Title: Profile of miRNAs in small extracellular vesicles released from glioblastoma cells treated by Boron Neutron Capture Therapy

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Abstract

[Purpose] Boron neutron capture therapy (BNCT) is a tumor cell-selective particle-radiation therapy. In BNCT, administered *p*-boronophenylalanine (BPA) is selectively taken up by tumor cells, and the tumor is irradiated with thermal neutrons. High-LET α-particles and recoil ^{7}Li, which have a path length of 5-9 μm, are generated by the capture reaction between ^{10}B and thermal neutrons and selectively kill tumor cells that have uptaken ^{10}B. Although BNCT has prolonged the survival time of malignant glioma patients, recurrences are still to be resolved. miRNAs, that are encapsulated in small extracellular vesicles (sEVs) in body fluids and exist stably may serve critical role in recurrence. In this study, we comprehensively investigated microRNAs (miRNAs) in sEVs released from post-BNCT glioblastoma cells. [Methods] Glioblastoma U87 MG cells were treated with 25 ppm of BPA in the culture media and irradiated with thermal neutrons. After irradiation, they were plated into dishes and cultured for 3 days in the 5 % CO_2 incubator. Then, sEVs released into the medium were collected by column chromatography, and miRNAs in sEVs were comprehensively investigated using microarrays [Results] An increase in 20 individual miRNAs (ratio>2) and a decrease in 2 individual miRNAs (ratio<0.5) were detected in BNCT cells compared with non-irradiated cells. Among detected miRNAs, 20 miRNAs were associated with worse prognosis of glioma in Kaplan Meier Survival Analysis of overall survival in TCGA. [Conclusion] These miRNA after BNCT may proceed tumors, modulate radiation resistance, or inhibit invasion and affect the prognosis of glioma.

Keywords: glioblastoma, small extracellular vesicles, microRNA, microarray and boron neutron

capture therapy

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Introduction

Glioblastoma with isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2) wild-type is the most lethal among the primary brain tumors, with one of the worst overall prognoses across cancer entities. The median overall survival is 12 months despite multimodal therapy including surgery, radiation, and pharmacotherapy [1]. There is a desperate need for new treatments that extend survival, and clinical trials have been conducted worldwide. Boron neutron capture therapy (BNCT) is a form of tumor-selective particle radiation therapy that consists of two components. First, a boron-10 (^{10}B)-containing drug is administered to the patient to obtain a sufficient tumor ^{10}B concentration, and then, the tumor is irradiated with epithermal neutrons. The resulting $^{10}B(n,\alpha)^{7}Li$ capture reaction produces α- particles whose short path (5–9 μm) selectively kills tumor cells while sparing adjacent normal tissues [2]. BNCT has been applied to the treatment of malignant glioma and proved effective even for recurrent cases in a phase II study that used an accelerator as a new neutron source [3]. However, glioma recurrence is still inevitable after BNCT, locally in the original lesion or distantly in the cerebrospinal fluid (CSF) space as dissemination. In addition, the most frequent cause of death after BNCT is CSF dissemination, which occurs more frequently in the small cell subtype of $IDH1^{R132H}$ mutation-negative GBM [4]. The mechanism underlying glioma recurrence after BNCT remains unknown.

SEVs are small membrane vesicles with sizes ranging from 50 to 150 nm. They serve as functional mediators and promote intercellular communication during physiological and pathological

processes. This intercellular communication is involved in various pathophysiological processes, including migration, treatment resistance, and metastasis in cancer [5]. SEVs contain proteins, lipids, microRNAs (miRNAs), mRNA, and DNA, enabling cells to exchange genetic information [6, 7]. miRNAs are non-coding RNAs of 19 to 24 nucleotides, and they serve as hubs in gene regulatory networks by negatively regulating target protein-coding genes and posttranscriptional regulation of gene expression [8]. miRNAs are encapsulated in lipid membranes such as extracellular vesicles in blood and body fluids, exist stably, are taken up by the cells they reach, and act negatively on target genes, performing post-translational modification [9]. Importantly, serum miRNAs have been identified as useful biomarkers of the Glioma index, which discriminates diffuse glioma from non-cancerous controls, and the 3-Tumor Index, which discriminates among GBM, primary central nervous system lymphoma, and metastatic brain tumor [10].

