory complex that binds intragenic regions on insulator sequences.²⁷ A recent study showed that CTCF contributes to regulate alternative splicing. CTCF allows the insertion of exon five of the *CD45* gene and promotes the elongation of a weakly expressed RNA,²⁸ whereas DNA methylation alters DNA binding of the CTCF factor. Interestingly, the 150 bp sequence of TrkB-T1 3'UTR sequence has been picked up by a CTCF ChIP-seq experiment in human cell lines.^{29,30} Furthermore, other epigenetic modifications, such as H3K36me3,³⁰ which has been suggested to be an epigenetic mark contributing to alternative splicing regulation,³¹ have been found in this region. Characterizing CTCF binding sites and mapping H3K36me3 epigenetic marks on *TrkB* in the frontal cortex of suicide completers could highlight the mechanism by which DNA methylation on TrkB-T1 3'UTR sequence leads to a specific decrease of TrkB-T1 transcript in context of suicide.

Methylation, vulnerability factors to suicidal behavior, and astrocytic dysfunction

Table 3. Genotypic and allelic distributions at the rs1624327 for the initial and extended cohorts of controls and suicide completers

A	Initial cohort		
Genotype	G/G	A/G	A/A
Control	7	4	2
Suicide	7	3	1
B	Initial cohort		
Allele	G	A	
Control	18	8	
Suicide	17	5	
C	Extended cohort		
Genotype	G/G	A/G	A/A
Control	16	10	2
Suicide	20	11	2
D	Extended cohort		
Allele	G	A	
Control	42	14	
Suicide	51	15	

(A) Genotype at the rs1624327 for the initial cohort of 13 controls and 11 suicides with low-TrkB-T1 expression. (B) Allelic frequency at the rs1624327 for the initial cohort of 13 controls and 11 suicides with low TrkB-T1 expression. (C) Genotype at the rs1624327 for the extended cohort of 28 controls and 33 suicides with low TrkB-T1 expression. (D) Allelic frequency at the rs1624327 for the extended cohort of 28 controls and 33 suicides with low TrkB-T1 expression.

DNA methylation of the TrkB-T1 3'UTR sequence in the brain from suicide completers might be related to changes in the maintenance of synapses³² and/or to inflammatory mechanisms. There is substantial evidence suggesting that suicidal risk is related to molecular deregulations associated with early life events.⁴ Results of studies investigating gene expression profiles related to suicidal risk, notably in the context of childhood abuse,^{3,8} suggest that early-life adversity increases vulnerability to suicidal behavior by altering stress response later on in life, through modulation of genes that belong to HPA axis.^{33,34} Furthermore, stressful events and depressive disorders were shown to alter expression of genes that normally promote cell survival.³⁵⁻³⁷ Depression and suicidal behaviors were linked to inflammatory processes, as an increased level of kynurenine was found in the CSF of patients diagnosed with MDD and past history of suicide attempts compared with control.³⁸ In addition, our group has found a hypertrophy of astrocytes, cells that act as modulator of the immune response in the CNS,³⁹ in the BA24 of suicide completers.¹⁶ Finally, TrkB is part of an astrocytic gene network that has been reported as downregulated in the brain of suicide completers.¹⁴ Consequently, differential DNA methylation in the *TrkB* gene investigated in the current study could be related either to: (1) the molecular changes that translate stress responses; (2) inflammatory processes, which specifically involve astrocytes; or (3) a decrease of astrocytes or a decrease of astrocyte functioning linked to suicidal behaviors. Further studies need to be performed in order to support these hypotheses.

To conclude, the current study, based on the downregulation of the TrkB-T1 transcript observed in brain of suicide

completers, focused on the functional effect of methylation of DNA in the TrkB-T1 3'UTR. We found that DNA methylation in the TrkB-T1 3'UTR sequence has an effect on TrkB-T1 expression. Compared with previous findings, DNA methylation in this genomic region seems to be the most important mechanism regulating TrkB-T1 expression in the brain of a subgroup of suicide completers. Further studies need to be performed to investigate: (1) the mechanisms, molecular partners, and interactions by which methylation of 3'UTR DNA sequence affects TrkB-T1 expression and (2) the consequences of *TrkB* gene methylation on cell functioning and network properties, especially on morphology and astrocytic activation. This study reinforces the idea that astrocytic dysfunction occurs in suicide and depressive pathophysiology, potentially related to impairments in cell survival and communication and/or dysregulation leading to inflammatory processes.

