

Allelic imbalance of tissue-type plasminogen activator (t-PA) gene expression in human brain tissue

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SUMMARY

We have identified a single-nucleotide polymorphism (SNP) in the t-PA enhancer (-7351C>T), which is associated with endothelial t-PA release *in vivo*. *In vitro* studies demonstrated that this SNP is functional at the level of transcription. In the brain, t-PA has been implicated in both physiologic and pathophysiologic processes. The aim of the present study was to examine the effect of the t-PA -7351C>T SNP on t-PA gene expression in human brain tissue. Allelic mRNA expression was measured in heterozygous post-mortem brain tissues using quantitative TaqMan genotyping assay. Protein-DNA interactions were assessed using electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Significantly higher levels of t-PA mRNA were generated from chromosomes that harboured the wild-type -7351C allele, as compared to those generated from the mutant T allele (for the hippocampus, C to T allelic ratio of ~1.3, $P=0.010$, $n=12$; and for the cortex, C to T allelic ratio of ~1.2, $P=0.017$, $n=12$). EMSA showed reduced neuronal and astrocytic nuclear protein binding affinity to the T allele, and identified Sp1 and Sp3 as the major transcription factors that bound to the -7351 site. ChIP analyses confirmed that Sp1 recognizes this site in intact cells. In conclusion, the t-PA -7351C>T SNP affects t-PA gene expression in human brain tissue. This finding might have clinical implications for neurological conditions associated with enhanced t-PA levels, such as in the acute phase of cerebral ischemia, and also for stroke recovery.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is the main activator of the fibrinolytic system in the intravascular compartment. In the presence of fibrin, t-PA converts plasminogen to plasmin, which leads to clot dissolution. Previously, we identified a single-nucleotide polymorphism (SNP) in the enhancer region upstream of the t-PA gene, *i.e.* the t-PA -7351C>T SNP (1). Carriers of the mutant T allele showed reduced endothelial t-PA release *in vivo* (1). In line with the thromboprotective role of t-PA, we found that t-PA -7351T allele carriers have an increased risk of myocardial infarction (2), a finding that was later confirmed in the Framingham cohort (3). Our *in vitro* studies have shown that the t-PA -7351C>T SNP is functional at the level of transcription (4). The T allele binds the transcription factors Sp1 and Sp3 with decreased affinity, which in turn results in a reduced transcriptional activity with this allele (4). *In vitro* studies of human endothelial cells have also shown that this polymorphism affects the t-PA response to a wide array of stimuli (5).

In recent years, it has become increasingly apparent that the role of t-PA extends far beyond that of regulating vascular patency. In the brain, t-PA is produced by neurons, microglia, cerebral endothelial cells, and astrocytes (6). Under physiologic conditions in the adult brain, the parenchyma has a low level of t-PA expression, which is predominantly localized to the limbic system, including the hippocampus (6). This pattern of t-PA expression supports the observation that t-PA participates in processes of synaptic remodelling and plasticity (7, 8). However, under pathologic conditions in the brain, *e.g.* following an ischemic event or seizures, excessive release of t-PA can trigger proteolytic cascades, which subsequently may increase excitotoxicity and neurodegeneration (9, 10). Given the well-documented and multifaceted role of t-PA, the aim of the present study was to test the hypothesis that the t-PA -7351C>T SNP affects the expression of t-PA in

human brain tissue in a similar manner to that previously observed in the endothelium.

MATERIAL AND METHODS

Post-mortem human brain tissue

Cortical and hippocampal post-mortem tissues from 42 fresh-frozen brains were obtained from the Neurological Foundation of New Zealand Human Brain Bank. All the donated brains were collected with the full consent of the next of kin and in line with ethics approval from the University of Auckland Human Participants Ethics Committee. The t-PA 20099T>C SNP, which is located in a coding exon, was used as a marker for the t-PA -7351C>T SNP (described more in detail below under Quantitative TaqMan genotyping assay). Twelve subjects were heterozygous for both SNPs, and were thus selected for the subsequent allele-specific expression studies (described below). The mean age at death of these 12 subjects (5 females and 7 males) was 63 years (range, 21-83 years). The mean post-mortem interval delay was 13 h (range, 7-21 h). There were no evident neurologic injuries associated with the cause of death and assessment of all brains by a neuropathologist revealed no neurological abnormalities. The processing of fresh brain tissue was performed as described (11).

