이학석사 학위논문

Altered expression of exosomal microRNAs in *Toxoplasma gondii*-infected BV2 microglial cells

: Its role in regeneration of glioblastoma cells

톡소포자충이 감염된 BV2 미세교세포에서 엑소좀 miRNA의 발현 변화

: 신경교종세포 발달에 가능한 역할에 대해

2016년 2월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과

송 혜 미

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이 논문을 이학석사 학위논문으로 제출함

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송 혜 미의 이학석사 학위논문을 인준함

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위 원 장 _____ (인)

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ABSTRACT

Altered expression of exosomal microRNAs in *Toxoplasma gondii*-infected BV2 microglial cells : Its role in regeneration of glioblastoma cells

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Toxoplasma gondii (*T. gondii*) is an intracellular protozoan parasite that modulates the environment of the infected host. Exosomal microRNAs derived from various protozoan-infected host cells are known to be a regulator for survival of parasites through inhibition of microbicidal functions of the infected host cells. The favorable environment manipulated by *Toxoplasma* resembles tumor microenvironment and may help to induce the incidences of brain tumors. However, the possible mechanisms involved in *Toxoplasma* infection inducing brain tumors are not well understood. In this study, in order to investigate the possible role of *Toxoplasma* infection in brain tumor development, the author isolated and characterized the exosomes from murine microglial cells (BV2 cells) infected with RH or

ME49 strain of *T. gondii*. The author analyzed exosomal miRNAs expression profiles by microRNA array in the BV2 cells and validated them by quantitative reverse-transcription PCR (qRT-PCR) in part. Fourteen miRNAs known to be related to tumor or host immunity were significantly altered in both RH and ME49 strain *Toxoplasma*-infected BV2 cells. The author used various web tools such as functional disease ontology analysis, i.e., FunDO, for the purpose to find out related diseases involved with the targeted genes of the significantly regulated miRNAs. As a result, altered miRNAs by *Toxoplasma* infection showed the positive correlations with tumor progression and the brain tumor was mainly related to the predicted genes of up-regulated miRNAs ($P < 0.05$). Furthermore, the author confirmed that *Toxoplasma*-infected BV2 exosomes could be internalized by host cells and induced the regeneration of U87 glioblastoma cells *in vitro*. These results supported the close relationship between *Toxoplasma* and glioblastoma regeneration. In conclusion, the author confirmed altered expression of exosomal miRNAs in *Toxoplasma*-infected BV2 microglial cells and certainly suggested that *Toxoplasma*-infected microglia cell-derived exosomes play an important role as a regulator for the regeneration of glioblastoma cells.

Keywords: *Toxoplasma gondii*, Microglial cell, Exosome, microRNA, glioblastoma

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INTRODUCTION

Toxoplasma gondii (*T. gondii*) is a universal parasitic microorganism that generally infects most warm-blooded animals like humans through various survival strategies (Tenter et al., 2000). The protozoan parasite was estimated to infect about 30% people worldwide and the entire human population is at a risk for exposure to infection (Tenter et al., 2000; Pappas et al., 2009). *T. gondii* is considered to be a serious pathogen that can invade cells of vital organs including the central nervous system (CNS); however, the symptoms caused by *T. gondii* are usually mild and asymptomatic in immunocompetent hosts. This phenomenon is probably due to an immune-evasion mechanism of the parasites in which the parasites seclude themselves into tissue cysts via stage conversion from the acute phase tachyzoites to the chronic phase bradyzoites (Aliberti, 2005).

Toxoplasma can also modulate the environment of infected hosts to disseminate and establish asymptomatic lifelong infection in its hosts (Lambert and Barragan, 2010) and has significant effects on host behavioral pathology through modulation of the cell cycle and transcription and evasion of immune responses (Blader and Saeij, 2009). Interestingly, the habitable environment during lifelong infection of *Toxoplasma* within its hosts is analogous to the tumor microenvironment. The correlation between *Toxoplasma* prevalence

and brain tumor incidence was supported by several previous reports (Thirugnanam et al., 2013). The first proposed some relationships between *Toxoplasma* infection and cancer development (Schuman et al., 1967) and presented sero-epidemiological evidences regarding to the association between toxoplasmosis and brain cancer which were consistently followed by several researchers (Vittecoq et al., 2012; Thomas and Lafferty, 2012; Cong et al., 2015). However, the mechanisms of *Toxoplasma* infection inducing brain cancers in its host are not well understood.

The tumor microenvironment is a cellular environment including a variety of cellular components containing immune cells, blood vessels and extracellular matrices (ECM) that surround the tumor cells. Tumor cells can influence the microenvironment by releasing tumor-derived molecules for promoting tumor angiogenesis and inducing peripheral immune tolerance. Especially, tumor-associated macrophage (TAM) has main roles in tumor metastasis, vascularization and immune modulation (Zhang et al., 2012). In addition, various tumor-derived molecules drive TAMs toward an anti-inflammatory phenotype. It is normally accepted that the TAMs have similar functions to the M2-like activated phenotype that is produced from alternative activation of macrophages (AAMs) (Solinas et al., 2009) and assist pro-tumorigenic and pro-angiogenic activities by producing various growth factors and pro-angiogenic cytokines (Komohara et al., 2008). Microglia, i.e., resident macrophages of the central nervous system,

make a variety of responses in defense from pathogen infection and remodel the tissue injury for sustaining the tissue homeostasis. Also, it substantially contributes to the tumor mass of glioblastoma and has been shown to play an important role in regulating glioblastoma growth and invasion (Li et al., 2012).

Toxoplasma preferentially infects host dendritic cells, monocyte/macrophage lineage cells such as microglia (Butcher et al., 2011) and promotes the differentiation of M2 phenotype macrophages which influence wound repair after pathogen infections and is also associated with a high degree of vascularization and granuloma fibrosis (Albina et al., 1990; Kodelja et al., 1997; Kreider et al., 2007; Raes et al., 2007). Moreover, the role of *Toxoplasma* modulating BV2 microglial cells in host environment resembles that of TAMs expressing arginase-1, interlukine-10 (IL-10), and transforming growth factor (TGF)-β in experimental human neoplasms model; these molecules reduce the anti-tumor activity of T cells and natural killer cells (Hao et al., 2013).

Exosomes are cell-derived vesicles that influence the tumor related biological process such as anti-apoptosis, angiogenesis, and metastasis within the tumor microenvironment (Azmi et al., 2013). Exosomes are known as cell-to-cell communicators delivering biological contents such as nucleic acid and protein from neighboring to distant cells and influence many immunological, cellular and metabolic activities in the recipient cells (Deolindo et al., 2013).

Furthermore, the level of miRNA content in exosomes was significantly regulated according to whether cells were affected on *Toxoplasma* (Pope et al., 2013). In addition, Thirugnanam et al. (2013) hypothesized that *Toxoplasma* promotes brain carcinogenesis by changing its expression on specific host miRNAs related to tumor development.

