Application Note

Global Gene Expression of Major Depression

Reveals regulated Spermine/Spermidine N1-acetyltransferase (SAT) gene expression associated with a genetic polymorphism

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Introduction

Major depressive disorder (MDD) is a life-threatening disease that affects up to 20% of women and 12% of men during their lifetimes and has a prevalence of nearly 20 million Americans annually. This Application Note demonstrates how scientists at Gene Logic have investigated patterns of gene expression in major depression and compared them to those patterns observed in normal controls. The BioExpress® System, a database of gene expression measurements from human samples, curated by board-certified pathologists, and the Genesis Enterprise System® Software, an extensive collection of warehousing, visualization, and analysis tools, were used to perform the analyses. Gene Logic has established collaborative relationships with leading research institutions to obtain high-quality specific brain tissues which are then processed to generate whole genome gene expression profiles and for inclusion in the BioExpress® System.

In this study, subjects were young males and consisted of three groups: (1) suicides without MDD, (2) suicides with MDD, and (3) matched (non-suicide) controls. Within the global gene expression profile of over 45,000 genes and fragments tested, a consistent pattern was found for Spermine/Spermidine N1-acetyltransferase (SAT), the rate limiting enzyme in the catabolism of polyamines. The expression pattern of SAT was also validated by RT-PCR and immunohistochemistry. Polymorphism of the SAT gene at the SAT342A/C locus, located in a regulatory region, revealed a significant effect of the genotype on expression levels of SAT with SAT342C in suicides. The data suggest a role for SAT in suicide and depression, whose expression may be differentially regulated as a consequence of a polymorphic variant of the SAT342 locus.

Definition and Background

MDD plays a role in about half of all suicide attempts, which result in more than 30,000 deaths per year in the United States. Suicide is the leading cause of death for men younger than 35 years of age in many countries. The economic impact of MDD is estimated to exceed $40 billion per year in the United States. The majority of individuals afflicted with MDD are unaware they have a potentially treatable illness and do not seek medical attention (1).

It has been increasingly recognized that individuals who commit suicide have a biological diathesis that is partially inherited (2). Psychopathology, particularly MDD, is commonly associated with suicide, but the genetic predisposition to suicide is likely independent of

underlying psychiatric disorders (3-6). To date, several possible biological markers for suicide and depression have been identified (7), especially among components of the serotonergic (2) and noradrenergic systems (2, 8, 9), but no clear understanding has yet emerged.

Post-mortem and neuroimaging studies of the brain in the past have implicated Broadman's Areas (BA) BA 8/9 (dorsolateral prefrontal cortex) and BA 11 (orbital cortex) in suicide and depression (10, 11). Polycationic compounds such as polyamines have been implicated in mood disorders such as major depression (12, 13, 14). Polyamines, especially spermine, are stored in synaptic vesicles and are released by depolarization like neurotransmitters (16). Considering the multiple processes in which CNS polyamines have been implicated, changes in polyamines levels may produce profound effects in psychiatric diseases. SAT is the single rate-limiting enzyme in the catabolism of the polyamines spermidine and spermine (15).

The purpose of this Application Note is to discern the molecular changes in major depression and furthermore, to explore the expression and polymorphism of the SAT gene in the context of this disorder.

Materials and Methods

Details of the Human Clinical Study

The sample sets for BA 11 consisted of depressed suicide victims (SD; n=8) who died during an episode of major depression; suicide victims (S; n=7) with no lifetime history of major depression; and matched controls (C; n=9) with no history of suicidal behavior or a major psychiatric diagnosis. Subjects were matched on the basis of age and post-mortem interval. All subjects died suddenly without a prolonged agonal (hypoxic) state or protracted medical illness. Psychological autopsies were carried out using structured interviews (SCID-I, SCID-II) by trained clinicians with at least one informant per family. Following the interview, a review of the coroner's notes and all relevant medical records was performed and a case report was written for the purpose of a best-estimate diagnosis. Best consensus DSM-IV axis I and II diagnoses were made by a panel of psychiatrists based on the analysis of the case reports.

