Exosomal natural antisense transcripts in culture supernatants released from colorectal cancer SW480 cells.

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Abstract

Introduction: Exosomes are extracellular vesicles, which are released from most cells, involved in cell-to-cell communication. Exosomes contain membrane and cytosolic components such as proteins, lipids, messenger Ribonucleic Acids (mRNAs), and microRNAs. However, the existence of other types of RNAs in these exosomes is unknown.

Methods: In the present study, the presence of Natural Antisense Transcripts (NATs) transcribed from reverse strand deoxynucleic acids of genes in exosomes released from colorectal cancer SW480 cells were investigated using a sense/antisense custom microarray.

Results and discussion: When signal intensities with values of more than 100 were selected, 6,218 NATs were detected in exosomes released from SW480 cells. Among them, NATs transcribed from transcription factor 7-like 1 genes, which had the lowest signal intensities, were detected using strand-specific reverse transcription-polymerase chain reaction. Thus, a large number of NATs as well as mRNAs and microRNAs are present in exosomes and are likely to be delivered to other cells.

Introduction

Natural Antisense Transcripts (NATs) are Ribonucleic Acids (RNAs) that are transcribed from the opposite deoxynucleic acids (DNAs) strand of a gene and overlap in part with sense transcripts. NATs have been discovered using the analyses of human and mouse genome sequence project and full-length complementary DNAs (cDNAs). Kiyosawa et al. classified sense/antisense transcript pairs according to overlap patterns and protein or non-protein-coding patterns [1]. The functional annotation of the mammalian genome transcriptome sequencing consortium identified NATs for more than 70% of transcription units, most of which represent non-protein-coding RNAs [2]. A number of mechanisms have been proposed for NATs-mediated regulation of sense transcripts, including messenger RNAs (mRNAs). NATs localized in the nucleus are involved in transcriptional interference regulation, chromatin induction and DNA epigenetic changes, genomic imprinting, X-chromosome inactivation and alternative splicing [3-7]. NATs in the cytoplasm are involved in mRNA stability regulation, translational inhibition and endogenous small interfering RNA formation [8-10].

Recently, some researchers have investigated the role of NATs in several diseases. Differential NAT expressions were observed between cancerous tissues and the corresponding non-cancerous tissues obtained from colorectal and breast cancers [11,12]. In addition, Matsui et al. discovered that NATs of inducible nitric oxide synthase (iNOS), an important gene in inflammatory diseases, increased the stability of iNOS mRNA [8]. Furthermore, Faghihi et al. reported that NATs of beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1) increased the stability of BACE1 mRNAs by forming RNA duplex [13]. Thus, it is suggested that NATs are associated with the development and progression of several diseases.

Exosomes are extracellular vesicles (approximately 40-200 nm in diameter) of endocytic origin released from cells and contain proteins, lipids, and nucleic acids (DNAs and RNAs) derived from the cell [14]. Valadi et al. discovered mRNAs and microRNAs inside exosomes released from mast cells, showing for the first time that mRNAs and microRNAs could be delivered from one cell to other cells [15]. ExoCarta (http://www.exocarta.org/) is a database of exosomal components identified by both published and unpublished studies [16]. At this time, proteins (4,563 entities), mRNAs (1,639 entities), and microRNAs (764 entities), and lipids (194 entities) have been registered in the database (release date: May 29, 2012). It was first identified NATs transcribed from leucine-rich repeat-containing 24 (LRRC24), MDM2 proto-oncogene E3 ubiquitin protein ligase (MDM2), and cyclin-dependent kinase inhibitor 1A (*CDKN1A*) genes in exosomes released from three human colorectal cancer cells [17]. However, the types of exosomal NATs are unknown till date.

In the present study, the types of exosomal NATs were investigated using human colorectal cancer SW480 cells and a custom microarray containing human sense/antisense probes for approximately 21,000 genes.

Materials and Methods

Cell cultures

The human colorectal cancer cell line SW480 (CCL-228) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). These cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Collection of exosomes from culture supernatants

SW480 cells were plated on collagen-coated 10-cm dishes at 1 × 10^6 cells per dish with the culture media described above. After 72 h, the culture medium was discarded, and the cells were washed three times in a serum-free culture medium. Then, 10 ml serum-free culture medium was added to each dish, and the cells were cultured for 48 h. Cell cultures from each dish were collected and subjected to a sequence of centrifugation steps. Cell cultures were first centrifuged at 300 × g at 4°C for 3 min to remove floating cells. These supernatants were then centrifuged at 2,000 × g at 4°C for 15 min and collected in new tubes. In addition, culture supernatants were centrifuged at 12,000 × g at 4°C for 35 min to remove cellular debris, and these supernatants were collected in new tubes. Supernatants were then filtered through a 0.22 µm filter. These filtrates were ultra-centrifuged at 120,000 × g at 4°C for 70 min to collect exosomes using the Optima TLX Ultracentrifuge (Beckman Coulter, Brea, CA). Exosome pellets were washed in Dulbecco's phosphate buffered saline (D-PBS) and further ultra-centrifuged at 120,000 × g at 4°C for 70 min. Finally, exosome pellets were re-suspended in D-PBS.

