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Tannic acid facilitates expression of the polypyrimidine tract binding protein and alleviates deleterious inclusion of *CHRNA1* exon P3A due to an hnRNP H-disrupting mutation in congenital myasthenic syndrome

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We recently reported that the intronic splice-site mutation IVS3-8G>A of *CHRNA1* that encodes the muscle nicotinic acetylcholine receptor α subunit disrupts binding of a splicing repressor, hnRNP H. This, in turn, results in exclusive inclusion of the downstream exon P3A. The P3A(+) transcript encodes a non-functional α subunit that comprises 50% of the transcripts in normal human skeletal muscle, but its functional significance remains undetermined. In an effort to search for a potential therapy, we screened off-label effects of 960 bioactive chemical compounds and found that tannic acid ameliorates the aberrant splicing due to IVS3-8G>A but without altering the expression of hnRNP H. Therefore, we searched for another splicing *trans*-factor. We found that the polypyrimidine tract binding protein (PTB) binds close to the 3′ end of *CHRNA1* intron 3, that PTB induces skipping of exon P3A and that tannic acid increases the expression of PTB in a dose-dependent manner. Deletion assays of the PTB promoter region revealed that the tannic acid-responsive element is between positions −232 and −74 from the translation initiation site. These observations open the door to the discovery of novel therapies based on PTB overexpression and to detecting possible untoward effects of the overexpression.

INTRODUCTION

Tannins are plant-derived polyphenols and are divided into two groups of hydrolyzable and condensed tannins (proanthocyanidins) (1). Hydrolyzable tannins are derivatives of gallic acid (3,4,5-trihydroxyl benzoic acid) in which a variable number of gallic acids are esterified to a core phenol. The simplest hydrolyzable tannins are gallotannins that are polygalloyl esters of glucose. The prototypical gallotannin is tannic acid or 1,2,3,4,6-penta-*O*-β-D-glucose (CAS 1401-55-4, $C_{76}H_{52}O_{46}$) comprised five galloyl esters and a glucose. Commercially available tannic acid additionally includes gallotannins with a variable number of galloyl esters. Tannins are rich in nuts, red wine, tea and coffee, but hydrolyzable tannins including tannic acid are not rich in tea (2,3) or red wine (4).

Polypyrimidine tract binding protein (PTB), encoded by *PTBP1*, is multifunctional, which participates in pre-mRNA splicing (5,6), internal ribosome entry site (IRES)-mediated translation (7), mRNA polyadenylation (8), RNA localization (9) and mRNA stabilization (10). PTB carries four RNA recognition motifs and each motif binds to a CU-rich element (11). In terms of a splicing *trans*-factor, CU-rich splicing *cis*-elements are located upstream and/or downstream of alternatively spliced exons. PTB bound to the 3′ splice site directly

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competes with binding of U2AF65 that recognizes the polypyrimidine tract (12–15). Alternatively, PTBs bound to the flanking introns repress splicing of the exon (16–20).

We recently identified a pathogenic intronic mutation in a congenital myasthenic syndrome, IVS3-8G>A of *CHRNA1* that encodes the muscle nicotinic acetylcholine receptor α subunit (21). We reported that IVS3-8G>A disrupts binding of a splicing repressor, hnRNP H, and causes exclusive inclusion of the downstream exon P3A. The P3A(+) transcript encodes a non-functional α subunit and comprises 50% of the transcripts in normal human skeletal muscle, but its functional significance remains unknown (22–24).

Here, we report that PTB binds to the polypyrimidine tract of *CHRNA1* intron 3 immediately upstream of the hnRNP H-binding site, and silences recognition of exon P3A in cooperation with hnRNP H. IVS3-8G>A, however, has no effect on the binding of PTB. Screening of chemical compounds revealed that tannic acid mitigates exclusive inclusion of exon P3A due to IVS3-8G>A by activating the promoter region of *PTBP1* and causes a dose-dependent increase of *PTBP1* mRNA.

RESULTS

Screening of off-label effects of 960 chemical compounds

In an effort to search for a potential therapeutic modality for aberrant inclusion of *CHRNA1* exon P3A due to IVS3-8G>A, we screened for off-label effects of 960 chemical compounds (GenPlus, MicroSource Discovery Systems). Most were FDA-approved drugs, and some were bioactive compounds or natural products. We constructed a chimeric minigene carrying the mutant exon P3A with its flanking introns in the middle of the firefly luciferase cDNA (pcDNA3.1-Luc-mtP3A) (Fig. 1). Skipping of exon P3A should generate luminescence, whereas its inclusion should not. After the first round of screening in duplicate, we narrowed the list to 80 compounds and performed the second round of screening in duplicate. Among the 80 compounds, 24 consistently exhibited beneficial effects (Table 1). We observed no shared feature among the 24 compounds. We hereafter focused on tannic acid, because tannic acid was expected to have less toxicity or untoward effects than the other compounds.