The accrual procedure followed strict IRB approval and standard operating procedures provided by Gene Logic. The samples were prepared and analyzed at Gene Logic following the procedural recommendations of the Affymetrix Expression Analysis Technical Manual. Gene Logic's scientists prepared optimized sample preparation or analysis methods, when needed, to ensure that high quality data were generated from each sample. Only those samples that passed Gene Logic's stringent quality control metrics were added to the BioExpress® System for storage and analysis using the Genesis Enterprise System® Software.

The BioExpress® System is a gene expression database containing more than 18,000 samples representing diseases such as inflammation and autoimmune, oncologic, metabolic, cardiovascular, and central nervous system disorders. The present study used a portion of the Central Nervous System (CNS) Data Suite, a subset of data in the BioExpress® System generated from the analysis of CNS samples.

The Genesis Enterprise System® Software enables the storage of the gene expression data as well as the clinical data associated with the samples such as patient history, diagnostic tests, medication, etc. The Genesis Enterprise System® Software also provides standard tools to allow researchers to perform common research tasks, such as building complex queries to identify related samples, visualizations to identify outliers within associated samples, principal component analysis to segregate samples into related groups based on gene expression measurements, and the ability to identify genes which discriminate a pattern of regulation. Importantly, the Genesis Enterprise System® Software enables researchers to rapidly reduce complex data sets to biological conclusions by projecting expression data onto over 400 biological pathways, coupling visualizations of clinical data, expression data, and reference data, and providing the ability to quickly correlate gene expression changes to clinical parameters, including currently used markers.

Selection of Arrays

Principal Component Analysis (PCA) was used to identify outlier microarrays. Microarray quality control parameters included the following: noise (RawQ), consistent number of genes detected as present across arrays, consistent scale factors, and consistent ß-actin and GAPDH 5'/3' signal ratios.

Data Analysis

Expression data were stored and analyzed in the Genesis Enterprise System® Software. Genes were selected for analysis from the roughly 45,000 fragments on the microarray on the basis of "Present Calls" by MAS 5.0. Given certain detection p-value limits, the probe set is called either Present, Marginal, or Absent. In the current study, for a gene to be included, it had to be Present (detectable) in at least 75% of the subjects in at least one of the three groups. PCA was performed based on the initial, unfiltered gene sets and on the selected genes (according to our significance criteria). The PCA analysis based on the Affymetrix U133 A & B chipset did not discriminate the groups, whereas the PCA based on the selected genes showed discrimination of the three groups.

Results and Discussion

Analysis of demographic parameters revealed no significant difference in terms of age and post-mortem interval between the groups. Consistent with previous reports (20-22), analysis of post-mortem interval on RNA quality control parameters revealed no significant effects.

Statistical comparisons were performed on selected genes instead of on the total number of genes present in the chipset in order to reduce the number of comparisons and the chances of false positives. Using the criteria of a p-value $\leq$0.01 and fold change (FC)$\pm$ $\geq$1.3, differentially expressed genes were analyzed with three comparisons: depressed suicides versus control (SD-C), suicides versus controls (S-C) and depressed suicides versus suicides (SD-S). The fold change distribution table generated by the Genesis Enterprise System® Software summarizes the number of differentially expressed genes observed when comparing depressed suicides to controls (Figure 1).