DNA extraction and Genotyping

Genomic DNA (gDNA) was isolated from brain tissues using the E.Z.N.A Tissue DNA kit (OMEGA Bio-Tek, Norcross, GA, USA). Genotyping for the two SNPs (dbSNP reference IDs: rs2020918 and rs1058720) was performed using TaqMan assays as described (12). The assays used were: Assay-on-demand C_12108250_10 and C_3212004_10 (Applied Biosystems, Foster City, CA). Amplifications were performed in the Dual 96-well GeneAmp PCR System 9700 (Applied Biosystems), and probe signals (VIC and FAM) were recorded using the ABI PRISM 7900HT sequence detector system (Applied Biosystems). The genotypes in heterozygous samples were

verified by sequencing. PCR was carried out in a 5 μ l mixture that contained 10 ng DNA, 10 μ mol of each primer and AmpliTaq Gold 360 Master Mix (Applied Biosystems). The PCR primer sequences are listed in Table 1. Amplifications were performed using a touchdown PCR protocol with the following cycling conditions: 95°C for 5 min, followed by 20 cycles of 94°C for 15 s, 65°C to 55°C for 30 s and 72°C for 30 s, followed by 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. The PCR products were purified with the AMPure PCR purification system (Agencourt, Beverly, MA, USA). Sequencing reactions were carried out in a 10 μ l volume that contained 6 μ l PCR product, 0.25 μ l BDT (v 3.1), 1.875 μ l Buffer ABI (5x) and 1.6 μ mol of each primer. The conditions used for the sequencing reaction were as follows: 96°C for 1 min, followed by 40 cycles of 96°C for 10 min, 50°C for 5 s, and 60°C for 2 min. The products of the sequence reaction were purified with the CleanSEQ dye-terminator removal kit (Agencourt) before being analyzed on a 3730 DNA analyzer (Applied Biosystems).

Quantification of allele-specific gene expression

Complimentary DNA (cDNA) synthesis

Total RNA was isolated from brain tissues using an RNeasy mini kit (Qiagen, Hilden, Germany). To ensure removal of gDNA, RNA samples were treated with DNase I (RNase-Free DNase Set, Qiagen, Valencia, CA). DNA and RNA concentrations were determined in the Nanodrop (ND-1000 Spectrophotometer, Thermo-Scientific, Wilmington, DE, USA). First-strand cDNA synthesis was performed with the TaqMan Reverse Transcription kit (Applied Biosystems) in a final volume of 20 μ l. The cDNA was stored at -20°C until use.

Quantitative TaqMan genotyping assay

The t-PA -7351C>T is located in a non-coding part of the gene and can thus not be used for the quantitative TaqMan analysis. However, we have shown that the t-PA -7351C>T SNP is in strong linkage disequilibrium with a coding SNP, the t-PA 20099T>C SNP (a D' value of

0.93) (1, 13). Thus the t-PA 20099T>C SNP was used as a marker of the t-PA -7351C>T SNP. Allele-specific gene expression differences were analyzed by the TaqMan approach (14), using the same primer and probe sets used for genotyping of the t-PA 20099T>C SNP, with the modification that the PCR was performed and recorded in the ABI PRISM[®] 7900HT Sequence Detection System (Applied Biosystems) and included either 10 ng of gDNA or 10 ng of cDNA. To correct for subtle differences in the amplification rates of the two alleles, a standard curve for each fluorophore was created by mixing genomic DNA from two homozygous individuals in different allelic ratios, *i.e.* 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. A linear regression line (VIC or FAM versus the allelic ratios) was created. The cDNA and gDNA allelic ratios of each heterozygous sample were then extrapolated from the standard curves. Assuming that the two alleles are present in equal amounts in gDNA (15), the cDNA ratios were adjusted for the mean gDNA allelic ratio. The same experimental and analytical conditions were used for the cDNA and gDNA samples. Each heterozygous cDNA sample and its corresponding gDNA sample were assayed in three separate reactions, and each experiment was repeated on two different occasions. Mean intra-assay coefficient of variation (CV) was 4.1%.

Cell culture

Native human astrocytes (Clonetics, Walkersville, MD) were cultured in astrocyte growth medium (Clonetics) that was supplemented with 3% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. The medium was refreshed every 2-3 days. The astrocyte cultures were >95% glial fibrillary acidic protein-positive. Human umbilical vein endothelial cells (HUVECs) were prepared and cultured as described previously (4). Human astrocytes and HUVECs were cultured for no more than six passages. NT2 neuronal-like cells (16) (kindly supplied by Paul Ekert, WEHI, Melbourne, Australia) were grown in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY) that was supplemented

with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 µl/ml).

Preparation of nuclear extracts and labeling of oligonucleotides

Nuclear extracts from astrocytes and NT2 cells were prepared from cultures at 80-90% confluency, as previously described (4) or using a nuclear/cytosol fractionation kit (BioVision Inc, Mountain View, CA). The sequences of the oligonucleotides used in the electrophoretic mobility shift assay (EMSA) are listed in Table 2. Labeling of HPLC-purified oligomers (100 ng), annealing, and oligomer processing and preparation for EMSAs were performed as previously described (4).