In this study, we aimed to investigate miRNAs in sEVs secreted from glioblastoma cells after BNCT using microarray, which may explain the undefined and insightful mechanism of local recurrence or CSF dissemination after BNCT.

Materials and Methods

1. Cell culture

We used U87MG *delta EGFR* cells (kindly gifted by Dr. Hiroyuki Michiue at Okayama University,

Japan) cultured in Dulbecco's Modified Eagle's medium containing 10% sEV-depleted fetal bovine serum (FBS) (Exo-FBS™, System Biosciences, CA, USA) in 5 % CO_2 incubator. More than 3×10^6 cells were prepared on the day of irradiation.

2. Boron compound

A stock solution of *the p*-^{10}B-para-boronophenylalanine (BPA)- fructose complex was used as previously described [11]. BPA was purchased from KatChem Ltd. (Prague, Czech Republic), prepared by dissolving in distributed water as a complex with 3 % fructose and sterilizing with a filter (0.2 micro-meter pore size). The ^{10} B concentrations were measured by prompt gamma-ray spectrometry using a thermal neutron guide tube installed at Kyoto University, and the value was approximately 1000 ± 4.55 ppm. The day before irradiation, BPA was dissolved in the cell culture medium at a concentration of 25 ppm.

3. Neutron irradiation

The cells were rinsed with PBS, trypsinized, and collected after centrifugation. The Cell suspensions in 1.5-ml plastic tubes were irradiated for 70 min at room temperature using a neutron beam at the Heavy Water Neutron Irradiation Facility installed in the Kyoto University Reactor (KUR-HWNIF). The operating power of the reactor was 1 MW. After irradiation, the cells were disseminated in 10 cm dishes (3×10^5 cells/dish). Thermal neutron fluencies were measured using gold foil (3 mm in diameter and 0.05 mm in thickness) activation analysis. The gamma-ray dose, including secondary gamma rays,

was measured using a thermoluminescence dosimeter (TLD). The TLD used was beryllium oxide (BeO) enclosed in a quartz glass capsule. BeO is sensitive to thermal neutrons. The chemical composition of the cells was assumed to be H = 11.1, C = 12.7, N = 2.0, O = 74.2 weight percent, and the density was assumed to be 1.0 g/cm^3[12].

4. Sample collection

Three days after irradiation, the harvested culture supernatant of the three 10 cm dishes was collected in a 50 ml centrifugation tube and centrifuged at 2000 rpm at 4 °C for 20 min. Then, it was filtered (0.2 μm pore size) and stored in a deep freezer at -80 °C before use.

5. SEV isolation

The stored supernatant was dissolved and concentrated using a centrifugal ultrafiltration filter unit (Amicon Ultra-15, 100kDa, Merck Millipore, MA, USA) at 4000 × g at 4 °C for 2 h to obtain a final volume of 500 μL. The 500 μl of concentrated supernatant was overlaid on qEV 35 original size exclusion columns to collect particles in size from 35-200nm (Izon Science Ltd, Christchurch, New Zealand), and sEV-containing fractions were collected in 500 μL of PBS.

6. Tunable resistive pulse sensing (TRPS)

The size distribution profile of the sEVs was analyzed with TRPS (qNano, Izon Science Ltd.) using an NP100 nanopore. Carboxylated polystyrene beads (70 nm) were used to calibrate the concentration and size.

7. Total RNA extraction from sEVs and miRNA microarray analysis

Total RNA was extracted from the sEVs using Toray's 3D-Gene RNA extraction reagent (Toray Industries, Inc., Tokyo, Japan). Comprehensive miRNA expression analysis was performed using a 3D-Gene miRNA Labeling kit and 3D-Gene Human miRNA Oligo Chip Ver. 22 (Toray Industries, Inc.), according to the manufacturer's protocol to detect 2,565 human miRNA sequences. The expression levels of each miRNA were expressed as the background-subtracted signal intensities of all the miRNAs in each microarray. Signal intensity was calculated by setting the median value of the signal sum to 25 units. To identify robust miRNAs, miRNAs with normalized signal values of greater than 64 intensity units were selected. Raw and processed data from this analysis were deposited in the Gene Expression Omnibus repository (accession number: GSE243221). Heatmap in Fig. 2 was produced using the Log2 value of each sample when corrected so that the 75% percentile value of the total signal intensity was 1. The ratio in Table 1 was the value obtained by converting "Log2 ratio, which means a calculated value of “Log2 global normalization” difference between samples “ to an antilog number. The calculation was performed by GeneSpring GX (Agilent Technologies, Ltd. CA, USA).

