Heterogeneous Sequences of Brain Cytoplasmic 200 RNA Formed by Multiple Adenine Nucleotide Insertions

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https://doi.org/10.14348/molcells.2019.0108
www.molcells.org

Brain cytoplasmic 200 RNA (BC200 RNA), originally identified as a neuron-specific non-coding RNA, is also observed in various cancer cells that originate from non-neural cells. Studies have revealed diverse functions of BC200 RNA in cancer cells. Accordingly, we hypothesized that BC200 RNA might be modified in cancer cells to generate cancerous BC200 RNA responsible for its cancer-specific functions. Here, we report that BC200 RNA sequences are highly heterogeneous in cancer cells by virtue of multiple adenine nucleotide insertions in the internal A-rich region. The insertion of adenine nucleotides enhances BC200 RNA-mediated translation inhibition, possibly by increasing the binding affinity of BC200 RNA for eIF4A (eukaryotic translation initiation factor 4A).

Keywords: adenine nucleotide insertion, BC200 RNA, cancer cells, eIF4A, translation inhibition

INTRODUCTION

Brain cytoplasmic 200 RNA (BC200 RNA) was first identified as a non-coding RNA specifically expressed in human neuronal cells (Watson and Sutcliffe, 1987), where it migrates to dendrites and inhibits local translation by interacting with the translational factors eIF4A (eukaryotic translation initiation factor 4A), eIF4B (eukaryotic translation initiation factor 4B), and PABP (poly A binding protein) (Eom et al., 2011; Kondrashov et al., 2005; Lin et al., 2008; Muddashetty et al., 2002). This may lead to neural plasticity through formation of specific expression patterns of post-synaptic proteins (Briggs et al., 2015). In non-neuronal cells, on the other hand, BC200 RNA expression is highly suppressed. However, the tight control of BC200 RNA expression in non-neuronal cells fails in some diseases (Chen et al., 1997; Mus et al., 2007). In cancer of non-neuronal origins, BC200 RNA expression is activated (Chen et al., 1997), possibly by cancer-specific factors. Recent studies have shown that the abnormal expression of BC200 RNA is related to the development of cancer cells through various mechanisms (Booy et al., 2017; Hu and Lu, 2015; Peng et al., 2018; Ren et al., 2018; Samson et al., 2018; Shin et al., 2018; Singh et al., 2016).

Although BC200 RNA is transcribed from a single gene, biological and structural features of BC200 RNA may differ according to the cell in which it is expressed. Previously, we reported that BC200 RNA stability varies among cancer cells types (Kim et al., 2017), possibly owing to cell-specific biological or structural differences. Because RNA modifications can contribute to conferring distinct structural identities to the RNA itself, usually associated with cellular functions, we propose that BC200 RNA may be differentially modified in cancer cells. During the study of BC200 RNA biogenesis,

we found that BC200 RNA exhibits length heterogeneity. In this study, we investigated the underlying cause of this heterogeneity. We found various numbers of inserted adenine nucleotides in the internal A-rich region of BC200 RNA. Notably, BC200 RNA from cancer cell lines contained increased numbers of inserted adenine nucleotides compared with that from normal cell lines. We also found that insertion of adenine nucleotides enhanced BC200 RNA-mediated translation inhibition and increased the binding affinity of BC200 RNA for eIF4A.

MATERIALS AND METHODS

Cell culture
Cervical cancer (HeLa) cells, various breast cancer cell lines (MCF7, Hs578T, T47D, and MDA-MB-231), normal breast (MCF10A) cells, and normal keratinocytes (HaCaT) were grown as previously described (Kim et al., 2017). All cell lines were found to be mycoplasma free. For transfections, cells were seeded and incubated for 24 h prior to transfection of DNA using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's protocol.

Plasmid DNAs
Plasmids pT1010BC and pSUPER-BC200 (Kim et al., 2017; Shin et al., 2017a) were used for BC200 RNA overexpression in cells. BC200 RNA gene sequences were mutated using SOEing polymerase chain reaction (PCR), and PCR products were cloned into *BglII/XhoI* sites of the pSUPER vector (Oligoengine, USA) to generate pSUPER-BC200_A6, pSUPER-BC200_A11, and pSUPER-BC200_A24.