Tannic acid induces skipping of *CHRNA1* exon P3A in a dose-dependent manner

We next examined a dose-dependent effect of tannic acid. We added 0, 1, 10 and 100 μM of tannic acid to transfected HEK293T cells and examined its effect on splicing of exon P3A by real-time RT–PCR. Tannic acid exhibited no effect on splicing of wild-type exon P3A, whereas 100 μM tannic acid minimally but significantly increased the ratio of the P3A(−)-transcript (Fig. 2A and B). Lack of an effect on wild-type exon P3A may represent that the wild-type exon P3A is mostly spliced out even in the absence of tannic acid and that we cannot observe an effect of tannic acid in the wild-type construct.

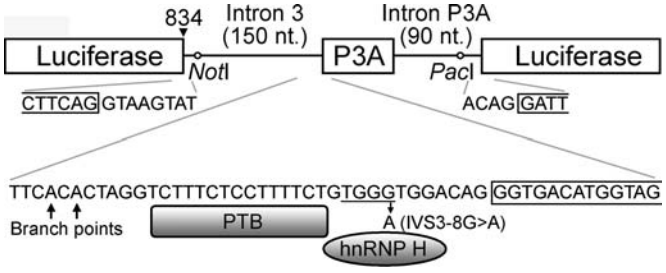

Figure 1. Chimeric *CHRNA1* exon P3A minigene for screening of chemical compounds. *CHRNA1* exon P3A and its flanking introns are inserted after position 834 (arrowhead) of the firefly luciferase cDNA. Skipping of exon P3A should generate an intact luciferase molecule, whereas inclusion of exon P3A should abrogate it. The hnRNP H-binding 'TGGG' motif is underlined.

Table 1. Twenty-four best compounds with averaged normalized relative luciferase activity ≥1.20 and CV% <0.20 ($n = 4$)

Compounds	Mean	SD
Tomatine	1.50	0.16
Tannic acid	1.47	0.20
Troxerutin	1.44	0.22
Camptothecin	1.43	0.12
Mexiletine hydrochloride	1.43	0.21
Halcinonide	1.42	0.07
Clobetasol propionate	1.40	0.21
6α-Methylprednisolone acetate	1.40	0.27
Flurandrenolide	1.33	0.19
Oxethazaine	1.31	0.11
Ketorolac tromethamine	1.28	0.09
Avocadynone acetate	1.27	0.10
Medrysone	1.26	0.19
Propafenone hydrochloride	1.26	0.10
Etodolac	1.24	0.11
Propylthiouracil	1.23	0.06
Pergolide mesylate	1.23	0.03
Hydrocortisone butyrate	1.22	0.10
5-Azacytidine	1.22	0.05
Alexidine hydrochloride	1.22	0.10
Fluconazole	1.21	0.10
Methoxyamine hydrochloride	1.21	0.09
N-Formylmethionylalanine	1.21	0.11
Dinitolmide	1.20	0.08

Among the 80 compounds that we employed for the second round of screening, 24 compounds consistently demonstrated beneficial responses. The values are the global mean and SD of the first ($n = 2$) and second ($n = 2$) rounds of screening.

Tannic acid has no effect on the expression of hnRNP H

We next searched for the molecular basis of the tannic acid-mediated amelioration of the aberrant inclusion of exon P3A. We previously reported that recognition of exon P3A is down-regulated by binding of hnRNP H to 'UGGG' at positions −11 to −8 of intron 3 (Fig. 1), and IVS3-8G>A attenuates its bindings ∼100-fold (21). We hypothesized that tannic acid increases the expression of hnRNP H and subsequently silences the recognition of exon P3A. We therefore analyzed the effect of tannic acid on mRNA levels of hnRNP H in HEK293T cells by real-time RT–PCR, but found no effect (data not shown).