EMSA

EMSAs were performed essentially as described previously (4). In brief, approximately 10 µg of nuclear extracts in 4 µl of Osborne buffer D were preincubated on ice for 15 min with 1 µl poly[d(I-C)] and 3 µl of SMK buffer. The ³²P-labeled probe was then added, and incubated for an additional 15 min on ice, before being applied to a native 5% polyacrylamide gel. For competition and cross-competition titration experiments, nuclear extracts were incubated with unlabeled double-stranded t-PA -7351C or T oligomer (0.5-, 5-, 50- or 500-fold molar excess) for 15 min on ice prior to addition of the probe. As nonspecific competitors, unlabeled scrambled oligonucleotides with the same base-composition as that of the corresponding probes were used at a 500-fold molar excess. Supershift experiments were performed using antibodies directed against Sp1 (#sc-420), Sp2 (#sc-643), Sp3 (#sc-644), and Sp4 (#sc-645) (Santa Cruz Biotechnology, Santa Cruz, CA), and 2 µl of specific antibody (4 µg in total) were added to the nuclear extracts for 1 h after the addition of the labeled probe.

Chromatin immunoprecipitation (ChIP)

Astrocytes and HUVECs were treated with retinoic acid (RA; 10⁻⁶ M) and the PKC activator phorbol 12-myristate 13-acetate (PMA; 10⁻⁷ M) for 6 h before ChIP, to induce t-PA gene transcription through the t-PA enhancer and

promoter, respectively (17-20). Chromatin was cross-linked by the addition of 1% formaldehyde, followed by 10 min incubation at 37°C. Cells were washed with ice-cold phosphate-buffered saline containing proteinase inhibitors. Chromatin was sonicated using a VibraCell Sonicator (Chemical Instrument AB, Sollentuna, Sweden), and ChIP was performed according to the Upstate Biotechnology ChIP assay kit protocol (Upstate Biotechnology, Charlottesville, VA). After immunoprecipitation with rabbit anti-Sp1 antibody (#07-645; Upstate Biotechnology) or rabbit IgG (Upstate Biotechnology), the DNA was purified by ethanol precipitation. The input DNA was not subjected to immunoprecipitation. Two fragments were amplified using sequence-specific PCR (primers listed in Table 3). This included an enhancer fragment, encompassing the polymorphic Sp1 binding site (at position -7355 to -7346) which has been implicated in RA-mediated t-PA induction (4), and a promoter fragment, encompassing two Sp1 binding sites (at position -71 to -65 and -48 to -42) that are involved in PMA-mediated t-PA induction (19, 20). The cycling conditions were: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C (promoter fragment) or 64°C (enhancer fragment) for 30 s, 72°C for 30 s, and 72°C for 4 min. The PCR products were visualized by separation on a 2% TAE-agarose gel that contained ethidium bromide.

Statistical analysis

Values are presented as mean and standard error of the mean (SEM), and a *P*-value of ≤0.05 or less was considered statistically significant. Differences in the allelic expression ratios for the cDNAs from heterozygous individuals were tested using a one-sample *t*-test. Correlation analysis was performed with Spearman's test. The SPSS software for Macintosh (SPSS Inc., Chicago, IL) was used for all statistical calculations.

RESULTS

Allelic imbalance of t-PA gene expression in human brain tissue

Significantly higher amounts of t-PA mRNA transcripts were generated from chromosomes that harboured the wild-type t-PA -7351C allele as compared to the mutant T allele, both in the hippocampal and cortical brain tissue samples (mean \pm SEM C to T allelic ratios of 1.31 ± 0.05 , $P=0.010$, $n=12$, and 1.20 ± 0.03 , $P=0.017$, $n=12$, respectively) (Fig 1). There was a significant correlation between the C to T allelic ratio in the two brain regions ($P=0.014$). As expected, the C to T allelic ratios of the gDNA samples did not differ significantly from 1 (mean \pm SEM C to T allelic ratio of 0.98 ± 0.02 , $P=0.094$).

The t-PA -7351C>T SNP affects protein binding affinity

Using the EMSA technique, we have previously shown that the t-PA -7351C>T SNP affects nuclear protein binding affinity when using nuclear extracts from HUVECs (4). We also showed preliminary results of a similar protein binding pattern for nuclear extracts from NT2 cells (4). In the present study, differences in binding affinities between the -7351C and T alleles in nuclear extracts from human astrocytes and NT2 cells were investigated. The t-PA -7351C and -7351T probes produced similar migration patterns (Fig. 2A-D, lane 2). The two most prominent complexes (marked with arrows in Fig. 2) were specific, as competition for binding was observed following the inclusion of an unlabeled (cold) specific competitor with the identical sequence as the probe (Fig. 2A-D, lanes 3-6), whereas the inclusion of a 500-fold molar excess of nonspecific competitor did not result in competition for binding (Fig. 2A-D, lane 11). Cross-competition experiments with the C probe and titrated amounts of cold T competitor (Fig. 2A and C, lanes 7-10) and T probe versus cold C competitor (Fig. 2B and D, lanes 7-10) both indicated an approximately 10-fold greater protein binding affinity to the C as compared to the T allele variant. Similar differences in binding affinity between alleles were observed when nuclear extracts derived

from astrocytes and NT2 cells were used (Fig. 2A-D).