Expression and purification of eIF4A
eIF4A was expressed and purified as a glutathione S-transferase (GST) fusion protein, as described previously (Jang et al., 2017).

RNA preparation
Total cellular RNA was prepared using an easy-BLUE Total RNA Extraction Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions.

In vitro transcription
Template DNAs for *in vitro* transcription were obtained by PCR-amplification of BC200 RNA or its derivative sequences from plasmids pSUPER-BC200_A6, pSUPER-BC200_A11, pSUEPR-BC200_A14, and pSUPER-BC200_A24 using the primer pair, BC200_T7_F (5′-GAA TTC TAA TAC GAC TCA CTA TAG GCC GGG CGC GGT G-3′) and BC200_R (5′-AAA GGG GGG GGG GGG TTG TTG CTT TG-3′). *In vitro* transcription was carried out using a RiboMAX Large Scale RNA Production System (Promega, USA). *In vitro* transcripts were gel-purified. When required, transcripts were 5′-labeled with [γ-^{32}P]ATP (PerkinElmer, USA) using T4 polynucleotide kinase (Enzynomics, Korea) after treatment with Antarctic phosphatase (New England Biolabs, USA).

Primer extension
Primer extension was performed with 10 μg of total RNA, as previously described (Han et al., 2010), using 5′-^{32}P-labeled BC200 RNA primer (5′-AAA GGG GGG GGG GGG TTG TTG CTT TG-3′).

RACE assays
RACE (rapid amplification of cDNA ends) analysis was performed as previously described, with some modification (Liu and Gorovsky, 1993). Total RNA (10 μg) was treated with tobacco acid pyrophosphatase (Epicentre, USA), and an RNA product approximately 200-250 nt in size was gel purified. The gel-purified RNA was used for RNA ligation without adaptors to generate self-ligated products. The ligated RNAs were subjected to reverse transcription-PCR (RT-PCR) using the primer pair, BC200_CF (5′-GAC TCA CTA TAG GCC GGG CGC GGT G-3′) and BC200_CR (5′-AAA GGG GGG GGG GGG TTG TTG CTT TG-3′). RACE products were cloned and sequenced.

Filter binding assay
In vitro-transcribed BC200 RNA was renatured as described (Jung et al., 2014). BC200 RNA-eIF4A complex formation was performed in 100 μl of phosphate-buffered saline (PBS) containing 0.3 nM BC200 RNA and 1-6 μg eIF4A for 30 min at room temperature. The reaction mixture was subjected to dot-blot analysis, as described previously (Jung et al., 2014), using two membranes of Hybond ECL membrane (GE Healthcare, USA) and Hybond-XL membrane (GE Healthcare) after which each membrane was washed two times with PBS. The Hybond-XL membrane was hybridized with ^{32}P-labeled anti-BC200 probe (5′-TTT GAG GGA AGT TAC GCT TAT-3′) and exposed to the BAS-IP imaging plate (Fujifilm, Japan) and analyzed on an FLA-7000 phosphor-image analyzer (Fujifilm). The amount of radiolabeled RNA on each filter was quantified using ImageJ software (NIH, USA).

In vitro translation assay
In vitro translation was performed using a TnT (transcription/translation)-coupled rabbit reticulocyte lysate system (Promega). Total reaction volumes of 10 μl containing luciferase expression plasmid DNA, rabbit reticulocyte lysate, [^{35}S] methionine, TnT reaction buffer, T7 RNA polymerase, amino acid mixture (-Met), RNase inhibitor and increasing amounts of RNAs were incubated for 90 min at 30°C according to the manufacturer's recommendations. Proteins in reaction mixtures were resolved by SDS-PAGE. Gels were then dried, placed on the BAS-IP imaging plate (Fujifilm), and analyzed on an FLA-7000 phosphor-image analyzer (Fujifilm).