Results

1. Component and dose of the mixed neutron beam in BNCT

The neutron fluences were 6.46 x 10^{12} cm^{-2} for the thermal neutron range (less than 0.5 eV), and 1.2 x 10^{12} cm^{-2} for the epithermal neutron range (0.5 eV to 10 keV). The total absorbed doses were calculated as the sum of the absorbed doses attributed primarily to $^{1}H(n,n)^{1}H$, $^{14}N(n,p)^{14}C$, $^{10}B(n, alpha)^{7}Li$, and the contaminating gamma rays. The dose-converting coefficients and details of the calculation method have been previously described [12]. The thermal neutron dose was 0.86 Gy. The epithermal neutron dose was 0.095 Gy. The fast neutron dose (over 10 keV) was 0.66 Gy. The gamma ray dose was 1.27 Gy. Estimated ^{10}boron dose was 11.8 Gy. The percentages of dose of the thermal neutron, the epithermal neutron, the fast neutron, the gamma ray and the ^{10}boron dose were 5.8 %, 0.65 %, 4.5 %, 8.6 % and 80.3 % respectively. We have obtained the survival fraction (SF) by a colony formation assay from another experiment after the same BPA treatment and at the same neutron fluences and SF was 2.39 x 10^{-6}, which means almost dead.

2. Size distribution of the sEVs

SEV size distribution was assessed by TRPS (qNano, Izon Science Ltd), and sEVs isolated from non-treated or BNCT-treated U87MG cells had a typical diameter range of 70–150 nm (Fig. 1A and 1B).

3. miRNA differentially expressed in BNCT-treated glioblastoma cells

When miRNAs with intensities higher than 64 were extracted and compared between BNCT-treated and non-treated U87 MG cells, the number of upregulated (ratio >2) or downregulated (ratio <0.5)

miRNAs in BNCT-treated U87 MG cells was 20 (Table 1A) and 2 (Table 1B), respectively.

4. miRNAs that correlate with the life prognosis of glioma in TCGA project

Among the detected miRNAs, miRNAs that correlate with the prognosis of diffuse low grade glioma using Kaplan Meier Survival Analysis of overall survival in The Cancer Genome Atlas (TCGA) (Hazard ratio: HR <1 or >1 and p <0.05) according to the database from Cancer MIRNome [13] were shown in heatmap (Fig. 2). Most miRNAs were related to worse prognosis (HR >1) except the three miRNAs of miR-4763-3p, miR-128-2-5p and miR-1275 (HR <1). The purple clusters in the dendrogram indicate that miRNAs that were originally not present have appeared in the EV, and the green and yellow clusters in the dendrogram indicate that the number of miRNAs that were originally present has tended to increase.

Discussion

In this study, we identified for the first time the profiles of miRNAs in sEVs released from glioblastoma cells after high-LET radiation, BNCT. Some miRNAs that are differentially expressed after BNCT (Table 1) are involved in the regulation of various cancers, as well as gliomas. For example, in the up-regulated miRNAs shown in Table 1A, miR-650 expression has been proven to be a significant prognostic indicator in glioma in a study that enrolled 168 glioma patients and 21 normal

control brain specimens [14], and also a promising marker to detect gastric cancers in combination with carbohydrate antigen 211 [15]. Furthermore, miR-4725-3p targeting stromal interacting molecule 1signaling is involved in xanthohumol, a prenylated flavonoid extracted from the hop plant Humulus lupulus L., inhibition of glioma cell invasion [16]. miR-3147 may serve an oncogenic role in vulvar squamous cell carcinoma by targeting Smad4, a tumor suppressor gene [17]. miR-4270 is lower in radio-sensitive nasopharyngeal carcinoma patients in the GSE 139164 datasets provided by National Center for Biotechnology Information, and modulates X-ray-sensitivity of nasopharyngeal carcinoma cells through modulating of p53 *in vivo* [18]. On the other hand, mir-4270 suppresses hepatocellular carcinoma progression [19] and acts as a tumor suppressor in osteosarcoma cells [20]. Among the downregulated miRNAs listed in Table 1B, miR-1827 represses MDM2 to positively regulate the tumor suppressor p53 and suppress tumorigenesis in colorectal tumors [21]. Referring to the above reports, miRNAs in sEVs that are significantly increased or decreased by BNCT may promote tumors, and tumor resistance to radiation, but some suppress tumor invasion or progression on the contrary.