Therefore, the author especially focused on the altered exosomal miRNAs in *Toxoplasma*-infected host microglial cells and perceived its possible role in regeneration of glioblastoma. This findings are expected to provide a framework of relevance between *Toxoplasma* infection and glioblastoma and reinforce the need for further *in vivo/vitro* researches for observing the effect of miRNAs contained in exosomes.

MATERIALS AND METHODS

1. Cell culture

BV2 murine microglial cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene), 4 mM L-glutamine, 0.2 mM penicillin, 0.05 mM streptomycin (Welgene). U87 human glioblastoma cells were cultured in Roswell Park Memorial Institute medium (RPMI; Welgene) containing 10% FBS, 4 mM L-glutamine, 0.2 mM penicillin, 0.05 mM streptomycin. These cells were cultured at 37°C in a 5% CO_2 incubator.

2. *Toxoplasma gondii* infeciton

RH strain

Tachyzoites of *T. gondii* (RH strain) were collected from the peritoneal cavity of 6 week olds BALB/c mice that had been previously injected 3-4 days before. The tachyzoites were washed 3 times with phosphate-buffered saline and purified by centrifugation over 40% Percoll (Sigma-Aldrich, St. Louis, Missouri, USA).

ME49 strain

T. gondii, ME49 strain, was maintained by injecting cysts isolated from the brains of infected C57BL/6 mice (Orient Bio Animal Center, Seongnam, Korea) intraperitoneally. The brains of mice infected with ME49 strain were harvested at 3 months post-infection and minced to isolate cysts. Cysts were then isolated under a microscope and that of 15 were inoculated intraperitoneally into each experimental mouse. Infected mice were raised under SPF conditions at Seoul National University College of Medicine.

Toxoplasma lysate antigens (TLA)

TLA was prepared as previously described with slight modification (Lim et al., 2012). Briefly, tachyzoites of *T. gondii* (RH strain) were obtained from the peritoneal exudates of infected mice. Exudates were passed twice through a 25-gauge needle and then 5 μm filter membrane to remove debris and host cells. Parasites were resuspended in PBS (pH 7.4), and sonicated on ice. The supernatant (TLA) was filter-sterilized through a 0.22 μm membrane, and the protein concentration in TLA was determined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Rockford, IL, USA). TLA was stored at -70°C until required.

3. RNA purification

Total RNA was extracted from BV2-derived exosomes or BV2 cells using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions including incubation time at elution step. The RNA concentration was determined by NanoDrop 2000 Spectrophotometer. RNA quality for microarray was assessed by Agilent 2100 bioanalyser (Agilent Technologies, Amstelveen, Netherlands).

4. Exosomes isolation

DMEM with 10% exosome-depleted FBS was prepared by ultracentrifugation at 100,000g for 16 hours at 4°C with filtration through a 0.22-μm filter (Nalgene, Rochester, NY, USA). 5 $\times 10^5$ BV2 cells were cultured in DMEM with 10% exosome-depleted FBS and 1% antibiotics at 37°C and 5% CO_2. After 24 hours incubation, the tachyzoites of RH or ME49 strain (MOI=10) were added into the cells and non-invaded parasites were removed in an hour and refilled with the DMEM.

Exosomes from BV2 cells were isolated by differential centrifugation which is the most widely used method. In brief, the BV2 cell supernatants were harvested and centrifuged at 300g for 10 minutes at 4°C. The supernatants were transferred to new tubes and

centrifuged at 2,000g for 10 minutes at 4°C. The supernatants were transferred to new tubes and centrifuged at 10,000g for 30 minutes at 4°C. The supernatants were transferred to ultracentrifuge tubes and ultracentrifuged at 100,000g for 70 minutes at 4°C. For washing, the exosome pellet in each tube was resuspended in 1 ml PBS and collected the mixture into a centrifuge tube. The tube was filled with PBS completely and ultracentrifuged at 100,000g for 70 minutes at 4°C again. The pellet consisting of exosomes was resuspended in 300 μl of PBS for RNA or protein analysis. The concentration of isolated exosome was determined by BCA protein assay kit (Pierce, Rockford, IL, USA) and NanoDrop 2000 Spectrophotometer.

5. Characterization of exosomes

SDS-PAGE electrophoresis

The pellet from either BV2 cells or BV2-derived exosomes was lysated in PRO-PREP™ protein extraction solution (iNtRon Biotechnology, Seongnam, Korea) for 30 minutes on ice. The lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C and protein concentration was determined by BCA protein assay kit and NanoDrop 2000 Spectrophotometer. An equal amount 30 μg of protein was loaded on 10% SDS polyacrylamide gel. The gel was run at 120 V, 200 mA for 150 minutes and stained with Coomassie Brilliant Blue.

Western blotting

Proteins were separated by SDS-PAGE and transferred to PVDF membranes, which were then blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 hours. Protein bands were confirmed with Alix (Cell signaling Technology, Inc., Beverly, MA, USA) antibody as exosome marker. HRP-conjugated goat anti-mouse IgG (H+L) (Bethyl Laboratories, Montgomery, TX, USA) antibody was used as a secondary antibody and immune-reactive band was visualized using a luminescent image analyzer LAS-1000 plus (Fuji Photo Film, Tokyo, Japan).

Transmission electron microscopy (TEM)

TEM analysis was performed to examine the size or morphology of exosomes. Exosomes (6.40 μg/μl) isolated from BV2 cells were prepared on carbon-coating grid and negative stained using 2% uranyl acetate for 1 minutes as previously described with minor modifications (Perez-Hernandez et al., 2015). The prepared samples were observed using a JEOL 1200-EX II (JEOL, Tokyo, Japan) equipped with an ES1000W Erlangshen CCD camera (Gatan, Pleasanton, CA, USA) at an accelerating voltage of 80 kV.

6. Confocal microscopy

BV2-derived exosomes were stained using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, St.Louis, MO, USA) for 15 minutes at room temperature. 2×10^5 U87 cells were incubated with either PKH26-labeled exosomes (red) or without as negative control for 24 hours at 37°C in a 5% CO_2 incubator. The nuclei of BV2 cells were stained with DAPI (blue) and U87 cells were fixed in 4% paraformaldehyde for 15 minutes at 4°C before observing in Leica TCS SP8 STED CW confocal system (Leica Microsystems, Mannheim, Germany).

7. microRNA microarray

MicroRNA expression was analyzed with miRCURY^{TM} LNA microRNA Array, 7th generation-has, mmu and rno array (EXIQON, Vedbaek, Denmark), covering 1,119 well-characterized mouse microRNAs among 3,100 capture probes for human, mouse and rat miRNAs. In this procedure, 5'-phosphates from 250-1000 ng of total RNA was removed by treating Calf Intestinal Alkaline Phosphatase (CIP) followed by labeling with Hy3 green fluorescent dye. Labeled samples were subsequently hybridized by loading onto a microarray slide using Hybridization Chamber Kit (Agilent Technologies, Santa Clara, CA, USA) and Hybridization Gasket Slide Kit (Agilent

Technologies, Santa Clara, CA, USA). Hybridization was performed over 16 hours at 56°C followed by washing the microarray slide as recommended by the manufacturer. Processed microarray slides were then scanned with Agilent G2565CA Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA). Scanned images were imported by Agilent Feature Extraction software version 10.7.3.1 (Agilent Technologies, Santa Clara, CA, USA) and fluorescence intensities of each image were quantified using the modified Exiqon protocol and corresponding GAL files.