Wound-healing assay
HeLa cells were seeded in 6-well cell culture plates (SPL Life Sciences, Korea) at 2×10^5 cells/well and grown to confluence. The monolayer was scraped (wounded) with the tip of a sterile plastic micropipette, debris was removed by washing the cells with 1 ml of growth medium/well, and the medium was replaced with 2 ml of serum-free medium per well. Digitized images were collected at various post-wounding intervals using an inverted microscope (Eclipse TS100; Nikon, Japan) equipped with a digital camera (Digital Sight DS-Ri1; Nikon). Wounded areas were calculated using ImageJ soft-

ware, and results were used to estimate the degree of recovery.

Northern blotting

Total RNA was fractionated on a 6% polyacrylamide gel containing 7 M urea and electro-transferred onto a Hybond-XL membrane (GE Healthcare). The membrane was hybridized with ^{32}P-labeled anti-BC200 or anti-5S probe (5′-CAT CCA AGT ACT AAC CAG GCC C-3′) and analyzed as described above.

RESULTS AND DISCUSSION

Since the length of the BC200 RNA gene is 200 bp with no introns, the size of its transcript is expected to be 200 nt. Using primer extension analysis to map the 5′ terminus of BC200 RNA in HeLa cells, we found by chance that the expected band of the extended products on a gel was broad, containing more slowly migrating species (Fig. 1A), suggesting that the size of BC200 RNA is heterogeneous with some insertion sequences. To determine whether inserted nucleotides exist, we first gel-purified RNAs of about 200-250 nt from total RNA isolated from HeLa cells. The purified RNA was then intramolecularly self-ligated using RNA ligase and subjected to RT-PCR across the junction of the 5′ and 3′ ends. The PCR products, composed of the fixed primer sequences of nucleotides 163-197 of BC200 RNA and the reversed transcribed sequences in the remaining regions (nucleotides 1-162 and 198-200), were cloned and 9 clones were sequenced. We found sequence variations in the 1-162 region and the 5′-3′ junction, including the 198-200 sequence. We focused on sequence variation in the 1-162 region because variation in this region could explain the heterogeneity observed in primer extension analyses. We found extensive heterogeneity in sequences of the 1-162 region in BC200 RNA isolated from HeLa cells (Figs. 1B and 1C). These sequences deviated from the genomic sequence through ad-

dition of extra non-templated adenine residues (up to 18 nucleotides) in the internal A-rich domain of BC200 RNA. When cDNA clones of BC200 RNA were first sequenced in 1993, length heterogeneity in the A-rich region was also observed, but was interpreted as errors during cDNA synthesis (Tiedge et al., 1993). In our experiment, the insertion of non-templated adenine residues could result from errors during RT-PCR, because the error rate of the polymerase increases and causes sequencing errors in repeat sequences such as the internal A-rich region (Shinde et al., 2003). However, we found no more than one adenine nucleotide when in vitro-transcribed BC200 RNA was used for analysis. Therefore, it is unlikely that the extensive insertions of adenine nucleotides resulted from replication slippage during RT-PCR. When total RNA isolated from BC200-overexpressing HeLa cells was used, overexpressed BC200 RNA showed results similar to those obtained from endogenous BC200 RNA, suggesting that the extensive insertion of A residues is caused by non-templated nucleotide incorporation.