Material and Methods

Subjects

This study was approved by the Douglas Institute Research Ethics Board, and all participating families signed consent forms. Brain tissue was obtained from the Quebec Suicide Brain Bank (QSBB, www.douglasrecherche.qc.ca/suicide). The methylation arrays and validations were performed on a sample of 24 patients, which includes 11 suicides with low TrkB-T1 expression levels (mean age is 37 ± 13 and mean PMI is 26 ± 6), and 13 healthy controls not selected for TrkB-T1 levels (mean age is 33 ± 11 and mean PMI is 29 ± 6). Genotyping of the rs1624327 variant was performed on a total sample of 61 subjects including the 24 individuals that were part of the array study and 22 additional low TrkB-T1 expressors, as well as 15 healthy controls. The mean age of the 33 suicide completers was 39 ± 11 and the average PMI was 49 ± 26 . The mean age of the 28 controls was 42 ± 18 and the mean PMI was 32 ± 17 . All subjects were male and of French Canadian origin, a homogeneous population with a well-defined founder effect. All subjects died suddenly and could not have undergone any resuscitation procedures or other type of medical intervention, and thus did not experience prolonged agonal states.

Toxicological analyses at the time of death are systematically performed and past history of substance abuse and dependence are investigated. Among the suicide completers, 45% had a history of major depressive disorder ($n = 15$), 16% were diagnosed with depressive disorder not otherwise specified ($n = 5$), and 9% ($n = 3$) had substance dependence. One suicide completer was diagnosed with bipolar disorder and another with a psychotic disorder. Eight suicide completers (24%) did not have evidence of psychopathology and all controls were psychiatrically normal.

Diagnostic procedures

Diagnoses were obtained by means of psychological autopsies performed by trained clinicians using the SCID-I⁴⁰ with an informant best-acquainted with the deceased, as described elsewhere.⁴¹ Briefly, this is a structured diagnostic procedure to elicit diagnostic information by means of proxy-based interviews complemented

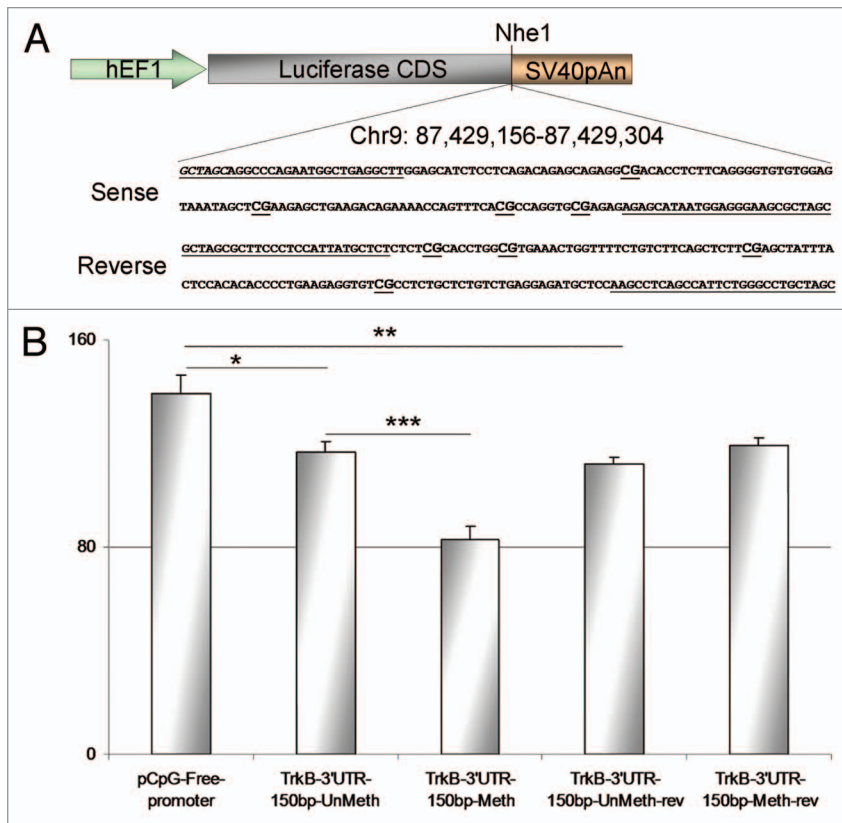


Figure 5. Functional analysis of site-specific methylation (A) Schematic representation of pCpGfreepromoter luciferase vector with 150 bp TrkB-T1 3'UTR DNA sequence inserted 3' to the luciferase coding sequences before an SV40 polyAn signal. Primers are underlined. CpG island are underlined and labeled in bold. (B) Luciferase activities in HEK293 cells, 24 h after transfection

with medical and coroner records, followed by a consensus diagnosis reached by a panel of clinicians^{42,43} using DSM-IV criteria.⁴⁴

DNA extraction

The DNA was extracted from 20 mg of brain tissue using the DNA extraction kit (Qiagen) and the procedure provided by the manufacturer.