8. Real-time RT-PCR

The cDNA was synthesized from miRNA with a Mir-X miRNA First Strand Synthesis and SYBR qRT-PCR Kit (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR with an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) used primers and templates mixed with the SYBR Premix. The sequence of the primers of miRNAs was designed based on miRBase (www.mirbase.org). These primers were pre-validated to generate single amplicon. DNA was amplified for 40 cycles of denaturation for 5 seconds at 95°C and annealing for 20 seconds at 60°C. The data generated from each PCR reaction were analyzed using the Data Analysis Module by iQ^{TM}5 optical system software (Bio-Rad). The specificity of reactions was

determined by melting curve analysis. The relative fold changes of gene expression between selected miRNAs and U6 were calculated by the standard curve method. Primer sequences are as follows; mmu-miR-21a-5p (MIMAT0000530), 5' TAGCTTATCAGACTGATGTTGA 3', mmu-miR-124-3p (MIMAT0000134), 5' TAAGGCACGCGGTGAA TGCC 3', mmu-miR-126a-3p (MIMAT0000138), 5' TCGTACCGTGAG TAATAATGCG 3', and mmu-miR-324-5p (MIMAT0000555), 5' CGCA TCCCCTAGGGCATTGGTGT 3'.

9. Cell migration assay

U87 glioblastoma cells were seeded and cultured up to 90% confluency in a 96-well plate. 5×10^4 U87 cells were incubated with serum-free DMEM overnight to synchronize and scratched with a 10 μl pipette tip. Then, U87 cells were incubated in 100 μl of the conditioned media with *Toxoplasma*-infected BV2-derived exosomes (50 μg/ml) or non-pulsed BV2-derived exosomes (50 μg/ml) as control for 8 hours. Cell migration was quantified as the average length of the elongation of wound edges by using Studio Lite version 1.0 (Better Light, Inc., Placerville, CA, USA).

10. Cell proliferation assay

3×10^4 U87 glioblastoma cells were seeded in a 96-well plate and incubated in 100 μl of the conditioned media with *Toxoplasma*-infected BV2-derived exosomes (50 μg/ml) or non-pulsed BV2-derived exosomes (50 μg/ml) as control for 12, 18, and 36 hours. Cell proliferation rates were determined with a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction.

11. Altered microRNAs analysis

Expression profiling of mature exosomal miRNAs in microglial cells by *Toxoplasma* infection was conducted and analyzed using various web-tools.

Targeted genes prediction

The miRNAs related with tumor development were identified experimentally by miRWalk2.0 (http://zmf.umm.-uni-heidelberg.de/apps/zmf/mirwalk2/index.html) and filtered more than 8.0 normalized signal intensity (log2). Among the miRNAs, differentially expressed miRNAs were determined through greater than 2.0-fold change filtering for analysis of miRNAs altered by *Toxoplasma* infection. Targeted genes of the miRNAs were predicted via three different miRNA target prediction web tools, miRDB (http://mirdb.org/miRDB),

TargetScan (http://www.target-scan.org) and miRanda (http://www.microrna.org) in common.

Disease ontology analysis by Functional Disease Ontology (FunDO)

The targeted genes of either 9 up-regulated or 4 down-regulated miRNAs were respectively analyzed for finding miRNAs-associated diseases by using the web tool, FunDO (http://django.nubic.northwestern.edu/fundo). The consequence of disease association was evaluated by Fisher's exact test.

RESULTS

1. Characterization of *Toxoplasma*-infected microglial cells-derived exosomes

Extracellular vesicles released from diverse cells are classified into three types like exosomes, microvesicles and apoptotic bodies (Deolindo et al., 2013). Exosomes are distinguished from other vesicles base on their different biophysical characteristics such as size, density and isolation condition. BV2-derived exosomes were obtained through differential centrifugation and confirmed using various methods to prevent contamination with either cellular components or other vesicles. SDS-PAGE showed the different pattern between extracted proteins from BV2 cells and exosomes (Fig. 1A) and the samples were evaluated for the presence of Alix, an exosomal protein marker in western blot analysis. Alix marker was detected in the exosomal proteins but not in the BV2 cell lysate proteins (Fig. 1B). In addition, purified RNA from BV2-derived exosomes was appraised by a bioanalyzer. The 18S and 28S ribosomal subunits were rarely observed in the RNA migration pattern, indicating that the purity of exosomal RNA was also reliable (Fig. 1C). Finally, electron microscopy revealed that the observed exosomes were shown in approximately

60-100 nm sizes with cup-shaped structure as typical exosome morphology (Fig. 2).

2. Expression profiling of mature exosomal miRNAs in microglial cells by *Toxoplasma* infection

The author performed microarray analysis to investigate the differences of miRNA expression profiles between *Toxoplasma*-infected and non-infected BV2 microglia-derived exosomes. Seventy-six predicted miRNAs related to host immunity and tumor development were predicted by miRWalk2.0 and hierarchical clustering was performed on top 30 up- and down-regulated miRNAs (Fig. 3A). Among 76 miRNA arrayed on RH/PBS, the expression of 14 miRNAs (10 up- and 4 down-regulated miRNAs) were significantly altered (>8 normalized signal intensity and >2-fold change) in *Toxoplasma*-infected BV2 cells, relative to non-infected cells. The author represented the considerably changed 323 miRNAs from the total analyzed 1,119 miRNAs in the Venn diagram which common region contained its selected miRNAs. This shared area showed that either host immunity or tumor development related 14 miRNAs were significantly changed in those following strains, RH or ME49, of *T. gondii* infection (Fig. 3B). The author visualized the expression of miRNAs related to the differentiation of the M2 phenotype and tumor-

angiogenesis factors in tumor microenvironment (Fig. 3C). This showed that the expression on the analyzed miRNAs such as miR-324-5p, miR-126a-3p, miR-124-3p, miR-17-3p and miR-21a-5p may indicate that the possible roles as the differentiation of the M2 phenotype and tumor-angiogenesis factors in tumor microenvironment based on the previous reports (Fig. 3C).

3. Validation of expression level of microarray results in exosomes through real-time RT-PCR.

Relative expression level of miR-21a-5p, miR-124-3p, miR-126a-3p and miR-324-5p was examined in the *Toxoplasma*-infected BV2 cells-derived exosomes by quantitative real-time RT-PCR. The data showed miR-21a-5p, miR-124-3p, miR-126a-3p and miR-324-5p exhibited significantly increased expression in exosomes. The results indicated that identical expression tendency of analyzed miRNAs by microarray. Error bars are standard error of the mean. $**P$ value $<$ 0.05 was obtained by student's t-test (Fig. 4).

4. Prediction of a correlation between significantly altered miRNAs by *Toxoplasma* infection and brain tumor in web-based tools.