As shown in the heatmap of Fig.2, we found almost 20 miRNAs that were significantly associated with the prognosis of diffuse gliomas (low grade glioma). These miRNAs were mostly related to worse prognosis and may remain candidates for predictive markers of prognosis even after BNCT, though their expression was not significantly different between the BNCT-treated and non-treated control cells. For example, the HR for miR-3154 and miR-4667-5p in the Kaplan Meier Survival Analyses of overall

survival in TCGA were 2.14 (p=2.02e-04) and 1.89 (p= 0.01) (Fig. 3A and 3B) [13].

To date, no comprehensive study has been conducted on sEV release caused by particle irradiation in cancer cells. However, the effects of X-rays on sEVs derived from cancer cells have been investigated and reviewed [22-24]. In the case of X-ray-irradiated U87MG cells, using a 1.33-fold change cutoff, there were 1308 mRNA changes at 24 h and 209 mRNA changes at 48 h in radiation-derived sEVs compared with non-irradiated controls. In contrast to the abundant mRNA changes, radiation-derived sEVs showed few changes in miRNA composition at both 24 and 48 h after irradiation. The miRNA targets that were downregulated in sEVs at 48 h were associated with cell movement as one of the top five molecular/cellular functions. Migration- and invasion-related mRNA, connective tissue growth factor, and insulin-like growth factor binding protein 2 were abnormally elevated in sEVs released from irradiated U87MG cells. Combined mRNA and protein array data were analyzed using functional networks showing cellular movement as the top function and nervous system development as the 3rd associated network function. Cellular growth and proliferation, cell signaling, and molecular transport are the 2nd, 3rd and 4th in molecular and cellular functions, respectively [25]. A previous study characterized the profile of exosomal miRNAs derived from bronchial epithelial cells (normal cells) irradiated with high charge and energy ions, ^{48}Ti, ^{28}Si, or ^{16}O ions at a dose of 1 to 2 Gy [26]. They reported that the miRNA profile was skewed toward a small number of species that were previously shown to be involved in cancer initiation and progression,

including miR-1246, miR-1290, miR-23a, and miR-205. Because of the differences in the type or dose of high LET radiation, it is difficult to compare, but miRNAs in sEVs that have similar functions regarding oncogenic properties and tumor progression, were observed in high charge and energy ions-irradiated normal lung cells and BNCT-treated glioblastoma cells.

This study used miRNA arrays to identify the profile of miRNAs in sEVs released from glioblastoma cells by BNCT. Future challenges will include verifying whether miRNAs in sEVs can undergo post-translational modification against known or unknown target genes in residual tumors, and microenvironment cells. If these findings become clear, it will be possible to reduce the risk of recurrence or a side effect event and further improve the quality of malignant glioma treatment using BNCT, leading to a longer prognosis for patients.

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Statements and Declarations

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Author Contributions: Natsuko Kondo, Manabu Natsumeda and Eishu Hirata contributed to the study conception and design. Material preparation, data collection and analysis were performed by Natsuko Kondo, Tadatoshi Kinouchi and Juntaro Matsuzaki. The first draft of the manuscript was written by Natsuko Kondo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

Data Availability: The datasets generated during this study are available in the Gene Expression Omnibus repository (accession number: GSE243221).

Figure legends

Fig 1. Particle size distribution of sEVs by TRPS analysis. **A.** non-irradiated U87MG cells. **B.** BNCT-treated U87MG cells.

Fig. 2. miRNAs related with prognosis in diffuse glioma. BNCT-treated and non-treated samples were plotted using unsupervised hierarchical clustering analysis with a heat map.

Fig. 3. The Kaplan Meier Survival Analysis of overall survival in TCGA. (**A)** for miR-3154 **(B)** for miR-4667-5p

Table 1. miRNAs differentially expressed after BNCT-treated glioblastoma cells.