Next, we examined whether adenine nucleotide insertions in BC200 seen in HeLa cells is a general phenomenon in cells expressing BC200 RNA. Accordingly, we analyzed the sequences of BC200 RNAs expressed in four breast cancer cell lines (T47D, MDA-MB-231, Hs578t, and MCF7), one normal breast cell line (MCF10A), and one normal keratinocyte cell line (HaCat). We also included HeLa cells as a control in this analysis. Sequences of about 20 clones of BC200 RNA molecules from each cell line were analyzed as described above. Although the number of inserted adenine nucleotides differed according to cell type, all cells examined showed adenine nucleotide insertions (Fig. 2), suggesting that adenine nucleotide insertion into BC200 RNA is a common phenomenon. The average length of inserted A residues in each cell type ranged from 4.75 to 9.70, with a rank order of T47D > HeLa > MDA-MB-231 > Hs578t > MCF7 > HaCat ≈ MCF10A, showing that cancerous BC200 RNA has longer adenine insertions than noncancerous BC200 RNA.
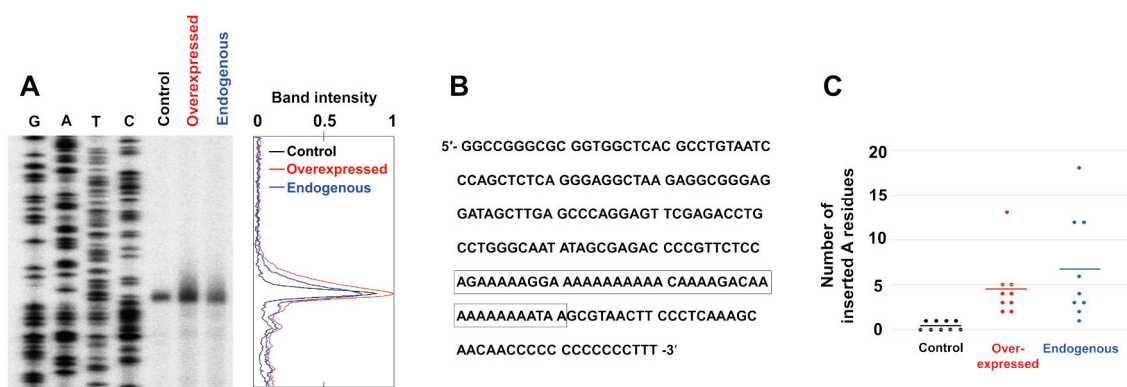

Fig. 1. Sequence heterogeneity of BC200 RNA in HeLa cells through adenine insertion. (A) BC200-specific primer extension products, generated from each RNA, were analyzed on 5% polyacrylamide sequencing gels containing 8 M urea. G, A, T, and C indicate the sequencing ladders obtained using the same primer. Band intensity was quantified. (B) The internal A-rich region is indicated on the primary sequence of BC200 RNA. (C) The number of inserted A residues in BC200 RNA was analyzed (n = 9). The straight bar in each lane indicates the average value. Control, in vitro transcribed BC200 RNA; Overexpressed, total RNA from HeLa cells transfected with pTC1010BC; Endogenous, total RNA from non-transfected HeLa cells.

The internal A-rich domain of BC200 RNA is a functionally important region involved in interaction with heterogeneous nuclear ribonucleoproteins (hnRNP) E1/E2, PABP, and eIF4A (Jang et al., 2017; Kondrashov et al., 2005). These interactions are closely related to the intracellular function and distribution of BC200 RNA (Eom et al., 2011; Shin et al., 2017b). We examined whether the length of the stretch of adenine residues affects the function of BC200 RNA. For this purpose, we synthesized BC200 RNAs with an extra 11 adenine nucleotides (BC200_A11) *in vitro* and analyzed its binding affinity for eIF4A, a key target protein of BC200 RNA that plays an important role in BC200 RNA-mediated translation inhibition
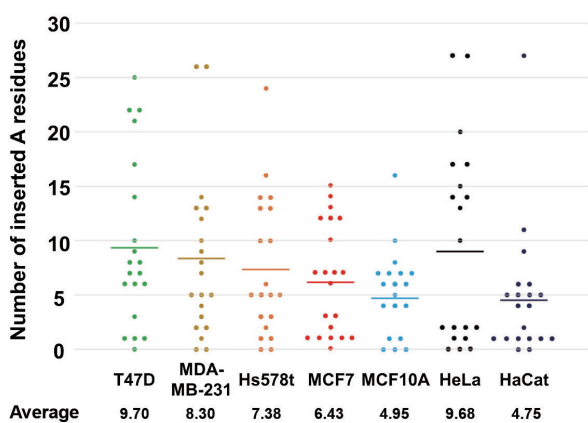
(Lin et al., 2008). Filter binding assays revealed that the affinity of BC200_A11 RNA for eIF4A was ~2.5-fold higher than that of control BC200 RNA lacking extra adenine nucleotides, suggesting that the presence of extra adenine nucleotides increases the binding affinity of BC200 RNA for eIF4A (Figs. 3A and 3B).

