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REVIEW

Measuring RNA Editing of Serotonin 2C Receptor

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Abstract—Pre-mRNA of serotonin 2C receptor (HTR2C, 5-hydroxytryptamine (serotonin) receptor 2C) undergoes A-to-I type RNA editing, which is a post-transcriptional event leading to the change of genomically encoded information. RNA editing generates various HTR2C isoforms, each of which has distinctive receptor activity. Postmortem, animal, and pharmacological studies have suggested that the altered RNA editing of HTR2C is involved in the pathophysiology of mental disorders, although results remain inconsistent. Here we review the techniques used for estimation of RNA editing of HTR2C. Among the techniques reported so far, a high-throughput sequencing-based method would be the most powerful method of choice for the large-scale experiments. Several different methods that were previously developed, such as pyrosequencing and capillary electrophoresis, should be suitable for validation as well as for rapid screening or exploratory purposes.

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RNA editing is a post-transcriptional modification event that modifies pre-mRNA sequences, leading to the change of information coded in the genomic DNA [1-3]. A-to-I type RNA editing includes conversion of adenosine to inosine by enzymes called ADARs (adenosine deaminases acting on RNA). Because the translation machinery regards inosine as guanosine, amino acid changes can occur depending on the sequence. One of the well-characterized A-to-I RNA editing targets is serotonin 2C receptor (HTR2C). *HTR2C* encodes a seven transmembrane-spanning, G protein-coupled receptor that activates phospholipase C. Mice with this gene knocked out were found to exhibit seizure and anxiety-related behaviors [4-6]. In addition, down-regulation of *HTR2C* was reported in brains of patients with bipolar disorder and schizophrenia [7, 8], suggesting its involvement in the pathophysiology of mental disorders [9].

RNA editing of *HTR2C* occurs at five positions (termed as sites A to E) that are located within the second intracellular loop of the receptor (figure). There are 32 transcripts in total, and they could generate up to 24 different protein isoforms. Each of the isoforms was found to have a distinct function and to be differentially distributed across brain regions [10-13]. To date, altered RNA editing has been reported in animal models of neuropsychiatric disorders and postmortem brains of patients with mental disorders, such as schizophrenia and major depression [9, 14]. However, owing to the limited sample size and resolution of analytical techniques, the results remain inconsistent. Therefore, estimation of the RNA editing of *HTR2C* in various samples in accurate ways is the first step towards understanding its role in complex neuropsychiatric disorders.

Measuring the RNA editing efficiency of *HTR2C* was more challenging than for other known RNA editing substrates. First, it contains multiple editing sites within a very short genomic region (i.e. five edited sites are located within 13 bp), and two of the five editing sites are adjacent. Second, not only the editing level of each site, but also the combination of edited sites, is clearly important as this produces different protein isoforms. Here we review the techniques used for estimation of RNA editing of *HTR2C*, focusing on recently developed techniques.

Direct sequencing of RT-PCR product. The simplest way of determining the RNA editing efficiency of *HTR2C* was direct sequencing of RT-PCR product that contains five editing regions. Comparison of the peak heights of

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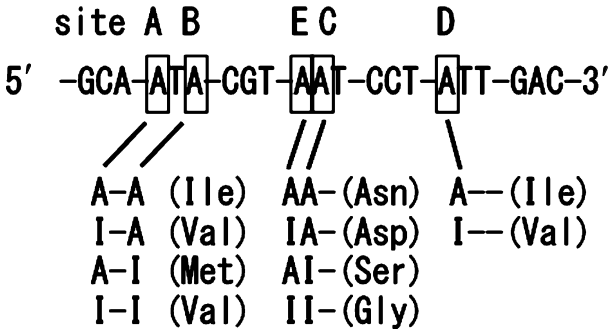

RNA editing of *HTR2C*. Pattern of amino acid change is shown

editing sites in the sequence chromatogram indicates the editing efficiency. This is particularly suitable for preliminary screening or detection of drastic change. For example, samples derived from ADAR2-knockout mice showed RNA editing efficiency that decreased to almost 0% at sites C and D [15]. However, this cannot be applied to detect subtle changes, and the overall accuracy is low compared with those of other methods. In addition, information on the editing pattern cannot be obtained. Standard curves using a mixture of fully edited and non-edited cDNAs will be helpful to monitor the linearity [16].

RT-PCR, cloning, and sequencing. A frequently used method is cloning–sequencing analysis of RT-PCR product. Instead of sequencing directly, the RT-PCR product is cloned to a bacterial strain so that each individual colony contains one cDNA molecule. Sequencing analysis is then performed for these colonies. The advantages of this method are its efficiency and the fact that results for a combination of editing can be obtained simultaneously. However, this method is time-consuming, and sequencing of a large number of bacterial clones is generally required for accurate data analysis. Some of the findings derived from a small number of colonies were not supported by recent high-throughput sequencing-based assays, as described below.