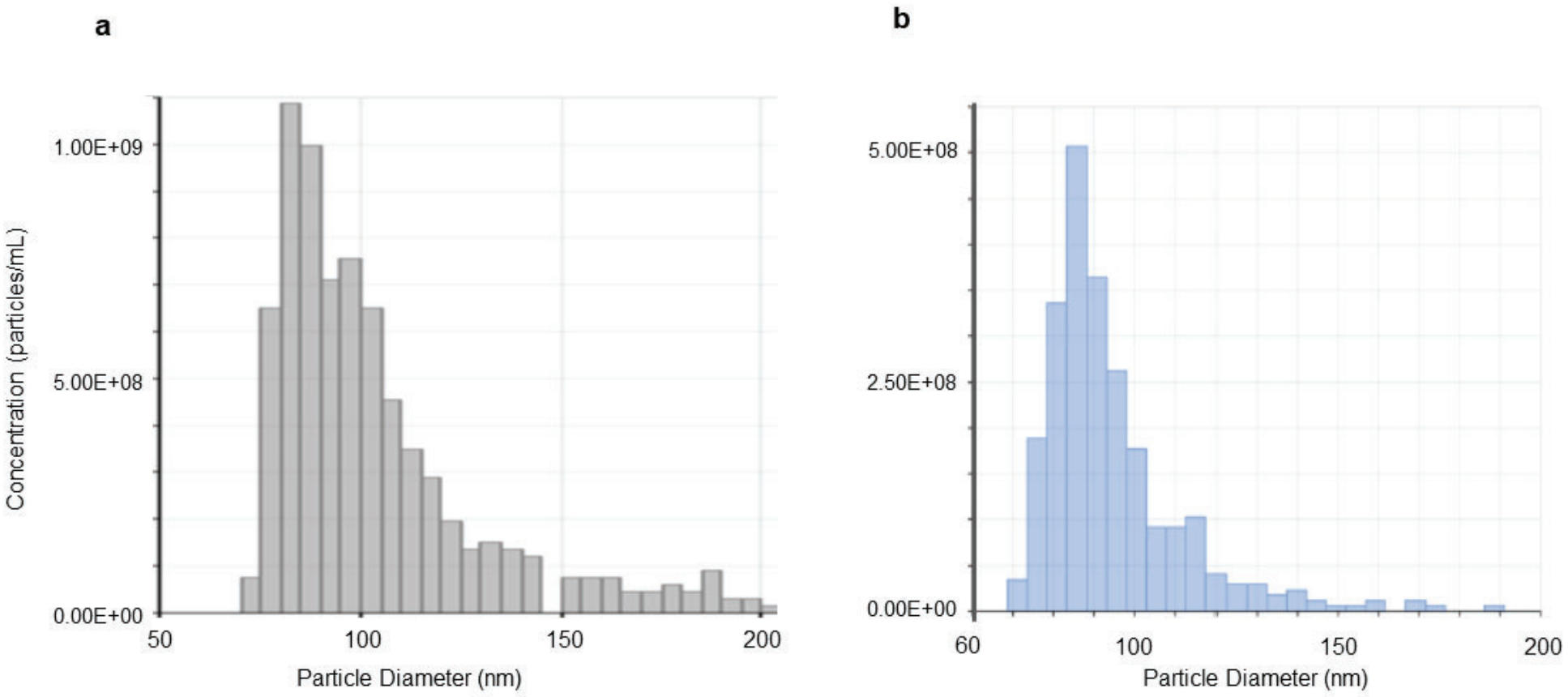
A. upregulated miRNAs

A. Up-regulated	control	BNCT	ratio
hsa-miR-7150	18	70	3.58
hsa-miR-6088	33	101	2.91
hsa-miR-1914-3p	28	85	2.90
hsa-miR-3196	23	71	2.87
hsa-miR-6834-5p	23	64	2.61
hsa-miR-1469	26	70	2.59
hsa-miR-3191-3p	26	71	2.56
hsa-miR-10396b-5p	55	142	2.47
hsa-miR-6885-5p	56	138	2.34
hsa-miR-650	31	77	2.32
hsa-miR-6891-5p	37	88	2.26
hsa-miR-4725-3p	28	66	2.22
hsa-miR-4751	30	69	2.21
hsa-miR-3937	45	100	2.11
hsa-miR-3147	47	104	2.11
hsa-miR-6724-5p	44	97	2.07
hsa-miR-6076	46	100	2.06
hsa-miR-4270	41	88	2.04
hsa-miR-513a-5p	35	75	2.02
hsa-miR-8052	49	104	2.01