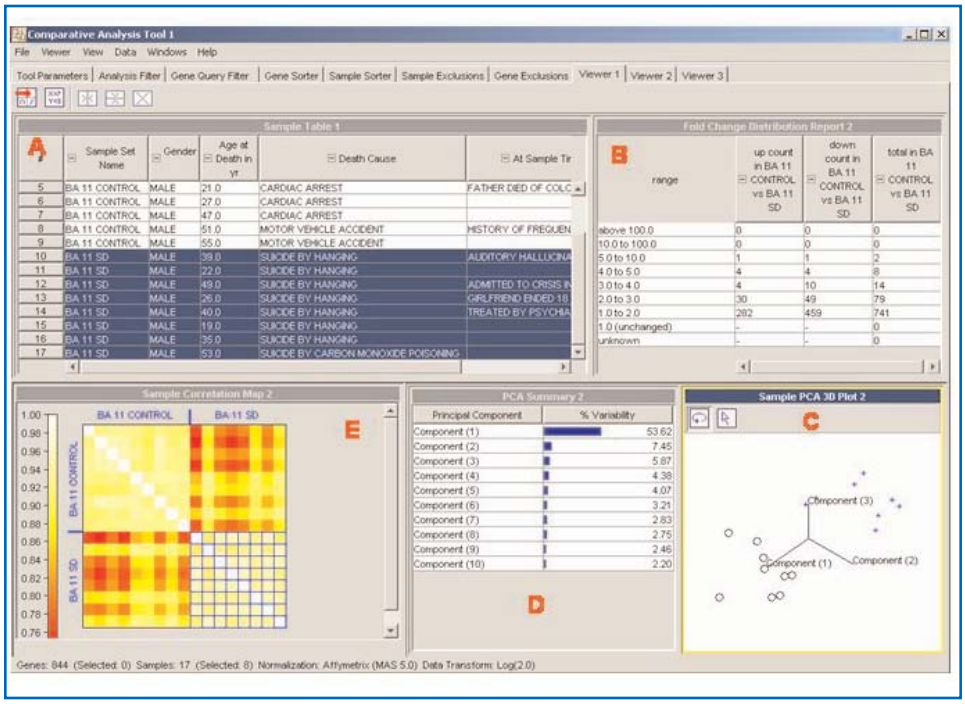

Figure 1. Comparative analysis summary for the control subjects compared with suicide with major depression subjects in the orbital cortex (BA 11). A. Clinical parameters of the suicide with major depression subjects (highlighted in blue). **B.** Fold change (FC; $\pm$ $\geq$1.3) distribution table listing the number of genes up- and down-regulated with P=0.01. **C.** PCA analysis of the differentially expressed genes in control and the suicide with major depression (highlighted in blue) groups showed a distinct spatial separation based on the first component. **D.** PCA summary showing that the separation is primarily in the first component. **E.** A sample correlation map showing the relationship of samples with respect to the results genes of the Comparative Analysis. Correlation is indicated by color according to the vertical scale.

Of the total of 844 genes up- and down-regulated, the greatest number of genes changing was in the 1 to 2 fold range (741 genes; $\pm$ $\geq$1.3), which is the typical range for CNS tissue. Similar observations were made for the suicide versus control group (54 genes; not shown) and the suicide versus depressed suicide (249 genes; not shown). As shown in Figure 1, PCA analysis of the differentially expressed genes in SD-C group showed a distinct spatial separation on the first component (53.62). The blue highlights the spatial separation of the SD's compared with the controls. This is confirmed by the correlation

map shown in Figure 1. Thus, the observed spatial discrimination demonstrates that the difference between the groups of subjects is based on genes that are differentially co-regulated between the groups.

Using the Contrast Analysis function of the Genesis Enterprise System® Software, we examined the differentially expressed genes across the three sample sets (control, suicide, and suicide with major depression) ranked by F-score (Figure 2). In the Gene Table (top left panel), with an F score >6.0, 638 genes were listed, where SAT was seventh on the list with an F score of 16.88. As illustrated by the e-Northern® Report (bottom left panel), in BA 11, SAT was significantly down-regulated in both depressed suicides and suicides in relation to controls with fold changes of -1.74 (P=0.003) and -1.35 (P=0.0008), respectively. SAT was also significantly down-regulated in two other brain regions, BA 4 and 8/9 (not shown).