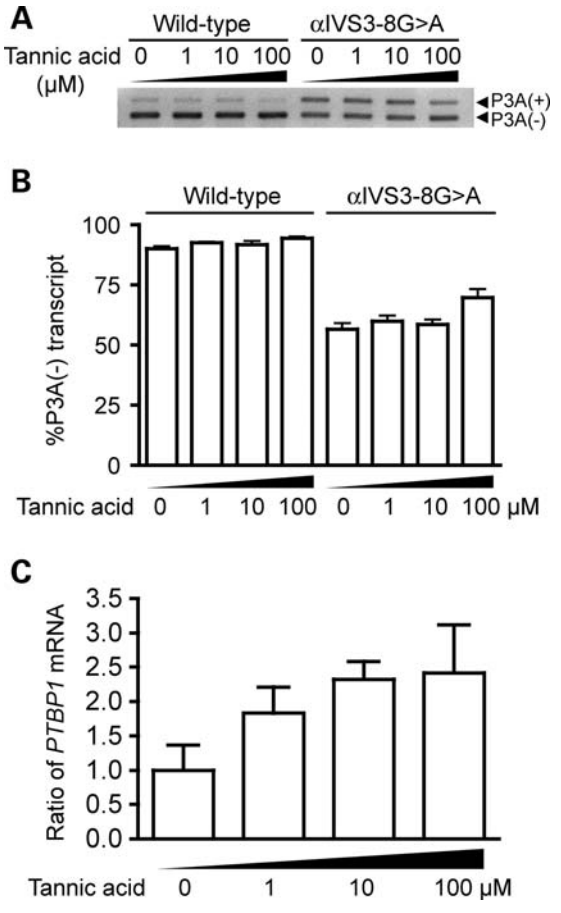

Figure 2. Tannic acid alleviates aberrant inclusion of *CHRNA1* exon P3A due to IVS3-8G>A by facilitating the expression of *PTBP1*. (**A**) RT–PCR of wild-type and mutant *CHRNA1* minigenes in HEK293T cells with increasing concentrations of tannic acid. (**B**) Real-time RT–PCR to quantify the ratio of P3A-skipped transcript arising from wild-type and mutant *CHRNA1* minigenes in HEK293T cells with increasing concentrations of tannic acid. The ratios are represented by the mean and SD. For the mutant minigene, 100 μM tannic acid increases the ratio of the P3A(−)-transcript. (**C**) Real-time RT–PCR to demonstrate a dose-dependent increase of *PTBP1* mRNA by tannic acid. The ratios are normalized to that in the absence of tannic acid. Means and SD are represented.

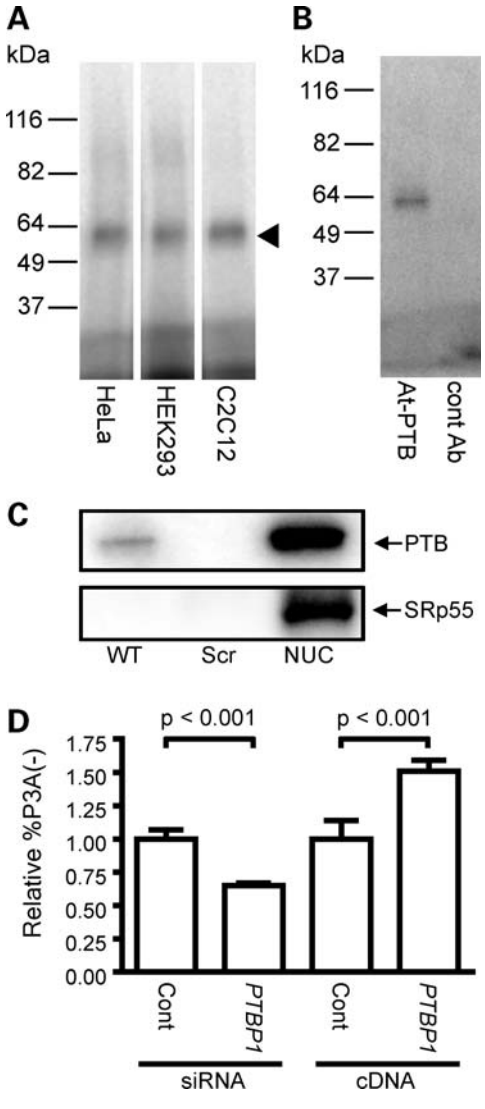

Figure 3. PTB binds close to the 3′ splice site of exon P3A and induces skipping of exon P3A. (**A**) ^{32}P-labeled RNA probe is incubated with nuclear extracts of indicated cells, followed by UV cross-linking and SDS–PAGE. Autoradiographs demonstrate a ∼60-kDa molecule (arrowhead). (**B**) ^{32}P-labeled RNA probe is incubated with nuclear extracts of HEK293T cells followed by UV cross-linking and RNase digestion. The bound molecules are immunoprecipitated with anti-PTB (At-PTB) or control antibody (cont Ab), and separated on SDS–PAGE. Only anti-PTB antibody precipitates a ∼60-kDa molecule. (**C**) Western blotting with anti-PTB or anti-SRp55 antibody of affinity purified HEK293T nuclear extract. Affinity purification also demonstrates the binding of PTB to the wild-type (WT) intron 3, but not to the scrambled RNA probe (Scr). NUC represents a lane loaded with nascent nuclear extract. (**D**) Real-time RT–PCR analysis of HeLa cells transfected with the wild-type minigene along with the indicated siRNA or cDNA. The %P3A values are normalized to those of control values. Down-regulation of *PTBP1* enhances, whereas overexpression of *PTBP1* silences the recognition of exon P3A. Bars represent the mean and SD of three experiments.