The specific targeted genes related to 13 differentially expressed miRNAs by *Toxoplasma* infection were predicted via three different web tools, miRDB, TargetScan and miRanda (Fig. 5). One thousand one hundred forty-four target genes of 9 significantly up-regulated miRNAs and 1,211 target genes of 4 significantly down-regulated miRNAs were identified through three different tools in common. The author investigated relevant diseases and the pathways of the predicted genes to understand a correlation with significantly altered miRNAs by *Toxoplasma* infection.

The target genes of 13 significantly altered miRNAs were analyzed by web tool, FunDO by which the author represented diseases having relevance to target genes. One hundred and ten genes among 1,144 predicted genes targeted by 9 up-regulated miRNAs and 110 genes among 1,211 predicted genes targeted by 4 down-regulated miRNAs were found to be associated with diseases. By FunDO analysis, 10 diseases were statistically enriched with up-regulated miRNAs targets (P value < 0.05), and 11 diseases were enriched with down-regulated miRNAs targets. The author could illustrate the top 5 diseases by showing map in which the number of selected genes was proportional to the sizes of the disease nodes (Fig. 6A,B). As predicted, the author confirmed that brain tumor is mainly related to predicted genes of up-regulated miRNAs (Fig. 6C,D). Our prediction results significantly coincided with previous reports that have been showing correlation

between *Toxoplasma* prevalence and brain cancer incidence (Table 1).

5. Cellular internalization of BV2-derived exosomes into U87 glioblastoma cells

The cellular internalization of BV2-derived exosomes into U87 human glioblastoma cells were visualized by confocal microscopy to represent the exosomes might be a key role in cells environment change like pathogen infection. PKH26-labeled exosomes were localized in the cytoplasm of U87 cells of which the nuclei were stained with DAPI (Fig. 7), implying that the BV2-derived exosomes could be internalized by host cells and would influence in the environment of recipient cells.

6. *Toxoplasma*-infected BV2-derived exosomes help to regenerate U87 glioblastoma cells *in vitro*

The author performed on cell migration assay and cell proliferation assay for investigating a role of *Toxoplasma*-infected BV2 cells-derived exosomes in U87 human glioblastoma development. U87 cells were treated with exosomes of *Toxoplasma*-infected BV2 cells and the results indicated that U87 cells which were stimulated with RH *Toxoplasma*-infected BV2-derived exosomes significantly increased

the migration rate (48.3%) compared to untreated U87 cells (23.5%) or *Toxoplasma*-uninfected BV2-derived exosomes treated U87 cells (25.8%) (Fig. 8A). Cell proliferation assay result also showed that *Toxoplasma*-infected BV2-derived exosomes induced the proliferation of U87 cells compared to control (Fig. 8B). Based on the data, the author could represent that *Toxoplasma*-infected BV2-derived exosomes play an important role in the regeneration of U87 glioblastoma cells. Error bars are standard error of the mean. * P value < 0.05 was obtained by student's t-test.

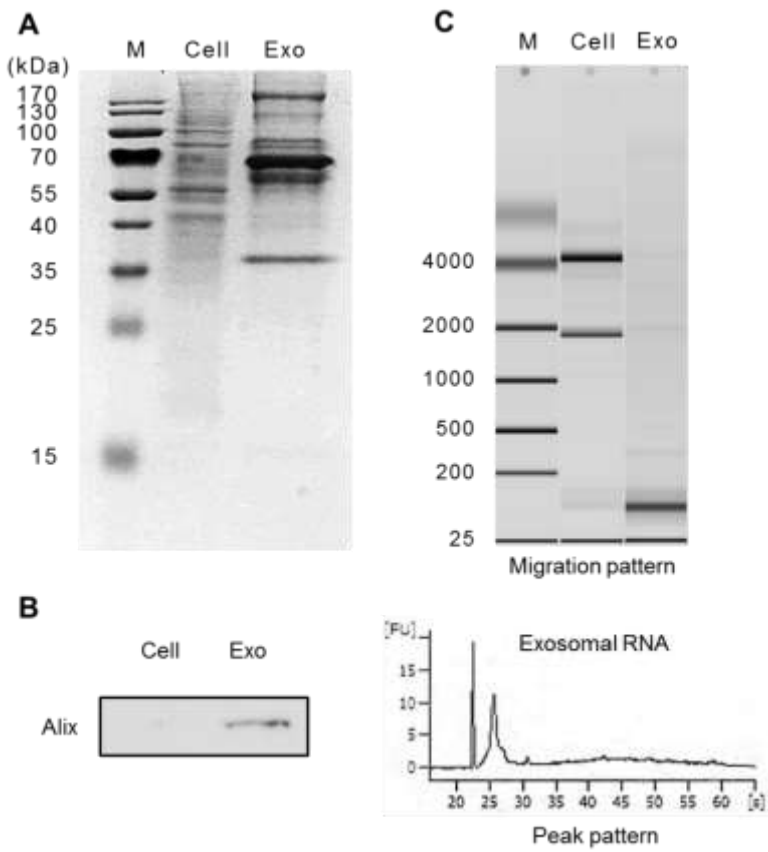


Figure 1. Characterization of BV2 cell-derived exosomes.

(A) SDS-PAGE image. Protein was isolated from either BV2 cells (Cell) or BV2-derived exosomes (Exo). An equal amount 30 μg of protein was loaded on 10% SDS polyacrylamide gel. The gel was run at 120 V, 200 mA for 150 min and stained with Coomassie blue. The pattern of proteins was different between cell and exosome. (B) Western blot analysis. Protein bands were confirmed with Alix antibody as exosome marker. Alix marker was detected in the exosomal proteins but not in the BV2 cell lysate proteins. (C) Purified RNA from BV2-derived exosomes was evaluated by a Bioanalyzer. As a result, the 28S and 18S ribosomal subunits were rarely observed in the RNA.

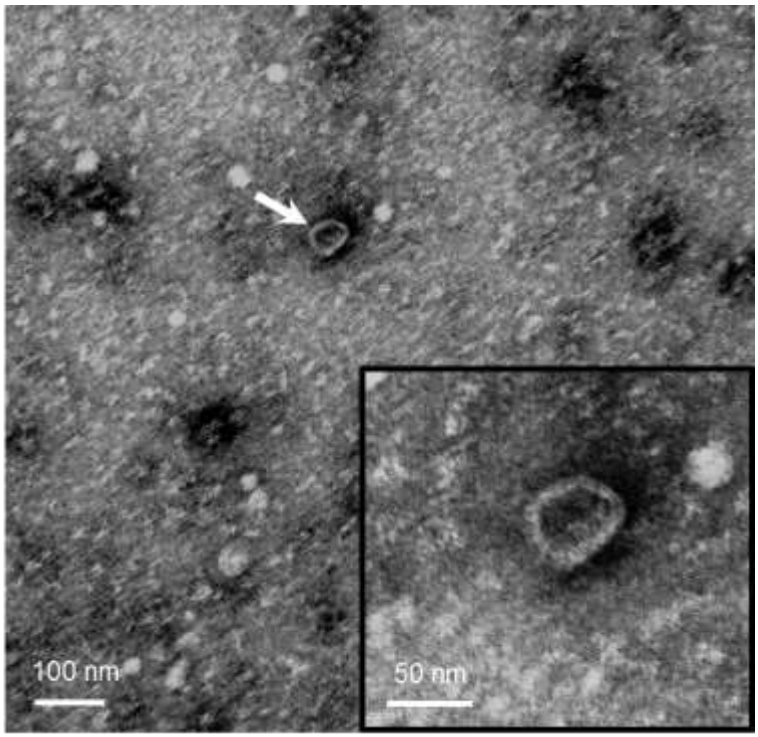


Figure 2. Electron microscopy of BV2-derived exosomes.