We then examined whether the extra adenine nucleotides affect BC200 RNA-mediated translation inhibition. In addition to BC200 RNA_A11, we synthesized two additional derivatives, one with six extra adenine nucleotides (BC200_A6) and one with 24 extra adenine nucleotides (BC200_A24), and analyzed their ability to inhibit translation in a rabbit reticulocyte system (Figs. 3C and 3D). We found that the translational inhibitory activity of BC200 RNA increased with increasing length of extra adenine nucleotides, suggesting that the adenine nucleotide stretch of BC200 RNA and its length play roles in enhancing BC200 RNA-mediated translation inhibition.

BC200 RNA is involved in promoting the tumorigenesis and development of cancer cells through various mechanisms (Samson et al., 2018; Shin et al., 2018). Since BC200 RNA knockdown reduces cell motility, we examined the effects of the extra adenine nucleotides on this cellular property. To this end, we knocked down endogenous BC200 RNA in HeLa cells, and then transfected cells with plasmids expressing BC200 RNA containing varying lengths of extra adenine residues. The migratory behavior of transfected HeLa cells was tested in wound-healing assays (Fig. 4). We found that all BC200 RNAs, regardless of the length of adenine nucleotide residues, rescued the BC200 RNA knockdown-induced reduction in HeLa cell mobility. The absence of additional effects of extra adenine nucleotides might indicate that these incorporated adenine nucleotides were sufficiently effective without additional incorporation; the observation that incorporation
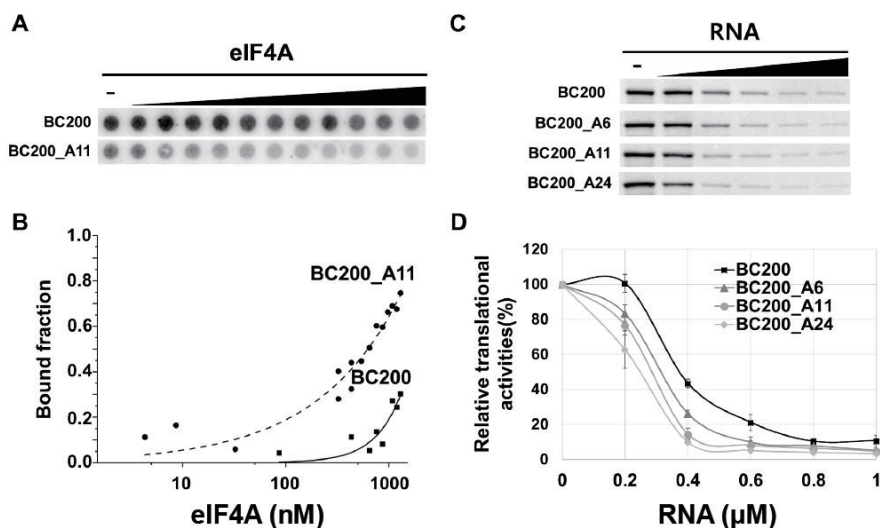

	T47D	MDA-MB-231	Hs578t	MCF7	MCF10A	HeLa	HaCat
Average	9.70	8.30	7.38	6.43	4.95	9.68	4.75

Fig. 2. Sequence heterogeneity of BC200 RNA in cancer cells. The number of additional adenine residues in BC200 RNA from various cell lines was analyzed. The straight bar in each lane indicates the average value.

