Detecting tissue-specific alternative splicing and disease-associated aberrant splicing of the PTCH gene with exon junction microarrays

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Mutations in the human ortholog of Drosophila patched (PTCH) have been identified in patients with autosomal dominant nevoid basal cell carcinoma syndrome (NBCCS), characterized by minor developmental anomalies and an increased incidence of cancers such as medulloblastoma and basal cell carcinoma. We identified many isoforms of PTCH mRNA involving exons 1–5, exon 10 and a novel exon, 12b, generated by alternative splicing (AS), most of which have not been deposited in GenBank nor discussed earlier. To monitor splicing events of the PTCH gene, we designed oligonucleotide arrays on which exon probes and exon–exon junction probes as well as a couple of intron probes for the PTCH gene were placed in duplicate. Probe intensities were normalized on the basis of the total expression of PTCH and probe sensitivity. Tissue-specific regulation of AS identified with the microarrays closely correlated with the results obtained by RT–PCR. Of note, the novel exon, exon 12b, was specifically expressed in the brain and heart, especially in the cerebellum. Additionally, using these microarrays, we were able to detect disease-associated aberrant splicings of the PTCH gene in two patients with NBCCS. In both cases, cryptic splice donor sites located either in an exon or in an intron were activated because of the partial disruption of the consensus sequence for the authentic splice donor sites due to point mutations. Taken together, oligonucleotide microarrays containing exon junction probes are demonstrated to be a powerful tool to investigate tissue-specific regulation of AS and aberrant splicing taking place in genetic disorders.

INTRODUCTION

Alternative splicing (AS) is one of the major mechanisms by which humans produce the complexity of the proteome. It has been estimated that greater than 55% of all genes and at least 74% of multi-exon genes are alternatively spliced in humans (1,2). Protein isoforms produced by AS can have antagonistic functions, such as anti-apoptotic Bcl-xL, versus pro-apoptotic Bcl-xS (3), or completely different amino-acid compositions due to different reading frames, such as p16(INK4a) versus p19(ARF) (4). In addition, AS is also implicated in pathophysiological processes and it has been estimated that at least 15% of point mutations that cause human genetic diseases affect splicing (5).

Nevoid basal cell carcinoma syndrome (NBCCS), also called Gorlin’s syndrome, is an autosomal dominant neurocutaneous disorder characterized by large body size, developmental and skeletal abnormalities, radiation sensitivity, basal
cell carcinoma (BCC) and an increased incidence of medulloblastoma (6). NBCCS is caused by inactivating mutations in the patched (PTCH) gene (7,8). The human PTCH gene contains 23 exons spanning ~65 kb and is predicted to encode a protein of 1447 amino-acid residues containing 12 transmembrane-spanning domains and two large extracellular loops (7). Heterozygous loss of PTCH found in certain sporadic and familial cases of BCC indicates that PTCH is also a tumor suppressor gene (9,10). In vertebrates, a second patched gene (PTCH2 in humans) was identified (11,12). So far, no mutations in PTCH2 have been reported in NBCCS although a limited number of mutations were found in BCC and medulloblastoma (11).

Recently, others and we have identified that PTCH undergoes complex AS between multiple first exons and a second exon (13–16). In addition, we also have identified additional mRNA isoforms downstream of exon 2. Therefore, to survey tissue-specific AS and disease-associated aberrant splicing of PTCH, we have developed oligonucleotide microarrays designed for profiling AS. Current conventional microarray technologies are limited in their ability to distinguish and analyze mRNA isoforms. Several groups have reported a survey of human AS using exon junction microarrays that can circumvent this problem (2,17–20). In this study, considering the volume and complexity of the data produced by genome-wide studies and the cost–benefit ratio of these arrays, we have developed microarrays focused on the PTCH gene in which probes were designed for individual exons and splice junctions including junctions derived from rare AS. Using these arrays, we demonstrate a detailed evaluation of the data on tissue-specific regulation of AS. In addition, we describe the use of DNA microarrays to identify the aberrant splicing taking place in a genetic disorder.