Transmission electron microscopy. The exosomes isolated from BV2 cells were negative stained using 2% uranyl acetate. The observed exosomes were shown in approximately 60-100 nm size with cup-shaped structure. White arrowhead points to exosome.

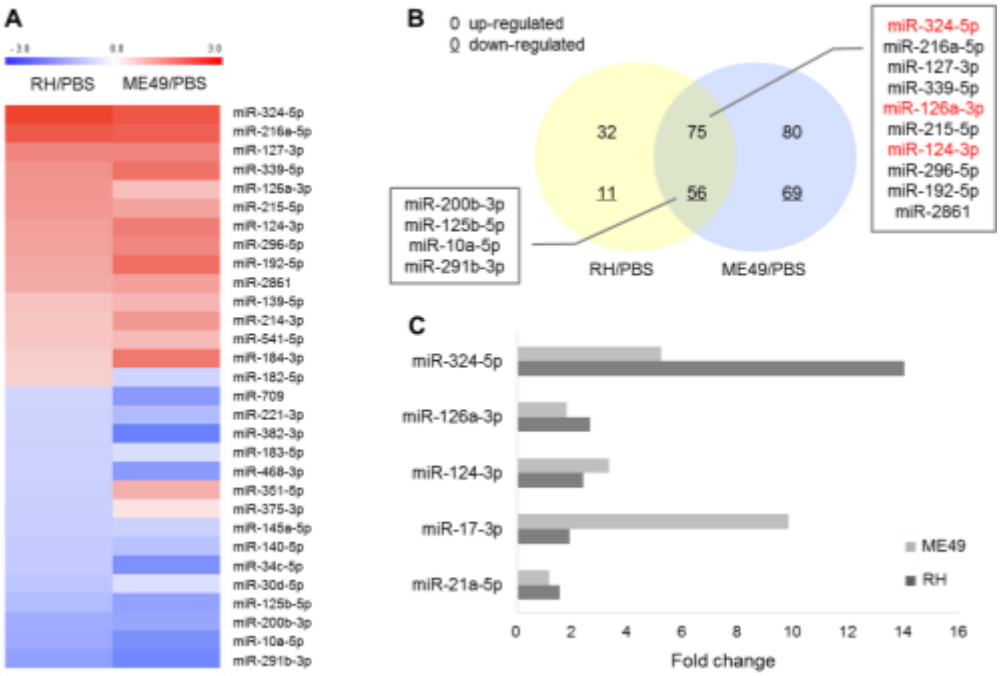


Figure 3. Expression profiling of mature exosomal miRNAs in microglial cells by *Toxoplasma* infection.

(A) Heatmap. Hierarchical clustering was performed on top 30 up- and down-regulated miRNAs related to host immunity and tumor development. (B) The common region of the Venn diagram showed the immunity or tumor development related 14 miRNAs were significantly changed in those following RH or ME49 strain of *Toxoplasma* infection. (C) Expression of miRNAs related to differentiation of M2 phenotype and tumor-angiogenesis factors in tumor microenvironment was visualized in the graph.

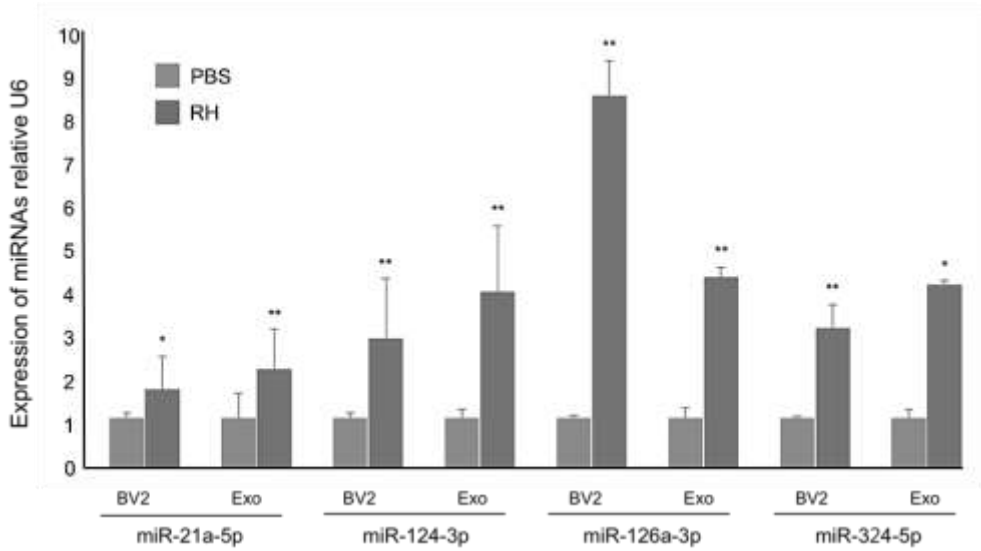


Figure 4. Validation of expression of microarray results in exosomes by real-time RT-PCR.

Relative expression level of miR-21a-5p, miR-124-3p, miR-126a-3p and miR-324-5p was examined in the *Toxoplasma*-infected BV2 cells-derived exosomes by real-time RT-PCR. The data showed that miR-21a-5p, miR-124-3p, miR-126a-3p and miR-324-5p exhibited significantly increased expression in exosomes. Error bars are standard error of the mean. * P value < 0.1 and ** P value < 0.05 was obtained by student's t-test.