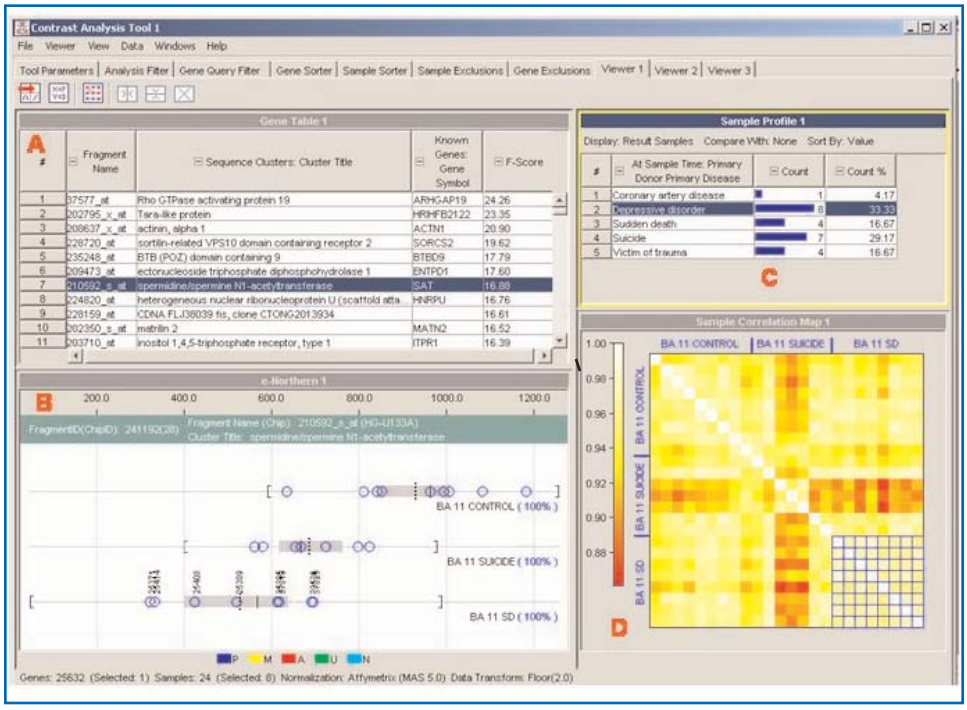

Figure 2. Contrast analysis function to examine the differentially expressed genes across the three sample sets (controls, suicide and suicide with major depression) based on F-score. A. Of the 638 fragments with an F score >6.0, the Spermine/Spermidine N^1-acetyltransferase gene (SAT) is highlighted. **B.** e-Northern® Report illustrating that the SAT gene was significantly down-regulated in both depressed suicides and suicides in relation to controls with fold changes of -1.74 (P=0.003) and -1.35 (P=0.0008). Each circle represents an individual sample. **C.** Number of samples in each sample set analyzed. [Note: Coronary artery disease, sudden death and victim of trauma samples all represent control samples for this study.] **D.** Sample correlation heat map showing the relationship of samples with respect to gene expression of the result genes of the Contrast Analysis. Correlation is indicated by color according to the vertical scale.

Further clarification of the role of SAT in BA 11 (control vs. SD) was quickly identified using gene ontology and pathway mapping functions (Figure 3). As illustrated in the gene profile table (top left panel), three Affymetrix fragments were identified for SAT (SAT being the HUGO symbol) linked to the arginine and proline metabolism. These are further highlighted in the Pathway Map (panel on right).