Sp1 and Sp3 bind to the polymorphic t-PA -7351 site

As this polymorphic site has previously been shown to bind Sp1 and Sp3 in nuclear extracts from HUVECs (4), antibodies directed against the Sp1-family of proteins were included in the supershift reactions. Using nuclear extracts from astrocytes, two specific DNA-protein complexes were observed in the control reaction (Fig. 3A, lane 1). Inclusion of the anti-Sp1 antibody resulted in a partial displacement of the slower migrating complex (Fig. 3A, lane 3). A slight weakening of this complex was observed after the addition of antibodies directed against Sp2 (Fig. 3A, lane 4) or Sp3 (Fig. 3A, lane 5). When the anti-Sp1 and anti-Sp3 antibodies were added simultaneously (Fig. 3A, lane 7), the complex was almost completely displaced, leaving only a less intense band, which probably represents the DNA-Sp2 complex. The faster migrating complex was almost completely supershifted following addition of the anti-Sp3 antibody. The anti-Sp4 antibody had no effect. Similar supershifted patterns were observed when nuclear extracts from NT2 cells were used, with the exception of an extra weak band between complex 1 and 2, that was supershifted by the Sp3 antibody (Fig. 3B, lane 4). Considering there are 4 isoforms of Sp3 (21), this band may represent an isoform not present in astrocytes. Inclusion of the anti-Sp1 antibody resulted in only a weak supershift (Fig. 3B). Therefore, additional sets of EMSAs were performed, and in most of the assays the Sp1 antibody produced a prominent supershift, and a representative result is shown in Figure 3C. Thus, Sp1 and Sp3 are confirmed to be the major transcription factors that bind to the -7351-site in nuclear extracts from both cell types, although the relative proportions of these two transcription factors and/or their isoforms seem to differ.

In vivo binding of Sp1 to the t-PA enhancer and promoter in human astrocytes and HUVECs

Using ChIP we found that an antibody directed against Sp1, but not the IgG antibody,

immunoprecipitated both the t-PA enhancer and the promoter elements in human astrocytes (Fig. 4), thereby indicating that Sp1 binds to these regions in the intact chromatin structure. Similar results were observed in HUVECs (data not shown).

DISCUSSION

In the present study, we have demonstrated that the t-PA -7351C>T enhancer SNP affects t-PA gene expression in hippocampal and cortical post-mortem brain tissue from human heterozygous subjects. By EMSA and ChIP we show that the polymorphism affects the binding of transcription factors Sp1 and Sp3, which likely serves as the molecular genetic explanation behind this allelic imbalance.

The wild-type C allele produced approximately 30% more t-PA mRNA transcripts than the mutant T allele in hippocampal brain tissue samples. A similar allele-specific effect was also observed for samples derived from the cortex. In the majority of individuals, the highest allelic expression ratio of t-PA was observed in the hippocampus, which is a region of prominent t-PA expression in the adult human brain (6). There was also a significant intra-subject correlation in the allelic expression ratios between the two different brain regions, which reduces the likelihood that the observed effect is due to random variation from sampling or measurement errors.

Our results showing an allele-specific imbalance of t-PA expression in the central nervous system are in line with our previous *in vitro* data demonstrating an effect of this polymorphism on endothelial t-PA gene expression (4, 5). In these studies we addressed the effect of this polymorphism on t-PA expression using transient transfections (4) and genotyped cells (5). There are some major limitations with these previous approaches. Firstly, in transient transfections, due to a lack of chromatin structure the regulatory element of interest is not in its normal genetic context, and secondly, when using cells divided into genotype groups,

expression may be affected by other factors than the genetic variation. Thus, the experimental design of the present study has a major advantage over that of our previous *in vitro* studies in that the relative expression of each allele is compared within the same sample, where one allele serves as an internal control for the other allele. Using this approach, we can control for confounding *trans*-acting and environmental factors, which otherwise create a high background, thereby allowing the detection of small differences in allelic expression levels. Therefore, in addition to demonstrating that the t-PA -7351C>T SNP affects the expression levels of t-PA mRNA in the human brain, the present results lend further support for a functional role for the t-PA -7351C>T SNP.