B. downregulated miRNAs

B. Down-regulated	control	BNCT	ratio
hsa-miR-1827	166		0.01
hsa-miR-4256	102		0.01

Figure. 1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5.00E+08
0.00E+00
Concentration (particles/mL)
50
100
150
200
Particle Diameter (nm)
b
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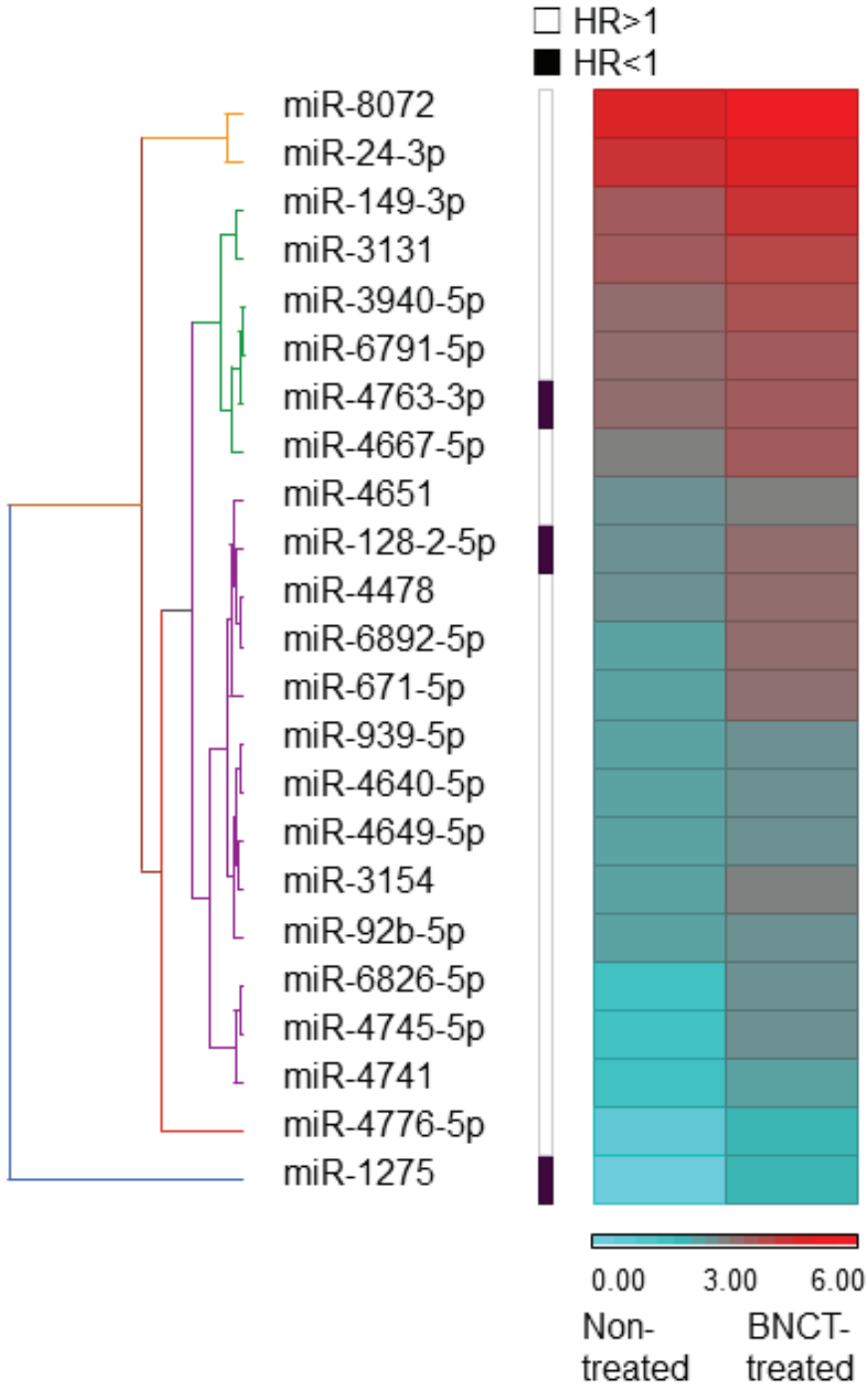
100
150
200
Particle Diameter (nm)