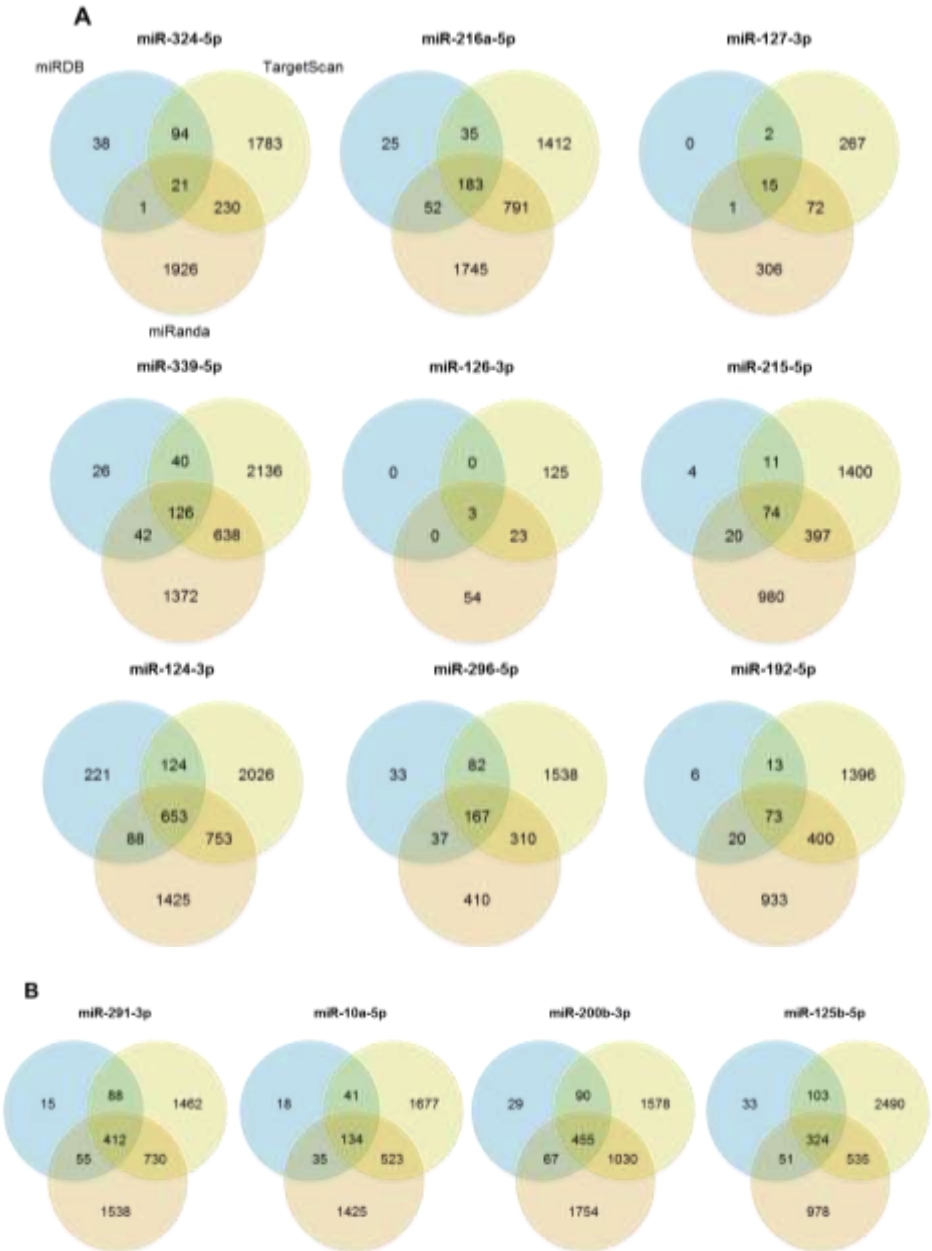
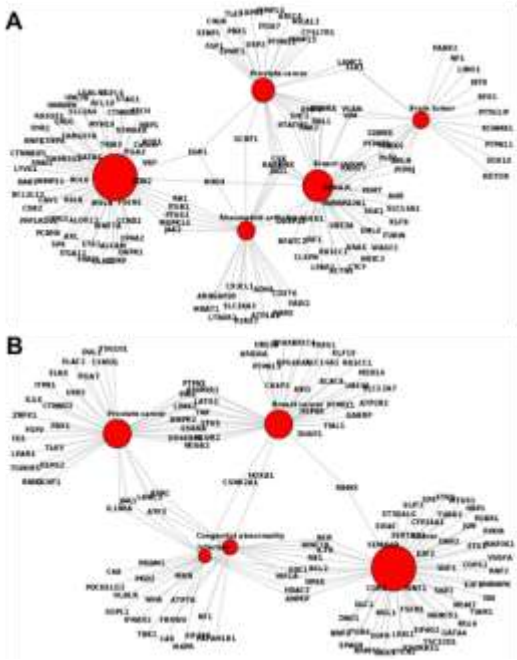


Figure 5. Prediction of genes targeted by miRNAs. The specific targeted genes related to 13 differentially expressed miRNAs, 9 up- (A) and 4 down-regulated (B) miRNAs, by *Toxoplasma* infection were predicted via three different web tools, miRDB, TargetScan and miRanda. The author used common genes for further analysis.



C

DO Term	Number of Genes	Bonferroni corrected *p*-value
Cancer	60	6.808E-11
Breast cancer	41	3.56E-09
Brain tumor	22	1.237E-07
Prostate cancer	31	0.00001487
Rheumatoid arthritis	24	0.000137
Obesity	16	0.004757
Leukemia	23	0.006415
Congenital abnormality	15	0.02549
Long QT syndrome	4	0.04129
Lung cancer	16	0.04305

D

DO Term	Number of Genes	Bonferroni corrected *p*-value
Cancer	59	2.138E-09
Prostate cancer	37	3.12E-08
Breast cancer	37	0.000002477
Infection	17	0.00001017
Congenital abnormality	20	0.00006247
Autistic disorder	12	0.0001233
Diabetes mellitus	27	0.00266
Lymphoma	11	0.002915
Asthma	15	0.006404
Leukemia	23	0.01492

Figure 6. Related diseases of miRNAs targeted genes by *Toxoplasma* infection.

(A, B) This map illustrated the top 5 diseases enriched with the target genes by using the web tool, FunDO. The targeted genes of either 9 up- or 4 down-regulated miRNAs which were controlled by *Toxoplasma* infection were predicted by miRDB, TargetScan and miRanda in common. Additionally, the number of selected genes was proportional to the sizes of the disease nodes. (C, D) In top 10 of the diseases, the amount of genes and *P* value followed in DO term.

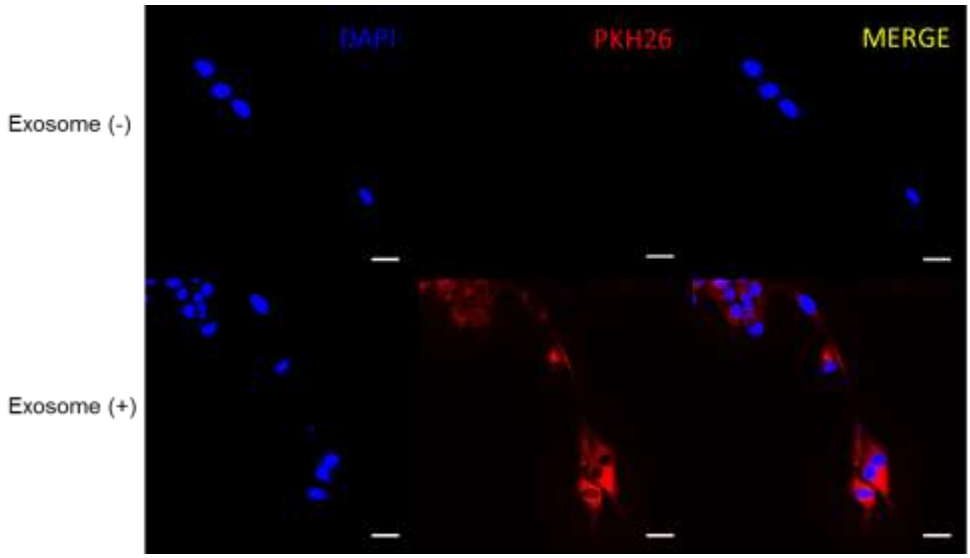


Figure 7. Cellular internalization of BV2-derived exosomes into U87 cells. Confocal microscopy. U87 cells were incubated with either PKH26-labeled exosomes (red) or without as negative control for 24 hrs. The nuclei of U87 cells were stained with DAPI (blue) before observing in confocal microscopy. These data represent that exosomes were uptaken by cells and were presented in cytoplasm (Scale bars: 5 μm, ×3000).