To determine whether the t-PA -7351C>T SNP affects binding of transcription factors in human brain-derived cells, as we have previously demonstrated in endothelial cells (4), EMSA experiments were performed. Our results demonstrate substantially reduced nuclear protein binding affinity to the probe that corresponds to the mutant T allele, as compared to that of the wild-type C allele, in nuclear extracts from both human astrocytes and NT2 cells. In line with our previous observations in endothelial cells (4), our cross-competition experiments using extracts from astrocytes and NT2 cells revealed that the binding affinity of nuclear proteins to the C allele variant was at least 10-fold higher than that observed with the T allele counterpart. Supershift experiments demonstrated that Sp1 and Sp3 are the two major transcription factors that bind to the polymorphic site in these cells. Therefore, our present and previous EMSA results indicate that all the cell types investigated, *i.e.* astrocytes, neuronal-like cells, and endothelial cells, contribute to the observed allelic expression of t-PA in human brain tissue.

Our previous transient transfection experiments have shown an allele-specific transcriptional effect of the t-PA -7351C>T SNP in response to treatment with RA (4), which acts through the multi-hormone responsive enhancer (18, 22). However, our further experiments on human

endothelial cells demonstrated an allele-specific effect also in response to stimuli that induce t-PA through the proximal promoter, *e.g.* PKC-activators (5). A common denominator for the effect of RA and PKC activators on t-PA expression is Sp1. Retinoic acid receptor and Sp1 functionally interact at the t-PA enhancer (4), while mutation of the two Sp1 sites in the proximal promoter has been reported to completely abolish the t-PA response to RA through the enhancer (23). Consequently, we have previously suggested that the allele-specific induction of t-PA involves an Sp1-mediated DNA-looping mechanism, which brings the distant t-PA enhancer into physical contact with the proximal promoter. The present results using ChIP support this hypothesis, as we show that Sp1 indeed binds to both the t-PA enhancer and the promoter region in the intact chromatin structure of human astrocytes and endothelial cells *in vivo*.

Regarding the putative biological significance of an allele-specific expression of t-PA in the human brain, it is reasonable to speculate that if the wild-type C allele confers an increased t-PA gene expression in response to factors involved in synaptic plasticity, this allele would be beneficial for learning and memory formation, as well as for functional recovery after cerebral ischemia. In contrast, high-level expression of t-PA may have deleterious effects in the acute phase of an ischemic event, as this could trigger proteolytic cascades and make neurons more vulnerable to excitotoxic damage. Therefore, although we have not detected any significant difference in allelic frequency between ischemic stroke patients and controls (24), the t-PA -7351C>T SNP may have an impact on the final infarct size following cerebral ischemia and/or for stroke recovery. It is thus of interest to study this SNP in relation to functional outcome after ischemic stroke. However, it is noteworthy that the mean allele-specific difference in t-PA expression was no greater than 30%, and that not all individuals showed an allele-specific expression of t-PA. However, the mean allele-specific effect observed in the present study is of similar magnitude to that previously reported for most SNPs of human brain-expressed genes

(25). Furthermore, while we, in light of our previous data, presume that the difference seen on the mRNA level produces a commensurate increase in t-PA protein levels (5, 17), localized increases are likely to be greater since t-PA can accumulate on cell surfaces via lysine binding sites and can also bind at high concentrations to dead or dying neurons following cerebral injury (26). It is also of note that we have shown that the allele-specific effect is potentiated when the level of t-PA gene expression is enhanced, *i.e.* following stimulation (4, 5). Thus, the allele-specific difference in t-PA expression is likely to be greater than 30% when the t-PA gene is induced, *e.g.* during cerebral ischemia, brain trauma or seizures.

The present study has some limitations. First, the present approach does not allow us to exclude the possible involvement of other polymorphisms that may be in linkage disequilibrium with the t-PA -7351 SNP. However, when we previously re-sequenced the enhancer, proximal promoter, exons, splice sites and the 3' untranslated region of the t-PA gene in subjects with contrasting endothelial t-PA release rates, we did not identify any other putative regulatory SNPs (1), which makes this scenario less likely. Second, the coding marker SNP is not in complete linkage disequilibrium with the t-PA enhancer SNP due to the fact that some subjects homozygous for one of the SNPs are heterozygous for the other (1, 13). In the present study samples heterozygous for both SNPs were used, and it is thus unlikely that they do not have the expected haplotypes. Third, this study investigates the allele-specific effect on t-PA mRNA levels and not protein. However, our previous work on human endothelial cells and astrocytes show that t-PA mRNA and protein are regulated in parallel in response to a wide array of stimuli (5, 17). Still, it cannot be excluded that this is not the case in other cell types.