RESULTS

Detection and validation of AS by oligonucleotide microarrays

So far, at least five alternatively used first exons have been reported in the PTCH gene (13–16). Recently, using RNA from various human tissues, we have identified additional mRNA isoforms generated by AS involving downstream exons (exons 2, 3, 4, 5 and 10) and an alternative exon named 12b by RT–PCR and sequencing (Fig. 1A and B). The numbering of exons is according to Johnson et al. (7) with GenBank accession no. U59464. Among these isoforms, the one skipping exon 10 has been briefly described previously (21) and the one skipping exons 4 and 5 has been deposited in Genbank (accession no. AB209495) but not discussed in a prior publication. The rest of the isoforms are novel. Wicking et al. (22) reported our exon 13 as exon 12b. However, as this is a constitutive exon, it is named as exon 13 in most publications and databases and the following exons are numbered by counting from 5′ to 3′. No AS involving exon 13 or exons further downstream could be identified in any tissues examined. If alternative exons can be spliced independently, then the PTCH locus potentially encodes 483 isoforms. To monitor splicing events of the PTCH gene, we designed oligonucleotide arrays on which 17 exon probes and 23 exon–exon junction probes as well as a couple of intron probes were placed in duplicate (Fig. 1A). Before analyzing the data obtained from various tissues and NBCCS patients, we validated each probe using plasmids encoding various PTCH isoforms. For this purpose, each cDNA sequence encoding six PTCH isoforms was amplified by PCR followed by in vitro transcription and labeling with Cy-5 and then applied onto the microarrays. The intensity data prior to normalization represented as an intensity heatmap is shown in Figure 1B. Each probe’s intensity was then adjusted in two ways: first, for the mean probe intensity for each construct and then for each probe’s sensitivity. Finally, mean probe intensities that should be positive according to the plasmid and probe sequences were adjusted to 1, and those that should be negative were adjusted to zero. As depicted in Figure 1C, all constructs showed profiles expected from exon composition and could be clearly discriminated from one another. No intensities in-between were observed. However, when placing probes at exon–exon junctions, there is little choice as to the underlying nucleotide composition. So, two junction probes, exon 9–11 and exon 12–13, were non-informative, e.g. saturated, constant intensity and could lead to erroneous predictions, therefore excluded from the figure.

Tissue-specific regulation of AS

To investigate tissue-specific regulation of AS using array data, we normalized probe intensities as described in Materials and Methods and in Figure 4. If no AS takes place at the exons or exon–exon junctions for which probes are designed, then the normalized relative probe intensities are near 1.0 in all tissues. In contrast, probes involved in AS should give intensities with large standard deviations. As expected from mRNA isoforms we have identified so far, a marked variation in normalized probe intensities was observed in exons 2–5, 10 and 12b (Fig. 2A). The tissue-specific accuracy of these array data was confirmed by RT–PCR. For example, when we focused on exon 12b, in tissues in which elevated probe intensities for exons 12–12b, 12b and 12b–13 were observed (i.e. the brain and heart), RT–PCR products containing exon 12b were also evident (Fig. 2B, lanes 1 and 8). We next addressed the question of where in the brain exon 12b was highly expressed. The subsequent investigation demonstrated that exon 12b was particularly expressed in the cerebellum among various brain tissues (Fig. 2B, lane 2). Additionally, two independent methods, microarray analysis and RT–PCR, demonstrated a strong correlation (Fig. 2C), validating the method of array data normalization. Array data as to inclusion or exclusion of exon 10 was similarly compared with the RT–PCR results. As shown in Figure 2D, these two methods again correlated well with each other. Isoforms skipping exon 10 were the most highly expressed in the thymus and the lung based on both these methods.

As shown in Figure 1A, AS involving exons 2–5 is relatively complicated and cannot be explained simply by inclusion or exclusion of a single exon. When we focused on isoforms skipping exons 4 and 5, the data from the two methods still correlated (Fig. 2E). However, the degree of correlation represented by $R^2$ was weaker than that obtained with
probes for exon 12b or 9 and 10, because AS skipping exons 4 and 5 is a rare event [up to 3% of the authentic isoform (shown by X-axis in Fig. 2E)].