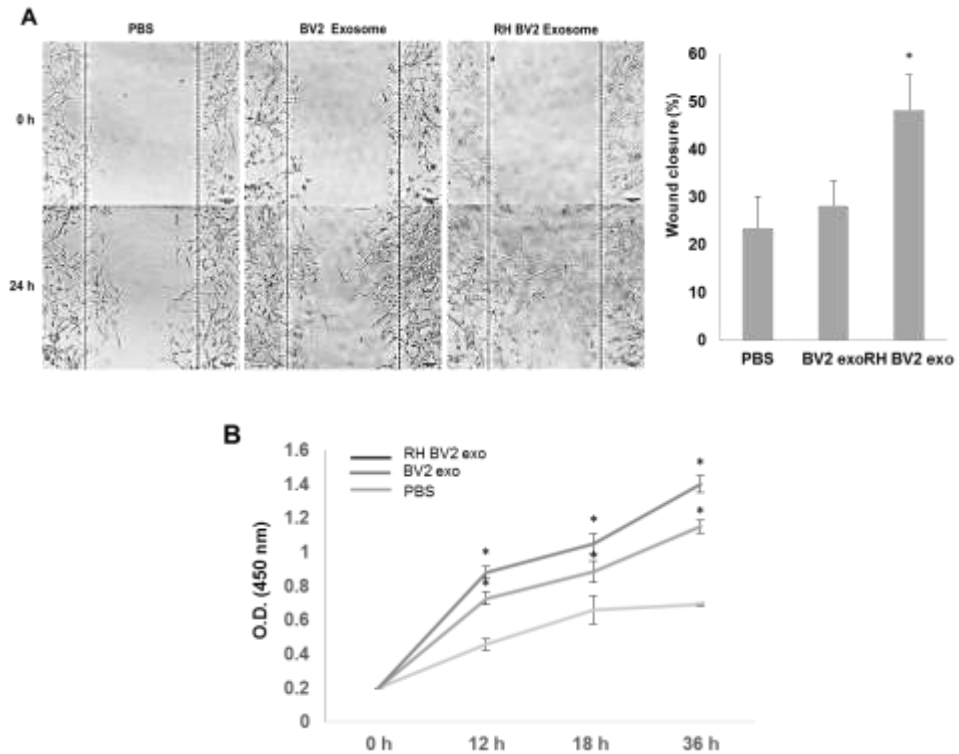


Figure 8. *Toxoplasma*-infected BV2-derived exosomes help regeneration of U87 glioblastoma cells *in vitro*. (A) Cell migration assay. The data indicated that U87 cells which were stimulated with RH *Toxoplasma*-infected BV2-derived exosomes significantly increased the migration rate (48.3%) compared to untreated U87 cells (23.5%) or *Toxoplasma*-uninfected BV2-derived exosomes treated U87 cells (25.8%). $*P$ value < 0.05. (B) Cell proliferation assay. The result showed that *Toxoplasma*-infected BV2-derived exosomes induced the proliferation of U87 cells compared to BV2-drived exosomes or PBS. * P value < 0.05.

Table 1. The prevalence of *T. gondii* among cancer patients and normal controls.

Year	Country	Cancer type	Methods	Results	Reference
1963-1964	USA	Glioma, Acoustic neuroma, Meningioma, Others	Sabin-Feldman dye-test	Cancer patients (n=126) : 56.3% Controls (n=126) : 41.3%	Schuman et al., 1967
2000-2002	Turkey	Hodgkin's lymphoma, Multiple myeloma, Leukemia, Others	ELISA (IgG)	Cancer patients (n=108) : 63.0% Controls (n=108) : 19.4%	Yazar et al., 2004
2006	China	Nasopharyngeal carcinoma, Rectal cancer, Others	ELISA (IgG)	Cancer patients (n=267) : 24.0% Controls (n=148) : 6.1%	Yuan et al., 2007
2008	Korea	Malignant neoplasms	LAT, ELISA (IgG)	Malignant neoplasms : 19.0% (16 cases/84 *T. gondii* positive cases)	Shin et al., 2009
2012-2014	China	Brain cancer, Lung cancer, Cervical cancer, Others	ELISA (IgG)	Cancer patients (n=900) : 35.6% Controls (n=900) : 17.4%	Cong et al., 2015
1979-2007	France	Brain cancer	Database	Brain cancer mortality rates increase with *T. gondii* seroprevalence in France.	Vittecoq et al., 2012
2008	37 Countries	Brain cancer	Database	Infection with *T. gondii* was associated with a 1.8-fold increase in the risk of brain cancers.	Thomas et al., 2012

DISCUSSION

Recently, researchers at the U.S Centers for Disease Control (CDC) observed the first notable case of a human developing cancer originated from a tapeworm called *Hymenolepis nana* and suggested a novel disease mechanism that links tapeworm infection and cancer (Muehlenbachs et al., 2015). Human multicellular parasites that live in host tissue generally have cellular manipulating mechanisms for host tissue invasion and immune evasion. The mechanisms would potentially be contributed in the process of malignant transformation within the host. Less than 20% of cancers are mostly caused by infectious viral pathogens, such as human papilloma, hepatitis B and C Viruses (Correia da Costa et al., 2014). Additionally, three carcinogenic helminthes such as *Opisthorchis viverrini*, *Clonorchis sinensis* and *Schistosoma haematobium* were classified as Group 1 carcinogens by International Agency for Research on Cancer (IARC) (Bouvard et al., 2009; de Martel et al., 2012; IARC, 2012). However, there is a lack of information about the several human parasites related with cancer compared to viral pathogen in current (Correia da Costa et al., 2014).

In this study, the author succeeded in the isolation and characterization of the exosomes derived from *Toxoplasma* (RH and ME49 strain)-infected BV2 microglial cells and analyzed the

expression profile of miRNAs in exosomes using microarray. The results provide possible effects of the *Toxoplasma*-infected microglial cell-derived exosome and the altered exosomal miRNAs expression profile in host environment. Especially, the changed expression of miRNAs was related with tumor-angiogenesis factors, anti-apoptotic pathway and also inducing differentiation of tumor-associated macrophages (M2 phenotype) in tumor microenvironment. The M2 form of tumor associated macrophages (TAMs) has been widely associated with poor prognosis, induction of angiogenesis, tissue re-modelling and enhancement on metastasis growth and seeding. Until now, there is no information about the role of *Toxoplasma* inducing exosomal miRNAs derived from host cells in tumor microenvironment.