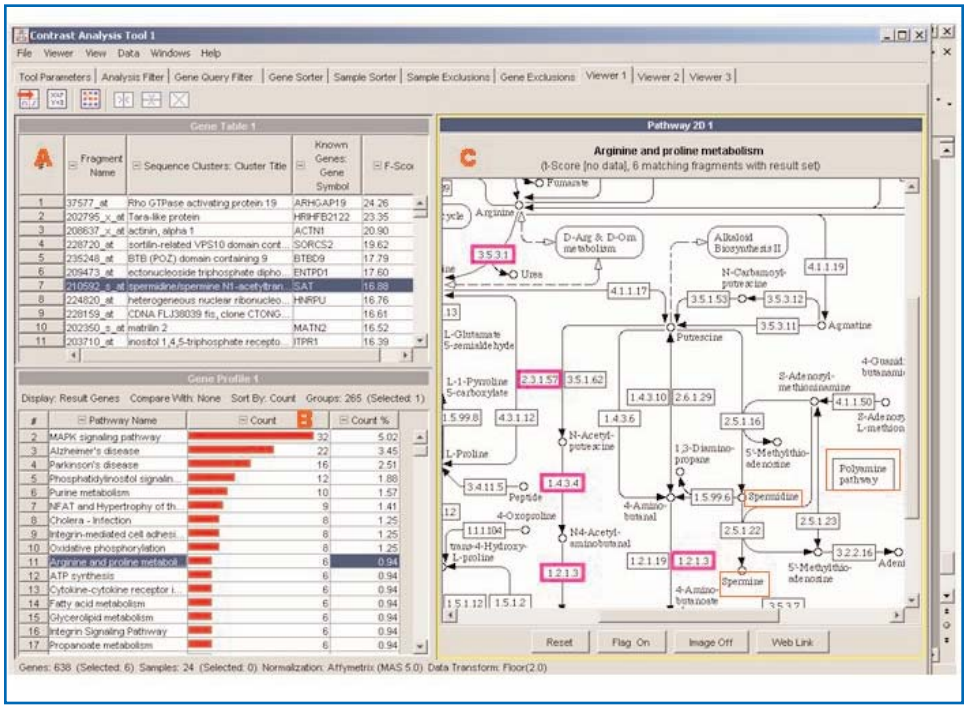

Figure 3. Pathway identification of the same gene table in Figure 2. A. Gene Table from Figure 2A. **B.** The highlighted SAT gene has three associated fragments and is identified in the Proline and Arginine metabolism pathway. **C.** Two of the SAT fragments are highlighted in pink. SAT is the rate-limiting enzyme in the catabolism of the polyamines, spermidine and spermine (highlighted in red).

SAT differential expression was validated by semi-quantitative RT-PCR (Figure 4A) on independent samples from the same individuals in all three brain regions (n=5 per group/region). In BA 11 SAT expression was 1.3-fold lower in suicides and 1.35-fold lower in depressed suicide than in controls ($F=4.17$; $df=2$; $P=0.024$). Lower expression of SAT was also confirmed in BA 4 for the suicides (FC = -1.3) and for the depressed suicides (FC= -1.3) when compared to controls ($F=3.84$; $df=2$; $P=0.032$). Similarily, in BA 8/9, SAT expression was lower among suicides and depressed suicides when compared to controls with fold changes of -1.38 and -1.27, respectively ($F=2.92$; $df=2$; $P=0.07$).

Altered expression at the transcriptional level does not necessarily lead to altered protein expression and SAT is known to undergo extensive post-transcriptional regulation (23, 24). Confirmation of the observed changes at the protein level was carried out by immunohistochemistry analysis in tissue sections prepared from the same brain regions

using a SAT polyclonal antibody (25). Figure 4B illustrates the observed changes in SAT immunoreactivity in BA11 of a control, a suicide and a depressed suicide. Quantification of immunopositive cells in a subgroup of subjects (n=3 per group) showed a lower SAT protein expression in both suicides with (FC=1.36; df=2; P=0.04) and without major depression (FC=1.35; df=2; P=0.005) when compared to controls. Thus, the microarray evidence, confirmed by the semi-quantitative RT-PCR, reflects relevant changes at the protein level and suggests a possible role of SAT in the pathophysiology of suicide and depression.

Recently, it was shown that SAT expression is closely regulated by a cis polyamine responsive element (PRE) located in the promoter region of this TATA-less gene (26). An analysis of the genetic variation at four loci in the SAT gene was undertaken to investigate their influence on SAT expression on these same brain regions. As SAT is an X-linked locus, males are hemizygous, and as all brains were from male donors, we could thus investigate the direct relationship between SAT allelic variants and the altered expression of SAT. SAT342A/C, the only polymorphic locus located in the PRE regulatory region, showed a significant effect on SAT levels in the BA4, BA8,9 and BA11 (F=5.34, degree of freedom (df) =1, P=0.024; not shown) with subjects having the SAT342A variant (SAT342C) showing greater expression in the two suicide groups.