Next, we evaluated the microarray data regarding the usage of the alternative first exons. RT–PCR was performed using the same forward primers for each alternative exon 1, as those used for the microarray analysis. As shown in Figure 2F and G, the two methods showed a good correlation regarding the usage of exon 1b. However, isoforms starting from exons 1a, 1d and 1e failed to demonstrate significant

Figure 1. Detection and validation of the known isoforms of PTCH. (A) Exon structure of the PTCH gene. Positions of the exon probes and the exon junction probes are indicated. (B) Probe intensities obtained with Cy-5-labeled RNA from constructs encoding various isoforms of PTCH. The labeling reaction was performed with T7 RNA polymerase by using the PCR product as a template obtained from the constructs. Each matrix point shows the S/N ratio of one exon or exon junction probe in one construct. Probes are ordered horizontally, 5′–3′, together with some probes for infrequently spliced junctions such as exon 2–6. Exon 3–4 is an exon junction probe between exon 3 and the alternative splice acceptor site located in intron 3. Probe sequences for most 5′ exons were not included in some of the constructs and were excluded from this figure. Hybridization samples form the vertical axis of each matrix. Exon compositions of the expression plasmids are briefly depicted at the bottom. (C) Normalized probe intensities. The mean positive intensity and the mean negative intensity are adjusted to 1 and 0, respectively.
correlations (data not shown). This is probably because the expression of isoforms starting from exons 1a and 1e is much lower than that starting from exon 1b (average expression of exons 1a and 1e is 3.4 and 13.8% of exon 1b, respectively, when pooled S/N ratios are compared). In addition, the PCR efficiency of the exon 1d primer is significantly lower than that of the exon 1b primer (37% of the exon 1b primer) (Supplementary Material, Fig. S1).

Detection of aberrant splicing in NBCCS patients

We have been investigating *PTCH* mutations in NBCCS patients (23) and have detected mutations in 13 out of 17 cases analyzed so far. A list of all cases is presented in Supplementary Material, Table S1. Among 13 cases, G17 had a mutation, c.584G>A (as per GenBank entry NM_000264.2: the A of the ATG of the initiator Met codon is counted as nucleotide +1), on exon 3. This raised two possibilities that explain the effects on the coding for the *PTCH* protein. One is a missense mutation, p.R195K (as per GenBank entry NP_000255.1). However, as this point mutation was located at the 3′ end of exon 3, and potentially disrupts a splice donor site, we sought the second possibility that the mutation may affect splicing. The data of the microarray analysis showed a significant decreased intensity for the junctional probe exon 3–4, but otherwise the data looked normal indicating an abnormal splicing between exons 3 and 4 (Fig. 3A). The electrophoretogram of the RT–PCR product revealed an additional band which had a larger molecular weight indicating the presence of an aberrant splicing (Fig. 3B, left panel). Sequencing of the additional product demonstrated that abnormal splicing was indeed taking place in which a cryptic splice donor site located in intron 3 was activated, resulting in the insertion of a 37-bp
intronic sequence between exons 3 and 4 causing a premature termination of the PTCH protein (Fig. 3C). We therefore concluded that it was the aberrant splicing rather than the missense mutation that caused the disease phenotype in this patient.

Another case, G9, did not contain mutations in any of the coding exons of PTCH. However, the array data demonstrated a markedly decreased intensity for the junctional probe exon 6–7 (Fig. 3A). The detection of the smaller RT–PCR product in G9 (Fig. 3B, right panel) prompted us to sequence this RT–PCR product, which again revealed the presence of an aberrant splicing. But in this case, a cryptic splice donor site located in exon 6 was activated generating the deletion of an exonic sequence of 87 bp (Fig. 3C). As a result, 29 amino-acid residues located in the first extracellular loop important for Shh binding were deleted. Mutational analysis in intron 6 identified a point mutation, c.945+5G>T, that partially disrupts the consensus sequence for the splice donor site (Fig. 3C).