PTB binds to *CHRNA1* intron 3 and silences splicing of exon P3A

We next looked for a new splicing *trans*-factor that regulates recognition of exon P3A. UV cross-linking of nuclear extracts of HeLa, HEK293T and C2C12 cells to wild-type *CHRNA1* intron 3 disclosed a ∼60 kDa fragment (Fig. 3A). Its molecular weight prompted us to hypothesize that the bound molecule was PTB. Immunoprecipitation of the UV-cross-linked nuclear extract (Fig. 3B) and western blotting of affinity purified molecules (Fig. 3C) indeed identified that the bound molecule was PTB. Additionally, siRNA-mediated down-regulation of PTB enhanced recognition of exon P3A, whereas overexpression of PTB silenced it (Fig. 3D), which supports the notion that PTB binds to intron 3 and induces skipping of exon P3A.

Surface plasmon resonance analysis demonstrated that IVS3-8G>A had no effect on the binding of PTB (data not shown). As we previously found that hnRNP H binds close to the 3′ end of intron 3 and silences splicing of exon P3A (21), we asked if hnRNP H and PTB interact with each other. Anti-hnRNP H antibody did not co-immunoprecipitate PTB in a nuclear extract of HEK293T cells in the absence of substrate mRNA (data not shown). To summarize, splicing of exon P3A is down-regulated by both hnRNP H and PTB,

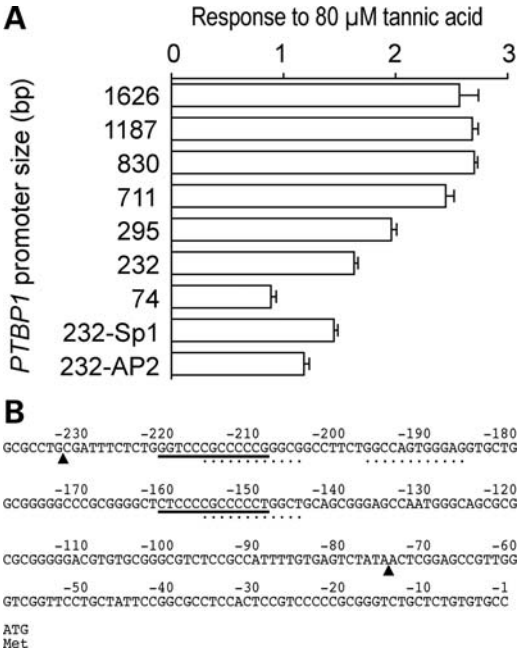

Figure 4. Scanning deletion analysis of the *PTBP1* promoter region. (**A**) Luciferase promoter assays to demonstrate responses to 80 μM tannic acid. Relative luciferase activities are first calculated by dividing the firefly luciferase activity by the cotransfected Renilla luciferase activity. The calculated relative luciferase activity in the presence of 80 μM tannic acid is then divided by that in the absence of tannic acid to estimate the response. Means and SD ($n = 4$) are represented. The sizes of the *PTBP1* promoter region before the translation initiation site are indicated. '232-Sp1' and '232-AP2' indicate a 232-nucleotide construct lacking two Sp1 elements [underlined in (B)] or three AP2 elements [indicated by dots in (B)], respectively. (**B**) *PTBP1* promoter region from position −239 to +3. Arrowheads point to the 5′ ends of the 232-nucleotide and 74-nucleotide constructs in (A).

and IVS3-8G>A only compromises binding of hnRNP H but not of PTB.

Tannic acid induces the expression of PTB by activating its promoter region

We next asked if tannic acid exerts its effect by inducing expression of PTB, and found that tannic acid increased the expression of *PTBP1* mRNA in a dose-dependent manner (Fig. 2C). We further asked how *PTBP1* is transcriptionally induced by tannic acid, and examined the effect of tannic acid on the *PTBP1* promoter region using the luciferase reporter assay (Fig. 4). We measured responses to 80 μM of tannic acid in constructs harboring 1626, 1187, 830, 711, 295, 232 and 74 nucleotides upstream of the translation initiation site of the human *PTBP1* gene. Tannic acid increases luciferase activity ∼2.5-fold when the promoter length is 711 nucleotides or longer (Fig. 4A). This degree of response is similar to that observed with the native *PTBP1* gene (Fig. 2C). The 295- and 232-nucleotide constructs also respond to tannic acid but to a lesser extent. The 74-nucleotide construct exhibited no response to tannic acid. We then asked why the 232-nucleotide construct responds to tannic acid, whereas the 74-nucleotide construct does not. We deleted two putative Sp1 sites and three putative AP2 sites from the 232-nucleotide construct (Fig. 4B). Both deletion constructs minimally reduced the responses to tannic acid but not to the level of the 74-nucleotide construct. Real-time RT–PCR also revealed that 1, 10 and 100 μM of tannic acid did not increase the expression level of the *SP1* gene encoding Sp1 or the *TFAP2A* gene encoding AP-2α (data not shown). To summarize, tannic acid-responsive *cis*-element likely resides between positions 232 and 74 of *PTBP1*, but the exact transcriptional *cis*-element remains unidentified.