In conclusion, we show for the first time that the t-PA enhancer -7351C>T SNP affects the expression of t-PA in human brain tissue. An allele-specific expression of t-PA in the brain may have clinical implications for

neuropathologic conditions associated with enhanced t-PA levels, such as in the acute phase cerebral ischemia, and also for stroke recovery.

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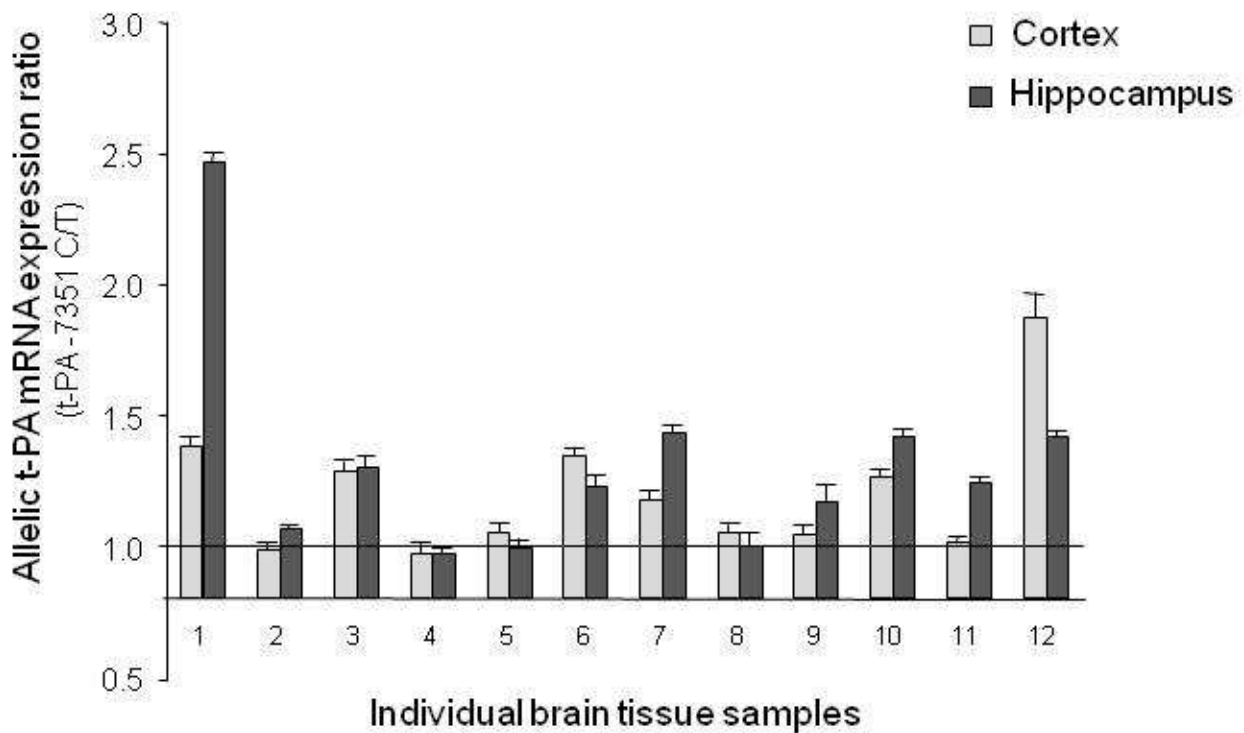


Figure 1. Individual allelic ratios of t-PA gene expression in human brain tissue.

Cortical and hippocampal samples heterozygous for the coding t-PA 20099T>C and the t-PA -7351C>T SNPs were investigated using the quantitative TaqMan assay. The horizontal line represents a ratio of 1, assuming equivalent expression of each allele. Each bar represents the cDNA allelic ratio (adjusted for the mean gDNA allele ratio) for each individual brain tissue sample. All samples were assayed in three separate reactions, and each experiment was repeated on two separate occasions.