The third case, G8, had a mutation, c.1526G>A, on exon 11. This mutation presumably results in a missense mutation, p.G509D. However, a growing body of evidence indicates that some single base changes may be capable of switching regulation from positive to negative or vice versa by disrupting or creating exonic splicing enhancers or exonic splicing silencers that are just beginning to be understood (24). Therefore, we investigated such possibilities using microarrays. As shown in Figure 3A, the splicing profile of G8 was similar
to that of the control and no significant changes in probe intensity were observed. So, we concluded that the mutation found in G8 was a bona fide missense mutation. Interestingly, substitutions of the same amino-acid residue have been reported in NBCCS (p.G509R and p.G509V) (25,26) and these mutations are predicted to disrupt the sterol-sensing domain of the PTCH protein (27). It has also been observed that the mutation p.G509V results in dominant negative activity in vivo in Drosophila (28). Two out of 17 patients, G7 and G13, did not have a mutation in the region we

Figure 3. Detection of aberrant splicing in NBCCS patients. (A) Normalized probe intensities obtained from NBCCS patients, G8, G9 and G17. Data were analyzed as described in Figure 2A. (B) RT–PCR analysis of AS in the PTCH gene. Exon 3 (G17) and exon 6 (G9) and flanking exons were amplified by RT–PCR and subjected to agarose gel electrophoresis. The positions of extra bands not observed in a control healthy individual (Cont) are indicated by arrows. (C) Schematic representation of abnormal splicing identified in G17 and G9. The positions of the point mutations are indicated by asterisks.
have sequenced, nor have any abnormal splicing profile by microarray analysis (Supplementary Material, Table S1). They may have a mutation in non-coding region important for the transcription of PTCH, or have a mutation in a gene other than PTCH. Altogether, these results demonstrate that exon junction microarrays can be used for detecting the mutations which result in aberrant splicing.

**DISCUSSION**

In this paper, we described the use of oligonucleotide microarrays containing exon junction probes to investigate tissue-specific AS and to detect disease-associated aberrant splicing. Our microarrays have been demonstrated to be a particularly powerful tool to detect the exon skipping/inclusion type of AS, the most common type (38%) of AS (29), in which even a rare AS event (no more than 3%) can be quantitatively detected using microarrays. Theoretically, previously unrecorded mRNA isoforms can be predicted with this method. If unexpectedly high variation is detected in certain probes, then AS is suggested in this region.

In this study, we found a novel alternative exon named exon 12b. High expression of exon 12b in the brain, particularly in the cerebellum, is intriguing, because proliferative effects of sonic hedgehog (Shh), the ligand of the PTCH protein, in the external granular layer of the cerebellum are well characterized (30) and patients with NBCCS are prone to develop the cerebellar tumor medulloblastoma (6). As exon 12b has an in-frame stop codon, the mRNA isoform containing this exon encodes a truncated PTCH protein that is presumably non-functional. However, this protein may have a dominant negative effect on the wild-type protein and such a possibility needs to be ruled out. Importantly, exon 12b was also found in mice and was also expressed in a brain- and heart-specific manner (data not shown). Recently, the splicing-regulatory element, UGCAUG, was reported to be evolutionarily conserved in introns that flank brain-specific alternative exons (31). Such an element was indeed identified in the intron between exons 12b and 13 both in humans and in mice (data not shown). In contrast, skipping of exon 10 leads to a 52 amino-acid in-frame deletion in the second and third transmembrane domains. The effect on the function of the PTCH protein is currently unknown and this PTCH isoform may be functional in some context.

Detection of AS using exon junction microarrays is limited in several ways. First, as the detection requires probes that match specific exons and splice junctions, probe selection is tightly constrained and some probes are non-informative due to cross-hybridization with somewhere else in human genes. In our study, two out of 42 probes, exon 9–11 and exon 12–13, gave constitutive high intensities and therefore were excluded from further study. Secondly, detection is based on differential expression. Therefore, if two isoforms are present in the same proportion in every tissue, no prediction will result, because normalized probe intensities will behave similarly to the pool. Third, precaution should be taken when interpreting the data on alternative usage of 5′-terminal exons, because not only expression levels but also other factors such as the sequences of forward primers constructed for 5′-terminal exons can have an effect on probe sensitivities.