RNA extraction

Isogen II (NipponGene, Tokyo, Japan) was used to isolate exosomal RNAs from exosome pellets. Exosomal RNAs were stored at -80°C until further use. The concentrations of RNAs extracted were examined using the Quant-iT RiboGreen RNA Reagent and Kit (ThermoFisher Scientific, DE, USA), according to the manufacturer's instructions. In addition, the size of the exosomal RNA was confirmed using the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Kit (Agilent Technologies, Foster City, CA), according to the manufacturer's instructions.

Microarray analysis

Exosomal RNAs (1 ng) derived from SW480 cells were amplified using Ovation Pico WTA System V2 (NuGEN

Technologies, San Carlos, CA). Amplified exosomal RNAs were purified using the QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA). Cyanine 3 (Cy3)-labelled cDNAs were synthesized using the Agilent Genomic DNA Enzymatic Labeling Kit (Agilent Technologies), according to the manufacturer's instructions. Cy3-labeled cDNAs were purified using the QIAquick Nucleotide Removal Kit (Qiagen). The concentration and quality of Cy3-labeled cDNAs were measured using the Agilent 2100 Bioanalyzer. Agilent 44 K × 4 human custom microarray slides (Agilent eArray Design ID=19052, produced by Tsukuba GeneTech Laboratory, Ibaraki, Japan) were hybridized with Cy3-labeled cDNA (5 µg) in hybridization solution and blocking agent (Agilent Technologies) at 65°C for 24 h using a hybridization oven. Microarray slides were washed in wash Buffer 1 and 2 (Agilent Technologies) including 0.005% Triton X-102. Washed microarray slides were dried using nitrogen gas. Cy3 fluorescence images of array slides were obtained using a DNA microarray scanner (Agilent Technologies) and were processed using Feature Extraction version 8.1 software based on instructions from Agilent Technologies.

Strand-specific reverse transcription polymerase chain reaction (RT-PCR)

First-strand cDNAs from NATs were synthesized using exosomal RNAs (1 ng), RT primer, and AMV Reverse Transcriptase (Promega, Madison, WI) according to the procedure recommended by Promega. The mixtures were then incubated at 50°C for 60 min. The resulting cDNAs were incubated at 99°C for 5 min and at 37°C for 60 min with ribonuclease A to digest RNAs. The first-strand cDNAs were subjected to ordinary PCR using forward and reverse primers.

The detection of NATs by PCR was performed using first-strand cDNAs, FastStart Universal SYBR Green Master (Roche Diagnostics, Basel, Switzerland), and primer pairs. PCR analyses were performed using the StepOne Plus Real-Time PCR system (RT-PCR; Life Technologies, Carlsbad, CA, USA) for 1 min at 95°C followed by 40 cycles each of 95°C for 15 s and 60°C for 60 s, following the manufacturer's instructions. The PCR products obtained above were electrophoresed using 4% agarose gels. The detection of amplified fragments was achieved by ethidium bromide staining using the ChemiDoc XRS (Bio-Rad) and the Quantity One software (Bio-Rad).

Results and Discussion

Detection of exosomal RNAs released from SW480 cells

To confirm that exosomes released from SW480 cells had been collected, exosomal RNAs were extracted from the precipitation of cells or exosomes obtained from supernatants by ultracentrifugation using Isogen II reagent. Exosomal and/or cellular RNAs obtained were electrophoresed using the Agilent 2100 Bioanalyzer. The 18S and 28S ribosomal RNAs were detected from the RNAs of SW480 cells (Figure 1A). On the

other hand, their ribosomal RNAs were barely detected in exosomal RNAs and small RNAs were detected in the size range of 25-200 nucleotides (nt) in these exosomal RNAs (Figure 1A). It was reported that several small RNAs were observed in exosomes [17]. This indicates that exosomes released from the supernatants of SW480 cells were collected in the present study.