DISCUSSION

PTB silences splicing of exon P3A

We identified that PTB binds close to the 3′ end of intron 3 and silences splicing of the downstream exon P3A. In our previous studies, Syproruby staining of the affinity purified nuclear extract disclosed binding of hnRNP H but not of PTB (21). In our current studies, UV cross-linking of the nuclear extract to a radiolabeled probe detected binding of PTB but not of hnRNP H. Binding of hnRNP H and PTB, as well as their silencing effects on splicing of exon P3A, have been confirmed by several other methods for both factors. For RNA-binding molecules, we frequently observe this kind of inconsistency. This is likely because an RNA-binding molecule recognizes the tertiary structure of RNA (25,26), and the tertiary structure likely differs depending on the size, position and the attached molecule of the RNA probe employed for the binding assay.

Although both PTB and hnRNP H bind to the similar intronic region and both silence splicing of exon P3A, we failed to detect a direct interaction of the two molecules. Markovtsov *et al.* (27) similarly found that hnRNP H/F and PTB/nPTB cooperatively enhance binding to a splicing *cis*-element of the c-*src* N1 exon, but did not demonstrate a direct protein–protein interaction. Further analysis is required to prove if the two molecules silence splicing of exon P3A cooperatively or independently.

Off-label effects of FDA-approved drugs

FDA-approved drugs and bioactive compounds have been employed for screening of off-label effects for neurological diseases, because the identified compounds can be readily applied to clinical practice (28,29). The identified off-label compounds include digitoxin, nerifolin, peruvoside and suloctidil for spinobulbar muscular atrophy (30); β-lactam antibiotics for amyotrophic lateral sclerosis and brain ischemia (31); and dorsomorphin for fibrodysplasia ossificans progressiva (32). Additionally, kinetin alleviates aberrant splicing of *IKBKAP* in familial dysautonomia (33). (−)-Epigallocatechin gallate also down-regulates the expression of hnRNP A2/B1 and normalizes the aberrant splicing of mutant *IKBKAP* (34). Similarly, sodium valproate corrects splicing of *SMN2* by increasing the expression of a splicing *trans*-factor, Htra2-β1, and also increases the expression of *SMN2* (35,36). Sodium valproate is a histone deacetylase (HDAC) inhibitor and activates a variety of genes (37). We identified that tannic acid works on the promoter region of *PTBP1* and facilitates its expression in a dose-dependent manner. Tannic acid, however, is unlikely to act directly on the promoter

region of *PTBP1*, but rather modulates unidentified cellular processes that culminate in the activation of the *PTBP1* promoter region. The exact activation mechanisms still remain to be elucidated.

Therapeutic effects of tannic acid in other diseases

Tannic acid has protein-aggregating, astringent, antimicrobial, anti-oxidant, anti-mutagenic and anti-proliferative properties (38). For example, the constringing action of tannic acid on mucous tissues is exploited to treat diarrhea in medical practice. Tannic acid inhibits the 3C-like protease encoded by the severe acute respiratory syndrome (SARS) coronavirus with IC_{50} of 3 μM (39). Tannic acid has anti-oxidant properties (40) and is protective against toxin-induced skin tumors (41). Tannic acid similarly suppresses skin tumor promotion induced by UV-B radiation by 70% in mice (42). Tannic acid is protective against spontaneous liver tumor development in mice by 87% (43). In addition, tannic acid raises the survival rate of mice bearing syngeneic tumors by 30% (44). Apoptosis-inducing activity of tannic acid is mediated by suppression of protease activity in tumor cells (45), reduction of ERK1, 2 phosphorylation by a cyclic adenosine 5′,3′-monophosphate-protein kinase A-dependent pathway (46) and also down-regulation of the superoxide dismutase (47). Up-regulation of PTB by tannic acid, however, has never been reported to our knowledge.

Potential clinical application of tannic acid

The LD_{50} of orally administered tannic acid is as high as 2260 mg/kg in rat (48). Rabbit has a higher LD_{50} of 5000 mg/kg (49). The human threshold, the tolerable daily intake (TDI), is extrapolated from the rodent threshold, the no observed adverse effect level (NOAEL), by dividing the NOAEL with the uncertainty factor (UF) (50). Assuming that the default UF representing the interspecies variation is 10 (51,52), the NOAELs of 2260 and 5000 mg/kg should give rise to the TDIs of 13.6 and 30.0 g of tannic acid for a 60 kg human, respectively. If these doses were completely absorbed and evenly dissolved in 60% body water, the tannic acid concentrations in body water would become 221 and 490 μM in human.