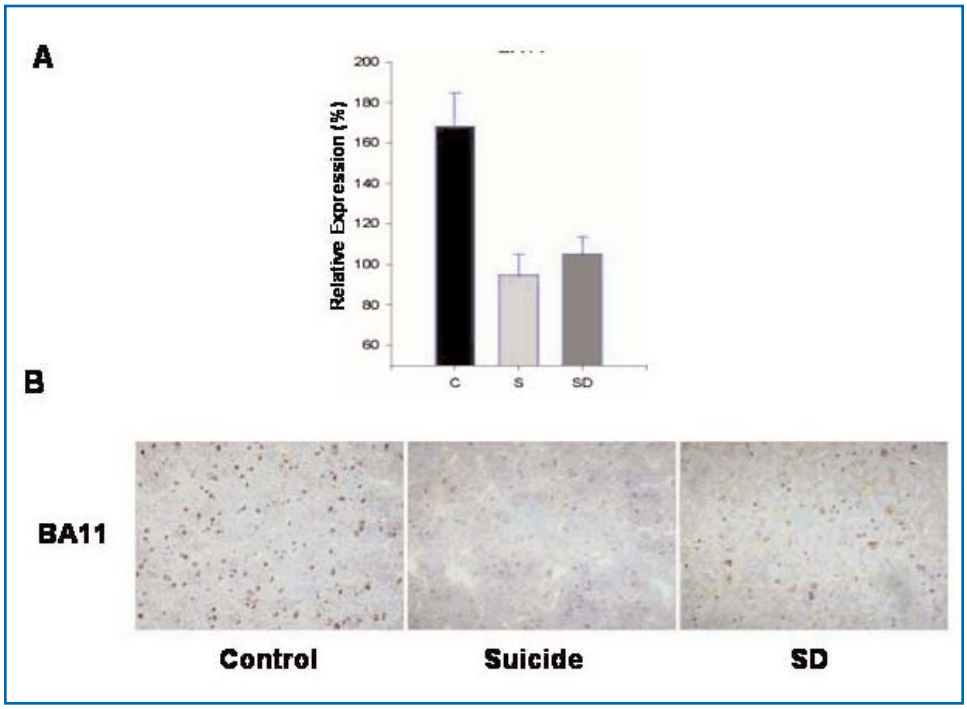

Figure 4. Semi-quantitative analysis by reverse transcription polymerase chain reaction (RT-PCR) mRNA levels, and immunohistochemistry analysis of SAT. A. Summary RT-PCR graphical representation of the relative (% of ß-actin) (to SAT mRNA levels in a group of controls (C), a group of suicides (S) and a group of depressed suicides (SD) in motor cortex (BA4), dorsolateral prefrontal cortex (8,9) and orbital cortex (BA11). **B.** Immuhistochemical staining photomicrograph of BA11 brain sections using a SAT polyclonal antibody (1:75) in a control, a suicide, and a depressed suicide.

Conclusions

Prevailing evidence implicates polyamines in mood disorders. This is based on several observations: Lithium prevents the stress induced PA response in rats (12-14). In addition, spermidine and spermine are able to block the serotonin transporter transient current in a way similar to fluoxetine and cocaine (24). Finally, glutamatergic neurotransmission is closely controlled by intracellular levels of polyamines, spermine, and spermidine being specific modulators of NMDA and AMPA receptors activity (25). Consequently, significant down-regulation of SAT would be expected to disrupt polyamine homeostasis resulting in regional increases in spermine, spermidine, or both. Considering the multiple processes in which CNS polyamines have been implicated, changes in polyamines levels may produce profound effects.

In this study, we examined the expression levels of genes on microarrays using the BioExpress® System and Genesis Enterprise System® Software in post-mortem cortical regions from subjects who died by suicide with and without major depression versus a group of controls. SAT was identified as a potential candidate mediating the risk for suicide. Confirmation of our results and further investigation of the role of SAT and other polyamine metabolizing enzymes in the neurobiology of suicide and major depression are warranted.

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