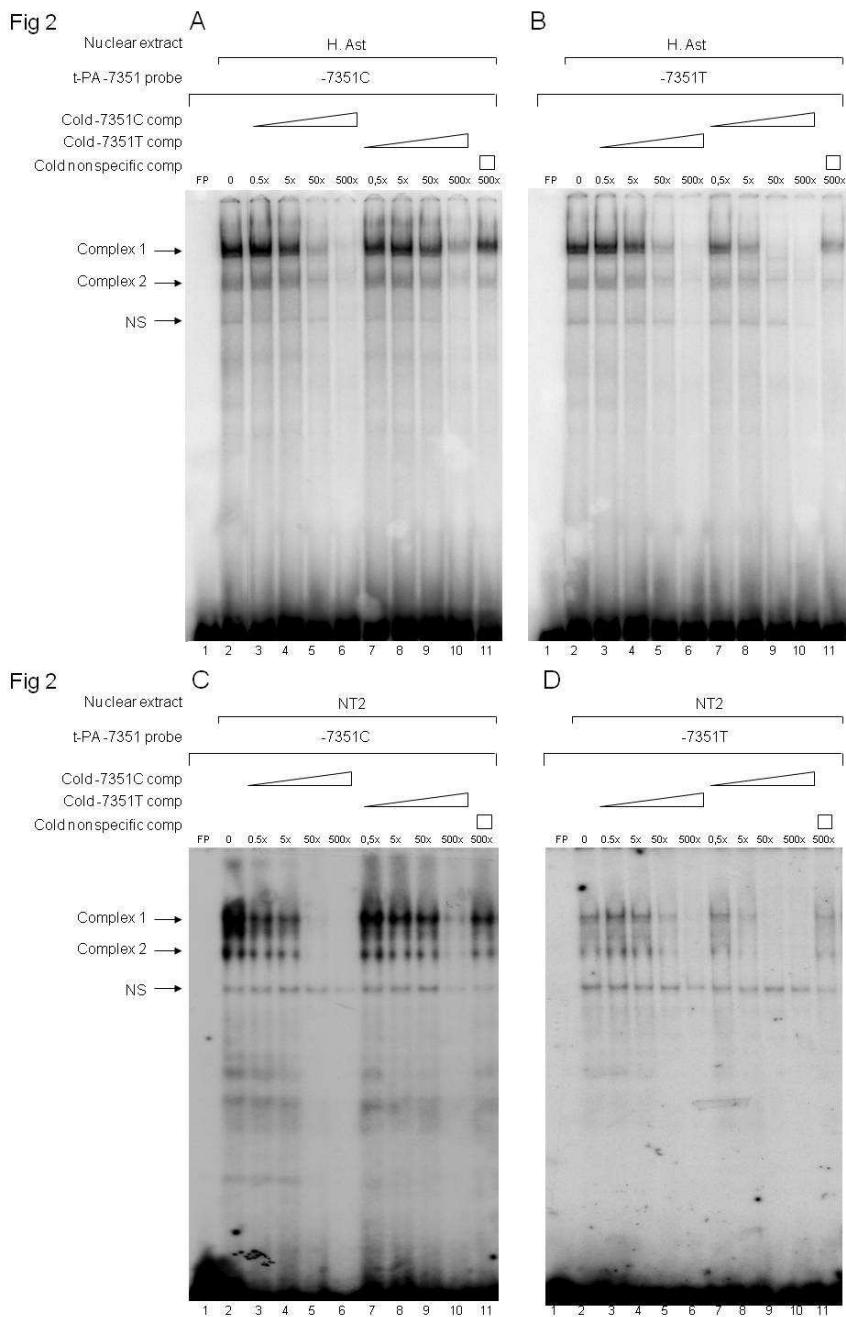


Figure 2. The t-PA -7351C>T SNP affects protein binding affinity.

(A-D) Competition and cross-competition EMSAs were performed using ^{32}P -labeled -7351C or T probes and unlabeled competitor oligomers. Nuclear extracts (10 μg) from human astrocytes (H. Ast, A-B) or NT2 cells (C-D) were included in the reactions as indicated, except for that in lane 1 (FP, free probe). Lane 2, Migration patterns produced in the absence of competitor. Lanes 3-6, Increasing concentrations of unlabeled oligomer of identical sequences to the labeled counterpart (self-competition, 0.1, 1, 10 or 100 ng, corresponding to approximately 0.5-, 5-, 50- or 500-fold molar excess, respectively). Lanes 7-10, Increasing concentrations of unlabeled variant oligomer (cross-competition, approximately 0.5-, 5-, 50- or 500-fold molar excess). Lane 11, Unlabeled nonspecific competitor (500-fold molar excess). The arrows on the left indicate the two specific complexes; NS, nonspecific. The results indicate a 10-fold higher protein binding affinity for the t-PA -7351C allele compared with the T allele variant.