In this study, we monitored a relatively small number of AS events of one gene in a relatively small set of samples. It should be noted, however, that this is the first report of microarrays used for the identification of aberrant splicings in a genetic disorder. In NBCCS, a considerable number of the patients do not have mutations within the coding region of PTCH (23,32). In such patients, exon junction microarrays will be a valuable tool for detecting mutations affecting the splicing event. According to the PTCH mutation database (http://www.cybergene.se/cgi-bin/w3-msql/ptchbase/index.html), at least 20 mutations have been reported to potentially result in abnormal splicing in PTCH and some of them have been proven experimentally (21,26). Thus, mutations having an effect on splicing events do not seem to be uncommon. Not only NBCCS, but also an increasing number of genetic diseases are known to be caused by mutations that alter splicing in cis (at least 15% of point mutations) (5 and reviewed in 24). Some of these mutations weaken or activate cis-acting element such as intronic splicing enhancers or intronic splicing silencers that are sometimes located in intronic sequences distant from exons. In such cases, mutations are not identified by the sequencing of coding regions, warranting the use of exon junction microarrays like the ones described in this study to rapidly search the candidate regions to be sequenced.

Apart from PTCH, germ-line mutations in the genes encoding Shh signaling components such as Shh or Gli3 are responsible for a variety of genetic disorders, most of which are accompanied by developmental anomalies in the central nervous system (33). In addition, somatic mutations of the genes involved in this signaling pathway, such as PTCH2 or Smootherned, are associated with various sporadic cancers (33). Moreover, distinct roles of PTCH2 splice variants in Shh signaling have recently been proposed (34). Therefore, oligonucleotide microarrays containing exon and exon junction probes widely covering these genes would be an attractive tool to study the pathogenesis of these disorders. In addition, these microarrays may also be useful to detect mutations in the dystrophin gene, because the removal of exon(s) is found in 60–65% of patients with Duchenne and Becker muscular dystrophies (35).

**MATERIALS AND METHODS**

**Plasmids**

The plasmid encoding myc-tagged full-length PTCH protein (exons 1b–23) (pMyc-Ptc1) was kindly provided by Dr Jeffrey Ming (15). The plasmids encoding other isoforms of PTCH were created by PCR-mediated mutagenesis as described previously (36) using pMyc-Ptc1 as a template. The details of construction are available on request.

**Oligonucleotide microarray construction**

All probes 34–76 bp in length were designed to have approximately the same annealing temperature (Tm). Splice junction probes between two exons were designed so that the Tm for the first exon sequence is the same as that for the second
exon sequence (Supplementary Material, Table S2). The oligonucleotide microarray, GenoPalTM (Mitsubishi Rayon Co., Ltd), was made in the following manner. Plastic hollow fibers were bundled in an orderly arrangement, and hardened with resin to form a block (Supplementary Material, Fig. S2). Oligonucleotide-capture probes were chemically bonded inside each hollow fiber with hydrophilic gel. The block was then sliced to make thin chips, each of which was set into a holder (http://www.mrc.co.jp/genome/e/index.html for details).

Preparation of labeled probe

Total RNA from a panel of human tissues was purchased from Ambion. Lymphoblastoid cell lines from NBCCS patients immortalized by Epstein-Barr virus were also used to obtain total RNA. All studies using patient samples were approved by the local ethic committee. The template for in vitro transcription was generated through a modified procedure using a SuperScript One-Step RT–PCR System with Platinum Taq (Invitrogen). In brief, 2.5 μg of total RNA was reverse-transcribed with SuperScriptIII RT/Platinum Taq Mix and T7-exon15 reverse primer, 5'-TAATACGACTCACTATA GGAGGTTCAGTACTAATGTC-3', for 30 min at 50°C. To evaluate the quality of RNA, T7-GAPDH reverse primer, 5'-TAATACGACTCACTATAGGG AGGAGGAGGAGATTCAGTGTGGT-3' was also added in the reaction. The RNA was degraded with the addition of RNaseH (Invitrogen) for 15 min at 37°C. After the addition of forward primers specific for each first exon (5'-AGCGCT TGTTTACCGAGGAG-3' for exon 1a, 5'-GGACCGGGACT ACCTGACC-3' for exon 1b, 5'-AAATGCCGCGCCGGG AGCAGCCT-3' for exon 1d and 5'-TTCTGGCGGGGT CCAGT-3' for exon 1e) and T7-exon15 reverse primer (final concentration 0.5 μM each) and the activation of Platinum Taq for 10 min at 94°C, PCR was run for 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min. Plasmid DNA encoding