The adverse effects of tannic acid have not been thoroughly investigated in humans. Koide *et al.* (44) report that 8750 mg/kg/day of dietary tannic acid for 35 days reduces the weight gain in mice, but causes no pathological changes in kidney, liver or lung. This would be equivalent to 52.5 g/day for a 60 kg human according to the NOAEL approach described above. Afsana *et al.* (53) report that 20 g/kg/day of dietary tannic acid for 3 weeks has no effect on the body weight gain or food intake in rats, but 10 g/kg/day or more of tannic acid for 3 weeks induces anemia by reducing the iron absorption, which would be equivalent to 60 g/day for a 60 kg human according to the NOAEL approach.

Clinically available tannic acid is albumin tannate (CAS 9006-52-4) composed of 50% tannic acid and 50% albumin. In clinical practice, 1–2 g of albumin tannate is given for diarrhea which is equivalent to 0.5–1 g of tannic acid. If these doses were completely absorbed and evenly dissolved in 60% body water in a 60 kg human, the tannic acid concentrations in body water would become 8–16 μM. Our studies using HEK293T cells demonstrate that even 1 μM of tannic acid up-regulates the expression of *PTBP1* but 100 μM of tannic acid is required to attenuate the aberrant splicing of exon P3A. As the absorption efficiency and elimination rate of dietary tannic acid has not been determined in humans, we cannot predict how much tannic acid would be required to treat patients, and whether it is toxic or not.

Diseases and physiological states in which the up-regulation of PTB is beneficial or detrimental

Raponi *et al.* (54) recently reported that PTB recognizes two splicing *cis*-elements in the pseudoexon of *NF1* activated by an A-to-G mutation 270 nucleotides upstream of the nascent intron/exon boundary. Up-regulation of PTB ameliorated the aberrant inclusion of the pseudoexon. Fred and Welsh (10) found that PTB stabilizes the preproinsulin mRNA by binding to its 3′-UTR and participate in the glucose-induced increase of preproinsulin mRNA. In both of these conditions, up-regulation of PTB by tannic acid might be beneficial.

On the other hand, PTB is overexpressed in tumor cells and promotes cell growth (55,56), although PTB itself does not transform cells (57). Viruses exploit PTB to facilitate the IRES-mediated translation initiation (58,59). PTB also silences the splicing of a cassette exon of the neuronal PTB (nPTB) and down-regulates the expression of nPTB in non-neuronal cells. In neuronal cells, microRNA miR-124 down-regulates the translation of PTB and hence up-regulates the expression of nPTB (60). In these conditions, up-regulation of PTB by tannic acid could to be detrimental.

Although tannic acid in the form of albumin tannate has long been used as a safe anti-diarrhea medication, our observations that tannic acid causes overexpression of PTB now opens the door to the discovery of beneficial as well as possible detrimental effects of PTB overexpression.

MATERIALS AND METHODS

Firefly luciferase minigene carrying chimeric *CHRNA1* exon P3A

We screened for off-label effects of 960 chemical compounds using chimeric minigenes carrying the firefly luciferase cDNA (Fig. 1). We first inserted the luciferase gene derived from the pGL3-Basic vector (Promega) into the CMV-based mammalian expression vector, pcDNA3.1 (Invitrogen), to make pcDNA3.1-Luc. Using the megaprimer-based site-directed mutagenesis method (61), we inserted a chimeric intron of 5′-GTAAGTA TCAAGCGGCCGCNNNNNTTAATTAATCTTACTGACAT CCACTTTGCCTTTCTCTCCACAG-3′ at position 834 of the luciferase cDNA, where position +1 represents the translation initiation site. The underlined are *Not*I and *Pac*I restriction sites. We next inserted *CHRNA1* exon P3A with its flanking introns of 150 and 90 nucleotides using the *Not*I and *Pac*I sites to make pcDNA3.1-Luc-wtP3A. With this construct, the nonsense-mediated mRNA decay is not provoked even when exon P3A is spliced in. We then engineered IVS3-8G>A

using the QuikChange site-directed mutagenesis kit (Stratagene), and made pcDNA3.1-Luc-mtP3A.

For all clones, the presence of the desired mutation and the absence of artifacts was determined by sequencing the entire insert with the CEQ8000 DNA analysis system (Beckman Coulter).