Expression profiles of exosomal NATs

Many researchers have shown that exosomes released from cells include microRNAs that are delivered to other cells [15,17-20]. Exosomal RNAs other than microRNAs have also been reported. Valadi et al. reported that exosomes released from mast cells included mRNA [15]. In addition, Lambertz et al. demonstrated that transfer RNAs (tRNAs) were detected in exosomes released from Leishmania [21]. In an earlier study, it was found that NATs of MDM2 and *CDKN1A* genes in exosomes released from human colorectal cancer cells [17]. However, the types of NATs in exosomes are unknown till date. In the present study, the types of exosomal NATs were investigated using human colorectal cancer SW480 cells and a custom microarray containing human sense/antisense probes.

Table 1. *Top 10 (of the 6,218 indicated) natural antisense transcripts in exosomes released from SW480 cells.*

Accession number	Gene symbol	Description	Signal value (g processed signal)
XM_001127227.1	*LOC728331*	PREDICTED: Homo sapiens hypothetical protein LOC728331 (LOC728331), mRNA	119623.8
NM_033301.1	*RPL8*	Homo sapiens ribosomal protein L8 (RPL8), transcript variant 2, mRNA	86993.31
NM_001015.3	*RPS11*	Homo sapiens ribosomal protein S11 (RPS11), mRNA	78607.87
NM_004559.2	*YBX1*	Homo sapiens Y box binding protein 1 (YBX1), mRNA	75369.76
XM_001126335.1	*LOC727826*	PREDICTED: Homo sapiens similar to ribosomal protein S11 (LOC727826), mRNA	73983.69
NR_002202.1	*FTHL7*	Homo sapiens ferritin, heavy polypeptide-like 7 (FTHL7) on chromosome 13	73893.05
XR_016327.1	*LOC338870*	PREDICTED: Homo sapiens similar to ribosomal protein S12 (LOC338870), mRNA	69479.47
XR_015536.1	*LOC731599*	PREDICTED: Homo sapiens hypothetical protein LOC731599 (LOC731599), mRNA	68422.79
NM_000973.3	*RPL8*	Homo sapiens ribosomal protein L8 (RPL8), transcript variant 1, mRNA	68120.92
NM_006401.1	*ANP32B*	Homo sapiens acidic (leucine-rich) nuclear phosphoprotein 32 family, member B (ANP32B), mRNA	67702.16

Table 2. *Bottom 10 (of the 6,218 indicated) natural antisense transcripts in exosomes released from SW480 cells.*

Accession number	Gene symbol	Description	Signal value (g processed signal)
NM_014028.3	*OSTM1*	Homo sapiens osteopetrosis associated transmembrane protein 1 (OSTM1), mRNA	101.94
NM_006184.3	*NUCB1*	Homo sapiens nucleobindin 1 (NUCB1), mRNA	101.8
XM_001127824.1	*LOC728574*	PREDICTED: Homo sapiens similar to retinitis pigmentosa GTPase regulator isoform C (LOC728574), mRNA	101.71
NM_022727.4	*HTF9C*	Homo sapiens HpaII tiny fragments locus 9C (HTF9C), transcript variant 1, mRNA	101.67
NM_001040445.1	*ASB1*	Homo sapiens ankyrin repeat and SOCS box-containing 1 (ASB1), mRNA	101.56
NM_147152.1	*ITSN2*	Homo sapiens intersectin 2 (ITSN2), transcript variant 2, mRNA	101.43
NM_152682.1	*RWDD4A*	Homo sapiens RWD domain containing 4A (RWDD4A), mRNA	101.38
NM_000292.1	*PHKA2*	Homo sapiens phosphorylase kinase, alpha 2 (liver) (PHKA2), mRNA	101.19

NM_031283.1	*TCF7L1*	Homo sapiens transcription factor 7-like 1 (T-cell specific, HMG-box) (*TCF7L1*), mRNA	101.19
NM_002269.2	*KPNA5*	Homo sapiens karyopherin alpha 5 (importin alpha 6) (KPNA5), mRNA	100.5