Despite the comprehensive studies on the roles of exosome as regulator in cancer, its roles between parasite and its host is still poorly understood. Microvesicles such as exosomes associated with parasitic infections need to better understand the meaningful interaction between parasites and host. So far, explanations about the immune-evasion mechanisms of protozoan parasites to survive against host attacks and establish to infection were based on a variety of modulating factors including secretory proteins (Deolindo et al., 2013). Recently, there has been an increasing concept that protozoa associated microvesicles and its miRNAs communicate with host cells for promoting the favorable environment to their survival (Twu et al., 2014).

In the first study of exosome associated with *Toxoplasma*, the exosome derived from *Toxoplasma* antigens pulsed-DC cells was reported an attractive potential tool for a cell-free vaccination strategy (Aline et al., 2004). Another following reports about *Toxoplasma* pulsed-exosome mainly mentioned the exosome as a potential vaccine. Pope et al. (2013) brought up the level of mRNA and miRNA content in exosomes was significantly regulated by *Toxoplasma* infection.

There are several reports about miRNAs affected by *Toxoplasma* infection until recently. The changed miRNAs in mouse spleen by *Toxoplasma* infection are associated with regulation of apoptosis and cancer (He et al., 2015). Recent studies have reported that altered miRNAs expression by the stimulation of pathogens plays a vital role in regulation of genes of the immune cells including macrophages/microglia, dendritic cells, and T cells (Nahid et al., 2011; Quinn et al., 2011).

Microglia are normally a resident macrophage among the mononuclear phagocytes in the CNS and have two main functions such as neuro-inflammatory response (M1-like pro-inflammatory activation phenotype) and wound healing (M2-like alternative activation phenotype) following phenotypic response to either pathogenic or cytokine stimulation (Freilich et al., 2013). However, there is little information about specific miRNAs related to the differentiation of M1 or M2 phenotype in microglia.

In our study, the author obtained that the altered miRNAs in *Toxoplasma*-infected BV2 cells-derived exosomes were related with the macrophage polarization and angiogenesis via microarray analysis. The miR-324-5p plays a crucial role in modulating macrophage function and is associated with both colitis and colon tumorigenesis in macrophages in patient with colon cancer (Chen Y et al., 2014). The miR-124 contributed to the differentiation of M2 phenotype, inhibiting the inflammatory response of macrophages infiltration and phagocytic capacity in developing microglia through targeting immune-related factors like TLR, P65 and TRAF6 (Ponomarev et al., 2011; Ma et al., 2014; Qiu et al., 2015). The miR-126 plays a crucial role in regulating the function of endothelial cells, angiogenesis and vascular integrity (Wang et al., 2008 and Fish et al., 2008) and induced anti-inflammatory effect and promote M2 macrophage polarization (Harris et al., 2008; Zhang et al., 2013; Hartmann et al., 2015). Although miR-124 and miR-126 has known the tumor suppressor miRNAs in several studies, the author suggested that the roles of miR-124 and miR-126 in the process of macrophage M2 phenotype polarization would be a tumor developmental strategy for evasion of anti-tumor immune response, inducing of angiogenesis and vascularization. For supporting our interpretation, the author need to set bounds to the changing expression level of miR-124 and miR-126 in the TAM but not in whole tumor grafts.

Most of miRNA species have various effects and even appear the

opposite effects in host similar microenvironment following external stimulus intensity or timing. Therefore, miRNA data obtained from microarray analysis need a direct physical experimentation to determine the exact biological effects of exosomal specific miRNAs derived from *Toxoplasma*-infected BV2 cells on the tumor development *in vitro* and *in vivo*.

In this study, the author confirmed altered expression of exosomal miRNAs in *Toxoplasma*-infected BV2 microglial cells and certainly suggested that *Toxoplasma*-infected microglia cell-derived exosomes play an important role as a regulator for the regeneration of glioblastoma cells. However, the further studies are needed for finding direct relation between changed exosomal miRNA and regeneration of glioblastoma by the exosomes.

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국문 초록

톡소포자충은 세포 내에 기생하는 기회감염성 원충으로 감염된 숙주의 환경을 생존하기 유리하도록 조절 하는 것으로 알려져 있다. 다양한 원충에 감염된 숙주세포로부터 유래된 엑소좀 microRNAs 는 숙주세포의 면역반응을 조절함으로써 기생충이 생존할 수 있도록 하는 조절 인자로 알려져 있다. 톡소포자충에 의해서 조절된 환경은 종양미세환경 (tumor microenvironment) 과 유사하여 종양 유발에 영향을 미칠 것으로 보여지지만, 톡소포자충 감염이 종양을 유발시킨다는 것에 대한 메커니즘은 아직 잘 밝혀져 있지 않다. 본 연구에서 저자는 톡소포자충 감염이 뇌종양 발달에 미치는 영향에 대해 조사하기 위해, 톡소포자충의 RH 혹은 ME49 종에 감염된 쥐 미세교세포 (BV2 세포) 에서 유래한 엑소좀을 분리하고 특성을 확인하였다. 그 후, microRNA array 를 통해 BV2 세포 유래 엑소좀 miRNAs 의 발현 양상을 분석하였고, 이러한 miRNAs 를 quantitative reverse-transcription PCR (qRT-PCR) 로 부분적으로 입증하였다. 종양 혹은 숙주 면역과 관련되어 있다고 알려져 있는 14개의 miRNAs 는 RH 와 ME49 종 모두에서 유의미하게 변화되었으며, 이러한 miRNAs 가 표적으로 하는 유전자와 연관된 질병을 찾기 위해서 Functional Disease Ontology analysis (FUNDO) 와 같은 다양한 웹 도구를 이용하여 분석하였다. 그 결과, 톡소포자충 감염에 의해서 변화된 miRNAs 는 여러 질병 중 종양과 양의 상관관계를 나타냈으며, 뇌종양이 주로 up-regulated miRNAs 의 타겟 유전자들과 관련성을

보여주었다 ($P < 0.05$). 더욱이, 저자는 톡소포자충이 감염된 BV2 유래 엑소좀이 숙주세포에 의해서 세포 내로 함입되는 것을 확인하였고, *in vitro* 실험을 통해 이 엑소좀이 U87 신경교종세포의 발달을 유도한다는 것을 확인하였다. 이러한 결과들은 톡소포자충과 신경교종세포 발달이 밀접하게 연관되어 있다는 것을 뒷받침했다. 따라서, 본 연구는 톡소포자충이 감염된 미세교세포에서 변화한 엑소좀 miRNAs 의 발현양상을 확인하였고, 그 엑소좀이 신경교종세포 발달의 조절자로 중요한 역할을 하는 것으로 제시하고 있다.

핵심어: 톡소포자충, 미세교세포, 엑소좀, microRNA, 신경교종세포

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