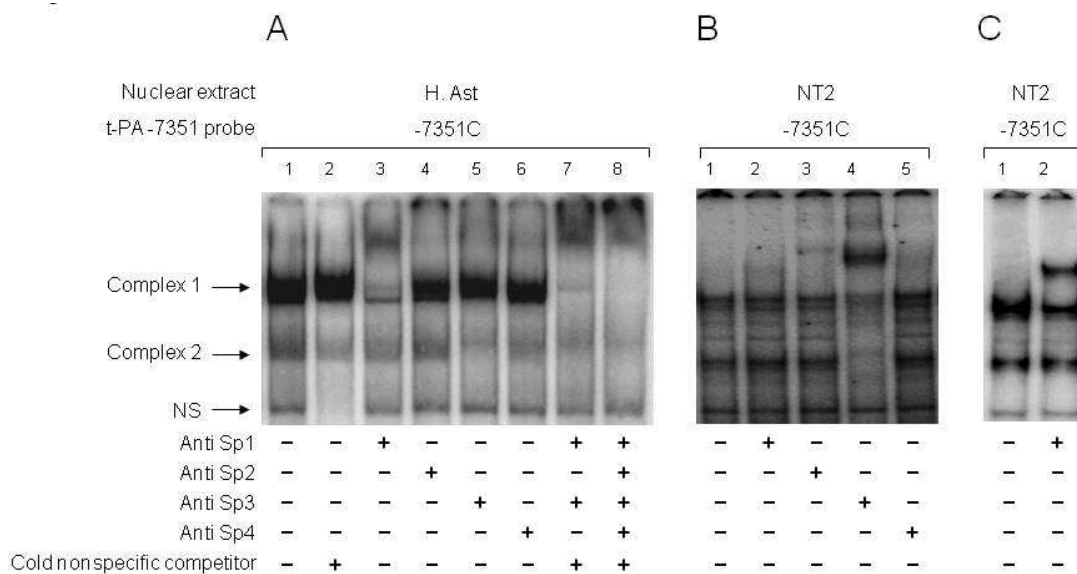


Figure 3. Sp1 and Sp3 bind to the t-PA -7351-site.

Nuclear extracts from human astrocytes (H. Ast; A) or NT2-cells (B) were incubated with the wild-type -7351C probe with or without the addition of antibodies directed against Sp1, Sp2, Sp3, or Sp4, as indicated. The EMSA was repeated with nuclear extracts from NT2-cells and antibodies directed against Sp1 (C). NS, nonspecific.

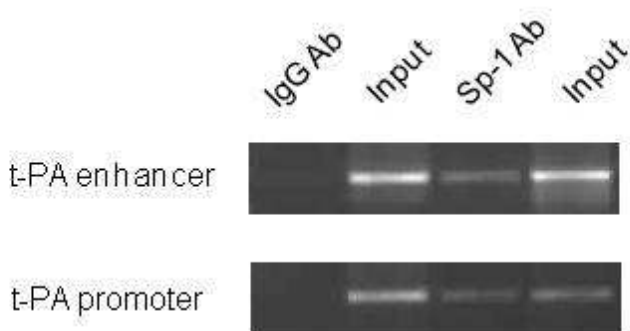


Figure 4. In vivo binding of Sp1 to the t-PA enhancer and promoter in human astrocytes.

Prior to chromatin immunoprecipitation (ChIP) experiments, human astrocytes (H. Ast) were treated with RA (10^{-6} M) and PMA (10^{-7} M) for 6 h. After immunoprecipitation with the anti-Sp1 antibody, DNA was purified, and sequence-specific PCR was performed using primer sets that cover the Sp1 sites in the t-PA enhancer and promoter, as indicated in Table 3. Anti-IgG antibodies were used as a negative control. ‘Input’ denotes non-immunoprecipitated DNA. A representative result from two separate ChIP experiments is shown.

Table 1. Sequences of primer pairs used for the DNA sequencing.

Primer sequence (5' - 3')	Amplicon length (bp)
t-PA -7351C>T SNP FP: GTGATCTCATTGCCGAGGTG RP: CTCCAGCCTGGGAGACAGAG	170 bp
t-PA 20099T>C SNP FP: GGCTGAATGAAAGGGGACTC RP: AGCTTCTCACAGGGGAATC	232 bp

FP denotes forward primer, RP reverse primer, bp base pairs, SNP single-nucleotide polymorphism.

Table 2. Sequences of oligonucleotides used in EMSA.

Name	Location	Sequence (5'-3')*
-7351C	-7363/C/-7339	CCCAAAGGAGCCCGCCCCAGACACA
-7351T	-7363/T/-7339	CCCAAAGGAGCCTGCCCCAGACACA
Scrambled -7351	-7363/scrambled/-7339	CGCAGACACCAGCCACGACGACCAC

The polymorphic nucleotide is indicated in bold type and the Sp1 binding sequence is underlined.

*Only forward sense strand is shown.

Table 3. Sequences of primer pairs used in ChIP experiments.

Primer sequence (5' - 3')	Amplicon length (bp)
t-PA enhancer FP: 5'-ATTGCCGAGGTGAATAGGG-3' RP: 5'-CCAGCCTGGGAGACAGAGTA-3'	161 bp
t-PA promoter FP: 5'-TGTCATCACAGGGTCCTGAA-3' RP: 5'-TAAAGCAGGGGAGGAAGTT-3'	151 bp

FP denotes forward primer, RP reverse primer, bp base pairs.