\[
P = \begin{pmatrix} P_1 & \cdots & \cdots & P_m \\ \vdots & \ddots & \vdots \\ \vdots & \vdots & \ddots \\ P_{m1} & \cdots & \cdots & P_{mn} \end{pmatrix}, \quad M = \begin{pmatrix} 1/\overline{P}_1 & 0 & \cdots & 0 \\ 0 & 0 & \cdots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & \cdots & 0 & 1/\overline{P}_n \end{pmatrix}, \quad \overline{P}_j = (\sum_{i} P_{ij})/m
\]

\[
PM = \begin{pmatrix} P_{11}/\overline{P}_1 & \cdots & \cdots & P_{1n}/\overline{P}_n \\ \vdots & \ddots & \vdots & \vdots \\ \vdots & \vdots & \ddots & \vdots \\ P_{m1}/\overline{P}_1 & \cdots & \cdots & P_{mn}/\overline{P}_n \end{pmatrix}
\]

\[
N = \begin{pmatrix} 1/\overline{p}_1 & 0 & \cdots & 0 \\ 0 & 0 & \cdots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & \cdots & 0 & 1/\overline{p}_m \end{pmatrix}, \quad \overline{p}_i = (\sum_{j} P_{ij}/\overline{P}_j)/n
\]

\[
NPM = \begin{pmatrix} P_{11}/\overline{p}_1\overline{P}_1 & \cdots & \cdots & P_{1n}/\overline{p}_1\overline{P}_n \\ \vdots & \ddots & \vdots & \vdots \\ \vdots & \vdots & \ddots & \vdots \\ P_{m1}/\overline{p}_m\overline{P}_1 & \cdots & \cdots & P_{mn}/\overline{p}_m\overline{P}_n \end{pmatrix}
\]

Figure 4. Matrix representation. The relationship between probes and tissues can be represented by an \(m \times n\) matrix \(P\). The total number of probes is \(m\), and \(n\) is the total number of tissues. Let \(M\) be an \(n \times n\) diagonal matrix where \(\overline{P}_j\) represents the mean \(P\) in tissue \(j\). The probe intensities normalized by total \(PTCH\) expression in each tissue can then be expressed as \(PM\). Let \(N\) be an \(m \times m\) diagonal matrix where \(\overline{p}_i\) represents the mean normalized \(P\) in probe \(i\). The matrix \(NPM\) therefore represents the probe intensities normalized by both factors described above and \(P_{ij}/\overline{p}_i\overline{P}_j\) is used in Figures 2 and 3.
various isoforms of PTCH was also subjected to PCR using the exon 2 forward primer, 5’-GCTGAGAGCGAAGTTTC AGA-3’, and T7-exon 15 reverse primer. PCR products generated from the reverse-transcribed cDNA and the plasmid DNA were purified with a PCR Purification kit (QIAGEN) according to the manufacturer’s instructions. Then, a Cy-5-labeled probe was generated by using the PCR product as a template with a MEGAscript T7 kit (Ambion). The reaction contained a 1:1.5 mixture of uridine triphosphate (UTP) and Cy-5-UTP (Amersham Biosciences). The product was purified with an RNeasy mini kit (QIAGEN) following the manufacturer’s instructions. One microgram of labeled probe was fragmented with RNA Fragmentation Reagents (Ambion) at 70°C for 3 min.

Hybridization and detection
Hybridization was carried out in a final volume of 0.1 ml injected into a hybridization chamber at 65°C for 16–24 h in 0.5× SSC with 0.2% SDS. The microarray chip was then washed twice in 0.5× SSC with 0.2% SDS at 55°C for 20 min and once in 0.5× SSC at 55°C for 10 min, before being slowly cooled to room temperature. GenoPal was then scanned and the image was captured with a cooled CCD-type Microarray Image Analyzer (Mitsubishi Rayon Co., Ltd). Fluorescent intensity was analyzed with software developed by Mitsubishi Rayon Co., Ltd. Fluorescence throughout the three-dimensional structure of each array feature can be efficiently captured due to the long focal depth of the optical system of the image analyzer (http://www.mrc.co.jp/genome/e/index.html).

Microarray data analysis
To analyze changes in the AS of a gene, changes in the total expression of a gene and differences in probe sensitivity should be separated and excluded. We used a simple and gene-specific method of deconvolution using a microarray platform.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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