Screening of off-label effects of 960 chemical compounds

HEK293T cells were maintained in the Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma-Aldrich). At ~50% confluency in a 75 cm^2 flask, we transfected 6 μg of either pcDNA3.1-Luc or pcDNA3.1-Luc-mtP3A along with 2 μg of phRL-SV40 encoding the Renilla luciferase (Promega) using 24 μl of the FuGENE6 transfection reagent (Roche). Sixteen hours after transfection, we removed the transfection reagent and split the cells into three 96-well plates containing 10 μM of each chemical compound (GenPlus) and 2% DMSO. After growing the cells for 24 h, we measured the firefly and Renilla luciferase activities with the Dual luciferase reporter assay system (Promega) using the Luminoskan Ascent luminometer (Thermo Scientific).

We first assayed effects of each compound on the transcription activity and on stabilities of the luciferase mRNA and protein using pcDNA3.1-Luc that codes for the nascent firefly luciferase and harbors no chimeric exon. We normalized the firefly luciferase activities with the cotransfected Renilla luciferase activities. We repeated the experiments five times for each compound. The coefficients of variation (CV%) of five assays were 9.4 ± 4.0% (mean ± SD) for all the 960 compounds. We next performed duplicate assays to determine the effect of each compound on splicing of *CHRNA1* exon P3A harboring IVS3-8G>A (pcDNA3.1-Luc-mtP3A) by measuring the relative luciferase activities in the presence of each compound. To estimate the effect of each compound on splicing of exon P3A, the relative luciferase activity of pcDNA3.1-Luc-mtP3A was normalized by dividing it by that of pcDNA3.1-Luc. The mean and SD of the normalized relative luciferase activities of 960 compounds was 1.05 ± 0.20, whereas the mean and SD of 192 negative controls without any compound was 1.00 ± 0.11. Eighty most effective compounds with normalized relative luciferase activities of 1.21 or higher (mean and SD, 1.36 ± 0.40) were subjected to a second round of screening in duplicate. The normalized relative luciferase activities of the 80 compounds in the second round of screening ranged from 0.63 to 1.92 with the mean and SD of 1.16 ± 0.25.

Analysis of effects of tannic acid on splicing of minigene carrying *CHRNA1* exon P3A

In order to analyze the effect of tannic acid on splicing of exon P3A, we employed the previously reported pSPL3-wtP3A harboring a 288-bp genomic fragment spanning IVS3-123 to IVS3A+90 of *CHRNA1* (21). We engineered IVS3-8G>A to make pSPL3-mtP3A. Transfection into HEK293T cells, RNA isolation and RT–PCR were performed as described (21). We also quantified the fraction of the P3A(−)-transcript by estimating the absolute copy numbers of P3A(+)- and P3A(−)-transcripts by real-time RT–PCR using Mx3000P (Stratagene) as previously described (21). Each real-time RT–PCR experiment was performed in triplicate. Patient cells were unavailable for the analysis of effects of tannic acid.

In vitro UV cross-linking and immunoprecipitation to isolate an RNA-binding protein

We synthesized [α-^{32}P]-CTP labeled RNA using the Riboprobe System (Promega) from a PCR-amplified fragment according to the manufacturer's instructions. The forward primer was 5′-*TAATACGACTCACTATA*GGGAGACAGG-3′, where the italicized is T7 promoter and the underlined is for annealing to the reverse primer. The reverse primer was 5′-CATGTCACCCTGTCCACCCACAGAAAAGGAGCCTG TCTC-3′. Nuclear extracts were prepared as previously described (62). The radioactively labeled RNA (1×10^4 cpm) was incubated at room temperature with 2 μl of nuclear extracts, 8 μg of yeast tRNA and 0.8 U of RNasin (Toyobo) in a final volume of 10 μl of the binding buffer (20 mM HEPES pH 7.8, 50 mM KCl, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA and 10% glycerol). After 20 min, samples were exposed to UV light (254 nm) for 10 min, digested with 50 U of RNase T_1, and subjected to SDS–PAGE (10% gel) and autoradiography. Immunoprecipitations were carried out under the same conditions but in 40 μl. After RNase T_1 treatment, we added 360 μl of IP buffer (0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, and 167 mM NaCl) and 80 μl protein G-Sepharose (GE Healthcare) precoated with yeast tRNA. After eliminating the protein G-Sepharose, we incubated the mixture at 4°C for 30 min and added 5 mg of anti-PTB antibody (ZYMED Laboratory) or isotype-matched control antibody to the supernatant. Samples were mixed overnight at 4°C before the addition of 60 μl protein G-Sepharose. We mixed the sample for additional 30 min at 4°C. After the beads were washed five times with the IP buffer, bound proteins were eluted and subjected to SDS–PAGE (10% gel) and autoradiography.