Methylated DNA immunoprecipitation, labeling, and hybridization

Methylated DNA was extracted following an adaptation of a methylated DNA immunoprecipitation (meDIP) method developed⁴⁵ using 5' methylcytosine antibody bound to sepharose beads. Input, unmethylated, and methylated fractions were purified by phenol-chloroform and precipitated in ethanol. Labeling, hybridization, and data extraction were performed following the manufacturer's (Agilent Technologies) instructions.

Every subject was hybridized on a separate microarray. Microarrays were scanned (High-Resolution C Scanner; Agilent Technologies), and data were extracted using commercial software (Feature Extraction; Agilent Technologies).

Microarray

A custom-designed 4 × 44K promoter tiling array was used for this study (Agilent Technologies). We tiled a 625 000 bp region centered at the transcription start site of *TrkB* gene. All regions

tilled contain one probe approximately every 100 bp. Extracted microarray intensities were processed and analyzed using the R software environment for statistical computing.⁴⁶ Data were corrected for multiple tests (FDR = 0.05).⁴⁷

Bisulfite treatment

The DNA sample were bisulfite treated using the following kit EpiTect Bisulfite Kit (Qiagen) and the protocol defined by the manufacturer.

Primer design and PCR

Primers (shown in Table 4) were design by methyl primer express software to generate three PCR products which covers a 1 kb region of TrkB-T1 3'UTR DNA sequence

Cloning

PCR product from bisulfite-treated DNA were cloned into a topo4 TA cloning vector and transformed into the Top10 bacterial strain. Plasmidic DNA from 20 to 30 individual colonies were extracted and sequenced by the Genewiz company according to the guidelines provided by the manufacturer. FASTA sequences and chromatograms were aligned and analyzed using Bioedit version 7.0.9.0 and Geneious version 5.5.2 software

Statistical analysis

Methylation data were analyzed using linear mixed models as implemented in SPSS 18. The best fit model was chosen comparing AIC and AICC scores. CpG were considered as repetitive elements and groups defined by the status control or suicide. The results were corrected for PMI and age as covariates when applicable. *P* values related to differences in site specific methylation between control and suicide were log transformed when required.

Allelic distributions were assessed by using Chi Square test. Comparisons of TrkB-T1 expression levels in controls and suicide group were also performed using a linear mixed model The graphic representation (Fig. 4) of the partial correlation between methylation of TrkB-T1 3'UTR sequence and TrkB-T1 expression was obtained performing a linear regression under SPSS and translating coordinates with a constant term in X and Y axes in order to get positives values for those two variables. Dot plots of TrkB-T1 expression were performed using GraphPad Prism 4 software.

Genotyping

Individuals were genotyped for the rs1624327 using a SNPpassay (Cat. # 4351379) provided by Life Technologies. PCR data were analyzed using SDS software version 2.2.1.

Functional analysis

To assess the functional effect of methylation on TrkB-T1 expression, we cloned in 3' position of the luciferase coding sequence, a 150 bp DNA fragment encompassing CpG6 to CpG9, into the pCpGfreepromoter luciferase vector in sense and antisense orientation using the primers (Table 4).

The construct was submitted to a 4-h treatment by Sss1 methylase (New England Biolabs) then purified.

This construct was co-transfected in HEK 293 cells with an equivalent amount of pGL3 control vector used for normalization. Luciferase activities were quantified using the dual luciferase reporter (Promega) kit 24 h after transfection in the cell medium and cellular extracts.

Statistical analyses were performed with SPSS 18 using ANOVA and post-hoc *t* test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:

www.landesbioscience.com/journals/epigenetics/article/29068

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Table 4. Primers used for to generate PCR products of TrkB-T1 3'UTR from bisulfite converted DNA and the constructs in pCpGfreepromoter lucia vector

Primer	Sequence	Length
TrkB-T1 3'UTRup1	TGTTTTTTTT TGTTGGGAAT T	261 bp
TrkB-T1 3'UTRdw1	ACCCCATTA AACATAAAT AA	
TrkB-T1 3'UTRup2	TTTATGGGTG TTTTAATGT GG	249 bp
TrkB-T1 3'UTRdw2	AAAAAAAAA ACCCTAAAT CAA	
TrkB-T1 3'UTRup3	AGGTTTAGAA TGGTTGAGGT TT	432 bp
TrkB-T1 3'UTRdw3	ATCAAATCA AACCAAAAAA AA	
TrkB-T13'UTRNHE1 up	GCTAGCAGGC CCAGAATGGC TGAGGCTT	150 bp
TrkB-T13'UTRNHE1 dw	GCTAGCGGCT TCCCTCCATT ATGCTCT	

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