Figure. 2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HR<1
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miR-149-3p
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miR-4640-5p
miR-4649-5p
miR-3154
miR-92b-5p
miR-6826-5p
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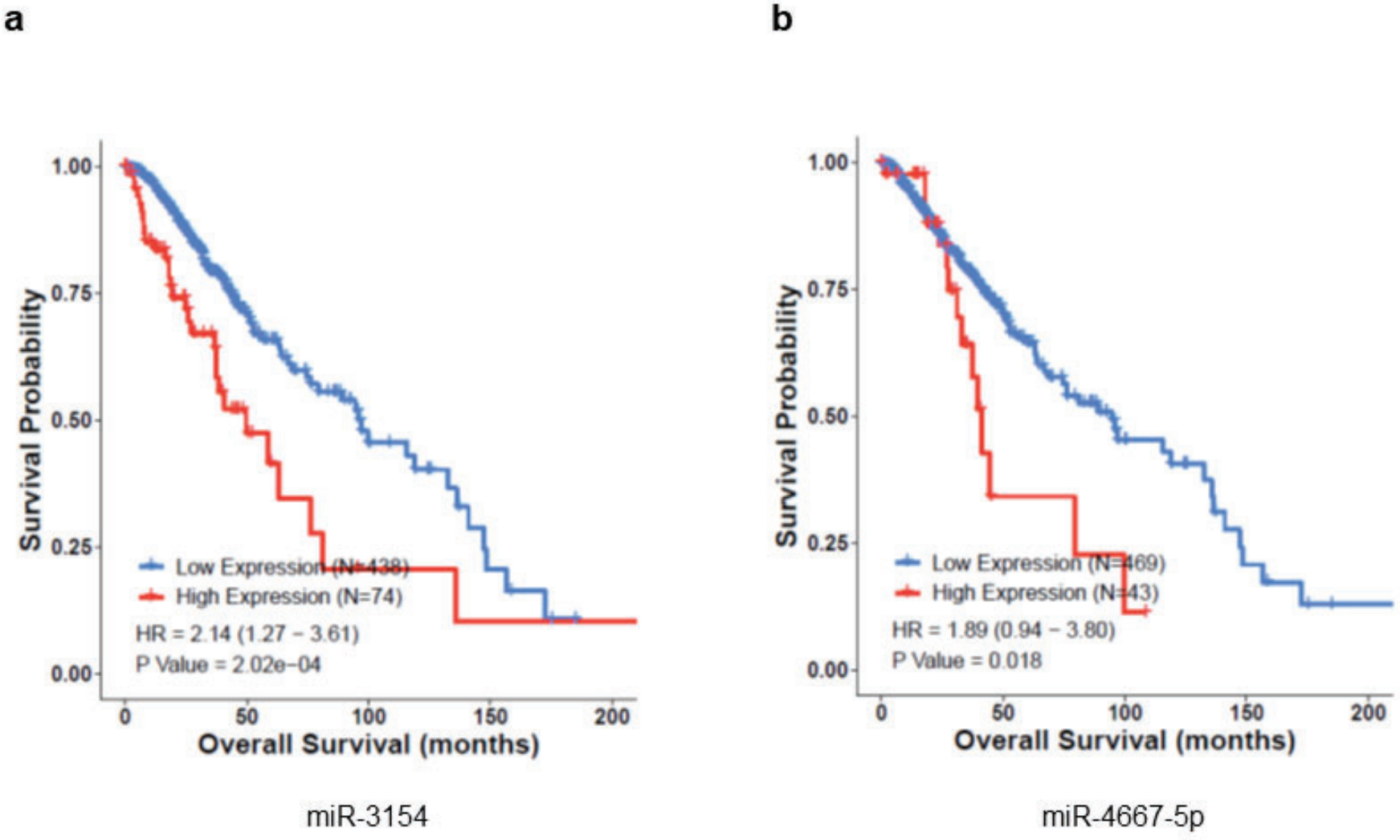
0.00
3.00
6.00
Non-treated
BNCT-treated

Figure. 3



miR-3154

miR-4667-5p