Surface plasmon resonance analysis

We measured the binding affinities of PTB for the wild-type and mutant *CHRNA1* ISS motifs by surface plasmon resonance using Biacore 3000 (GE healthcare). The synthesized RNA probe were 5′-UUUCUCCUUUUCUGUGGRUGGAC AGGGUGACAUGGUA-3′ with a biotin tag at its 5′ end (Hokkaido System Science). The underlined R was G for the wild-type probe and A for the mutant probe carrying IVS3-8G>A. The probe was comprised 25 nucleotides in intron 3 and 12 nucleotides in exon P3. The probe was immobilized onto a streptavidin-coated sensor chip (GE healthcare) at a concentration of 200 resonance units by injecting 0.1 pmol/μl of the RNA probe in HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% P20).

The human *PTBP1* cDNA was cloned into a pGEX6P1 vector (GE Healthcare) to make a GST fusion protein in bacteria. A fresh colony of *Escherichia coli* BL21 transformed with pGEX6P1-*PTBP1* was grown in 5 ml of LB medium containing ampicillin at 37°C for 12 h. The culture was transferred

to 200 ml of the LB plus ampicillin broth, and grown to OD_{600} of 0.1 at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM, and the culture was grown for another hour. The bacteria were harvested, resuspended in 10 ml of PBS and lysed by sonication. Bacterial debris was precipitated by centrifugation at 10 000*g*. We applied the supernatant onto a glutathione-Sepharose 4B column (GE Healthcare), washed the beads with PBS and eluted the bound protein with 10 mM glutathione.

Co-immunoprecipitation (Co-IP) to examine the binding of PTB and hnRNP H

We employed the Nuclear Complex Co-IP kit (Active Motif). We incubated 250 μg of a nuclear protein extract of HEK293T cells with 3 μg of anti-hnRNP F/H antibody (Santa Cruz) or isotype-matched control antibody in 500 μl of a low salt IP buffer (Active Motif) overnight at 4°C. We added 60 μl of protein G beads (GE Healthcare) and incubated for an additional hour on a rotary shaker. Beads were then washed six times with the low salt IP buffer. The immunoprecipitated proteins were dissolved in 20 μl of 1× SDS sample buffer, boiled for 5 min and analyzed by western blotting using anti-PTB antibody.

Real-time RT–PCR to quantify *PTBP1* mRNA

For quantification of the expression level of *PTBP1* mRNA in HEK293T cells, we employed Hs00259176_m1 for human *PTBP1* (TaqMan Gene Expression Assays, Applied Biosystems) with the Premix Ex *Taq* (Takara Bio) in Mx3000P. We also measured the expression level of *GAPDH* with a pair of primers, 5′-GTCAAGGCTGAGAACGGGAA-3′ and 5′-GTGAAGACGCCAGTGGACTC-3′, using the SYBR Premix Ex *Taq* (Takara Bio). We then normalized the expression level of *PTBP1* by that of *GAPDH*. Each experiment was performed in triplicate.

Gene silencing and overexpression of *PTBP1* mRNA

For down-regulation of *PTBP1* mRNA, we synthesized siRNA of 5′-GCCUCUUUAUUCUUUUCGGdTdT-3′. As a control, we employed the AllStar Negative Control siRNA 1027281 (Qiagen). HeLa cells were plated 24 h before transfection on 12-well plates. The transfection reagent included 300 ng of the minigene, 50 pmol of siRNA and 1 μl of Lipofectamine 2000 (Invitrogen) in 100 μl DMEM.

For overexpression of *PTBP1* mRNA, we purchased an IMAGE clone 3863892 encoding the transcript variant 1 of human *PTBP1* in a pCMV-SPORT6 vector from Invitrogen. As a negative control, we employed a CM-based pcDNA3.1(+) (Invitrogen). HeLa cells plated on 12-well plates as above was transfected with 100 ng of the minigene, 400 ng of a CMV-based vector and 1 μl of FuGENE6 (Roche) in 100 μl DMEM.

PTBP1 promoter assays

We introduced variable sizes of the human *PTBP1* promoter region immediately upstream of the translational start site into pGL3-Basic vector encoding the firefly luciferase gene (Promega). To delete three AP2 sites and two Sp1 sites from the *PTBP1* promoter region, we performed three and two rounds of the QuikChange site-directed mutagenesis kit, respectively. We transfected HEK293T cells in a 24-well plate with 0.3 μg of the pGL3 reporter vector and 0.1 μg of phRL-TK vector (Promega) using FuGENE6. Sixteen hours after transfection, we removed the transfection medium and added 0–80 μM of tannic acid (US Pharmacopeia and MicroSource Discovery Systems). We harvested cells 24 more hours and measured the firefly and Renilla luciferase activities. We performed all assays three or more times, and measured the luciferase activities in duplicate.

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Conflict of Interest statement. None declared.

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