A high peak of small RNAs was detected in exosomal RNAs from SW480 cells (Figure 1A). Because NATs include RNAs of longer lengths, it is necessary to amplify exosomal RNAs for the microarray analysis of NATs. Dafforn et al. reported small amounts of RNA amplification method, Ribo-SPIA, which generates micrograms of labelled cDNAs from 5 ng total RNAs in 1 day for microarray analysis [22]. Here I identified NATs using amplified exosomal RNAs by Ribo-SPIA method and a sense/antisense custom microarray. In addition, the reproducibility of this experiment was investigated. When signal intensities with a value of more than 100 were selected, 6,218 NATs were detected in exosomes released from SW480 cells (Tables 1 and 2). The amplified exosomal RNAs prepared using the Ribo-SPIA procedure indicate an accurate amplification of gene expressions ($R2=0.9985$) (Figure 1B). This result indicates that a large number of NATs were observed in exosomes released from SW480 cells. This data (GEO accession: GSE68979) is registered in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).
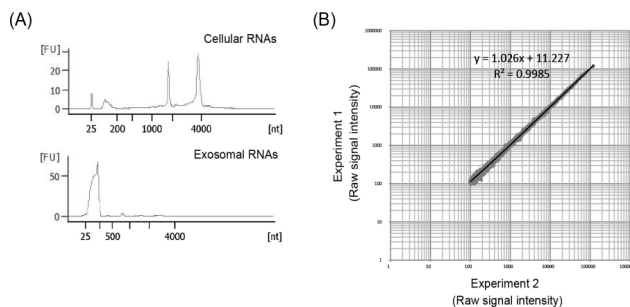

Figure 1. Detection of exosomal RNAs released from SW480 cells. (A) Detection of exosomal RNAs released from SW480 cells. Different types of small RNAs were observed in exosomes released from SW480 cells, whereas an Agilent 2100 Bioanalyzer barely detected the peaks of ribosomal RNAs (18S and 28S); (B) A scatter plot of exosomal NATs using two microarray data. The signal intensities between two microarrays are highly reproducible.

Detection of exosomal NATs of TCF7L1 gene

To validate these exosomal NATs as detected above, NATs transcribed from transcription factor 7-like 1 (*TCF7L1*) gene, which were found to have the lowest signal intensities, were investigated using strand-specific RT-PCR. It is difficult to specifically detect NATs by the reaction of reverse transcription using oligo dT primer or a random primer because a part of NATs has the complementary sequence within a region of mRNAs (Figure 2A). Therefore, I synthesized strand-specific cDNA derived from NATs of *TCF7L1* using RT primer (Table 3). The obtained cDNAs were subjected to RT-PCR using a SYBR green reagent and amplified. These amplicons were electrophoresed using 4% agarose gel and stained with ethidium bromide. Band of *TCF7L1* NATs detected using ultraviolet irradiation were 53 base pair (bp), respectively (Figure 2B). This indicates that NATs of *TCF7L1* are present in exosomes released from SW480 cells, suggesting that other higher-intensity NATs detected using microarray analysis are also present in these exosomes.
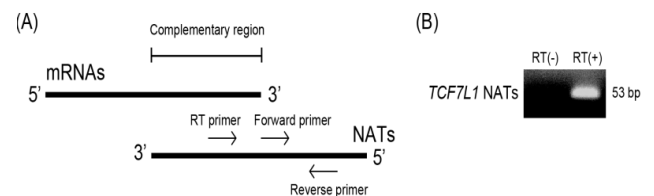

Figure 2. Detection of exosomal natural antisense transcripts (NATs) using strand-specific reverse transcription polymerase chain reaction (RT-PCR). (A) Schematic diagram used to detect NATs. Strand-specific cDNAs derived from NATs were synthesized using RT primer as shown in Table 3. To amplify cDNA derived from NATs, PCRs were performed using forward and reverse primers; (B) Detection of products amplified by PCR. The PCR products were electrophoresed using 4% agarose gels and stained using ethidium bromide.

Recently, the analyses of exosomal components were performed using next-generation sequencers. Vojtech et al. identified exosomal piwi-RNAs (small RNAs (26-31 nt) expressed primarily in germline cells) and Y RNAs (small RNAs (83-112 nt) involved in alternative splicing and regulating translation) from human semen exosomes using the HiSeq 2000 sequencing system by Illumina [23]. Their exosomal RNAs may be also delivered to other cells and functions in target cells. It is necessary to elucidate whether a number of NATs identified in the present study are delivered to other cells and their functions in target cells. In conclusion, a large number of NATs are present in exosomes released from colorectal cancer cells, suggesting that these NATs are also delivered to other cells.

Table 3. Primers for strand-specific reverse transcription polymerase chain reaction.

Primer name	Accession no.	Sequence (5′-3′)	Size (nt)	Amplicon size (bp)
TCF7L1 RT primer	NM_031283.1	AGCTTTCAGTGGAGAAAG	18	-

TCF7L1 forward primer	GGTCCTCTGAAATGGTTTC	19	53
TCF7L1 reverse primer	CACCTTGAGTCCCTTTAAATG	21	

Conclusion

A large number of NATs are present in exosomes released from SW480 cells.

Competing Interests

No competing interests exist.

Acknowledgement

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