Fig. 3. Effects of adenine nucleotide insertion in BC200 RNA on eIF4A-binding affinity and translation inhibition activity. (A) Increasing amounts of eIF4A were incubated with 0.3 nM BC200 RNA or BC200 RNA_A11. Dot-blot experiments showed that only unbound RNA was absorbed onto a Hybond-XL membrane. (B) The ratio of eIF4A bound to each RNA was analyzed. (C) Increasing amounts of RNA were added to the *in vitro* translation system, and expression levels of luciferase protein were analyzed by gel electrophoresis. (D) Relative translational activities are expressed as the ratio of protein levels in the presence versus absence of RNA.
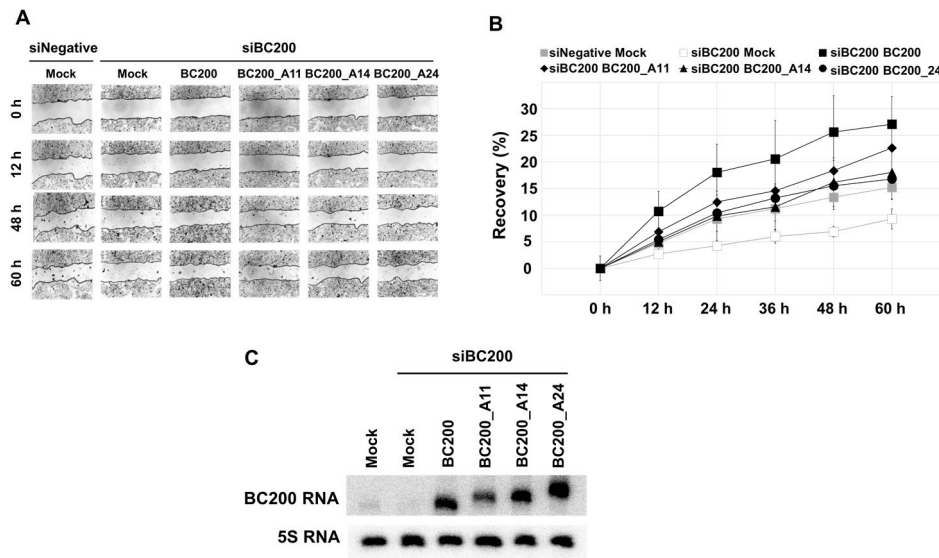
Fig. 4. Effects of multiple adenine nucleotide insertions on BC200 RNA-induced mobility of HeLa cells. (A and B) HeLa cells were transfected with small interfering RNA (siRNA) against BC200 (siBC200) or scrambled control siRNA (siNegative), incubated for 24 h, and then mock-transfected (Mock) or transfected with each form of BC200 RNA expression plasmid DNAs (pSUPER-200, pSUPER-BC200_A6, pSUPER-BC200_A11, and pSUPER-BC200_A24). Cells were scraped (wounded) 48 h after the initial transfection, and the degree of recovery was measured 0, 12, 24, 36, 48, and 60 h post-wounding. (A) Representative pictures, 40× magnification. (B) Quantitative analyses of wound-healing results. The percentage of recovery was measured and estimated based on the initial wound size of each sample (mean ± standard deviation; n = 3). (C) RNA expression from the corresponding plasmid DNAs was confirmed by analyzing total RNAs from transfected cells by Northern blotting.

of extra adenine nucleotides occurred even in BC200 RNA expressed from plasmids containing the BC200 RNA gene with no extra adenine residues (Fig. 1) may also be informative in this context. Although we did not observed the effects of extra adenine nucleotide addition in wound-healing assays, we believe that the presence of extra adenine nucleotides is likely somehow involved in cancer pathogenesis.

In this study, we found that extra non-templated adenine residues are inserted in BC200 RNA. Although it remains to be demonstrated how this occurs in the cell, we propose that extra adenine nucleotides are incorporated into BC200 RNA transcripts when RNA polymerase slips on the dA-rich region of the BC200 RNA gene during transcription. In bacteria, adenine nucleotide insertion frequently occurs during transcription of consecutive adenine sequences through transcriptional slippage (Baranov et al., 2005). When the *dnaX* gene is expressed in *Thermus thermophilus*, two types of proteins are expressed owing to a frame-shift caused by adenine insertion (Larsen et al., 2000). In human cells, adenine nucleotide insertion has also been observed at consecutive adenine residues of FAS mRNA (Wu et al., 2011). If this were the case for BC200 RNA, which is transcribed by RNA polymerase III (Kim et al., 2017; Tiedge et al., 1993), cancer cells would be more apt to exhibit transcriptional slippage during transcription of BC200 RNA by RNA polymerase III than normal cells.

Disclosure
The authors have no potential conflicts of interest to disclose.

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