Primer extension. Primer extension (PE) is often used to quantify site-specific RNA editing efficiency [10]. In this method, appropriate combinations of dNTPs or ddNTPs are incorporated into radio-labeled primer, which is designed to match a region proximal to the editing site, with T7 DNA polymerase. When ddNTP is incorporated, the extension reaction terminates. The products are then subjected to polyacrylamide gel electrophoresis to separate edited and non-edited products. This method is highly accurate and reproducible and is suitable for estimating the sites A and D located at the end of the RNA editing region. Although the use of carefully designed degenerate primers with dNTPs and ddNTPs theoretically enables internal editing sites such as sites B and E to be analyzed, the accuracy becomes relatively low [17, 18]. We developed a non-radioactive isotope method by PE combined with denaturing high-performance liquid chromatography (dHPLC) [19, 20]. In this method, PE reaction mixtures are separated and quantified by dHPLC. Unlike an RI-based method, this method allows high-throughput analysis. The main disadvantages of a PE-based method are considered to be the difficulty of measuring editing efficiency of the internal sites and the fact that a combination of editing patterns cannot be obtained.

Pyrosequencing. Pyrosequencing is a method based on the principle of sequencing by synthesis using four enzymes: DNA polymerase, ATP sulfurylase, apyrase, and luciferase [21, 22]. In this technique, after each dNTP is dispensed, DNA polymerase incorporates it into the primer that was annealed to the template DNA, and pyrophosphate (PPi) is released. ATP sulfurylase generates ATP with PPi. Luciferase then produces light using ATP. The light is detected and presented as a peak in a pyrogram. Each peak height is proportional to the number of dNTPs incorporated, allowing quantitative measurements. After the apyrase degrades residual dNTPs and ATP, the next dNTP is added, allowing incorporation of sequential dNTPs in a single reaction tube. Pyrosequencing can be used as an alternative method for sequencing of a large number of bacterial clones [23]. Using pyrosequencing technology, we developed a method to estimate the RNA editing efficiency of all five sites by optimizing the dispensation order using RT-PCR product directly [24]. In addition, our method cannot only estimate editing efficiency but also quantitatively estimate editing combination partially.

Capillary electrophoresis (CE). Both PE-based and pyrosequencing-based methods have limitations in that they cannot be applied to measure the editing combination. By utilizing the difference of electrophoresis mobility of different cDNAs, Poyau et al. developed a relative quantification assay using capillary electrophoresis (CE) [25]. This assay is based on the single-strand conformational polymorphism of the DNA. They showed that 30 out of 32 isoforms exhibited distinctive mobility by CE analysis. The remaining two isoforms, non-edited and site E-edited isoforms, showed indistinguishable mobility. However, considering that site E-edited isoform is rare, this may have little impact on the assessment. In this assay, RT-PCR of the editing region is performed with labeled primer and is subjected to CE. The mobility of the detected peaks is compared with that of predetermined reference isoform peaks. RNA editing efficiency is then estimated by calculation of the ratio of identified isoforms.

Quantitative real-time PCR and melting analysis. Lanfranco et al. reported a quantitative RT-PCR method for some of the HTR2C isoforms [26]. They designed four different TaqMan probes that specifically recognize different isoforms, which included non-edited, fully edited (ABECD), and partially edited (ABD or AD) isoforms. Chateigner-Boutin et al. reported melting analysis of PCR product for assessment of RNA editing status [27].

These methods may be useful, especially for rapid detection of alteration of RNA editing status.

High-throughput sequencing. Several groups have reported a high-throughput sequencing-based method focused on estimation of *HTR2C* RNA editing [28-31]. Abbas et al. used the Illumina Genome Analyzer II with sequencing primer designed for a region upstream of the first edited site [30]. They carefully filtered the sequence reads that had a sequence mismatch compared with the reference genome, ensuring the high reliability of data analysis. Importantly, they applied multiplex analyses by adding a unique tag sequence to each different sample. Morabito also reported multiplex analysis of editing status using a high-throughput sequencer [31].

A high-throughput sequence-based method substantially improves the accuracy and sensitivity of RNA editing analysis, and allows the statistical analysis of rare isoforms. For example, Abbas tested the RNA editing status of various brain regions derived from chronic drug-treatment mice. Among a total of 10 drugs tested, two antidepressants commonly increased the editing of A and B sites in hippocampus, whereas lithium increased the C and D sites in cortex [30]. In addition, the detected changes were very small, in the range of about 5% differences. Similarly, Morabito et al. tested the RNA editing status of brains derived from various inbred mouse strains and found that the differences were very small across strains [31].

Perspective. To date, several methods based on different principles have been reported for the study of RNA editing of *HTR2C*. It is important to determine the advantages and disadvantages of each method to enable better validation experiments using different methods to be performed. Although a high-throughput sequencing-based method is the first choice for large-scale experiments, it is still recommended that its results are validated by other methods to ensure accuracy. Several different methods, such as pyrosequencing and CE, should be suitable for such a purpose, as well as for rapid screening or exploratory purposes.

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