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Article

Lentinula edodes Cultured Extract Treated Mesenchymal-Derived Extracellular Vesicle Modulated Breast Cancer Stem Cells and Reprogramming of MicroRNAs

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Abstract

Background: Breast carcinoma represents the most frequent malignancy in women in Canada. The intrinsic or acquired drug resistance significantly contributes to increased risk of recurrence and metastasis. Although, front-line therapy is multimodal, chemoresistance remains a major hurdle in treatment and therapy. Intake of natural compounds issued from the fermentation processes are now considered a good strategy to help overcome chemoresistance. **Materials and Methods:** Extracellular vehicles (EVs) from Mesenchymal Stromal/Stem Cells (MSCs) pretreated with *Lentinula edodes* cultured extract (AHCC) were used to study the effect on reducing chemoresistance and modulating microRNAs in cell lines, MCF-7 and MCF-7/DOX cells by EVs derived from MSCs under AHCC treatment. Characterization of EVs was done by using nanoparticles tracking analysis. MicroRNAs and the formation of cancer stem cells were studied. **Results:** miRNA analysis revealed that AHCC remarkably affected the expression of several microRNAs, amongst which are miR-155, miR-34a, miR-Let7 and miR-200c. In vitro experiments showed inhibition of cancer stem cell proliferation after challenging the cells with EVs pretreated with AHCC. **Conclusion:** Our data demonstrated that AHCC may contribute to the modulation of tumor microenvironment, thus influencing the development of cancer stem cells.

Keywords: *Lentinula edodes*; extracellular vesicle; cancer stem cells; mesenchymal stem cell; microRNAs

1. Introduction

Breast cancer is the most common cancer in Canadian women, except for non-melanoma skin cancer [1]. It accounts for approximately 25% of new cancer cases and 13% of all cancer deaths in Canadian women. In 2024, an estimated 310,720 women in the United States will be diagnosed with invasive breast cancer [2]. It is estimated that breast cancer ranks the second most diagnosed malignancy in Canada after lung cancer, and first in cancer related to female. However, ranked as number one according to the Canadian cancer society statistics [3]. The current treatment protocol depends on the histology and stage of the cancer and consists of total resection of tumor mass followed by different modalities included: radical chemo-radiotherapy, hormone therapy, immunotherapy and targeted therapy. Patients may receive a combination or all of these modalities, and while some patients are responsive initially to this regimen, the likelihood of the recurrence is

extremely high [4,5]. Moreover, metastasis and development of drug resistance are the major challenges in breast cancer therapy.

Mounting evidence, suggests that a sub-population of tumor cells with the self-renewal properties is the main culprit of the drug resistance, metastasis and recurrence in many types of cancer [6]. Breast cancer also contains these subsets of cells functionally with stemness properties named "breast cancer stem cells" (BCSCs) [7,8]. This small tumor sub-population significantly contribute to the tumor reconstitution after therapy and resistance to therapy [9]. Furthermore, chemotherapy drugs used in treating breast cancer have significant side effects since they have the potential to harm healthy cells, leading to a range of adverse effects[10].

Hence, it is plausible that breast cancer patients may potentially benefit from including nutritional interventions alongside conventional therapies through specifically targeting the regulation of cancer stem cell proliferation [11,12]. There has been an increasing focus on the potential usage of non-toxic natural compounds as chemo-preventive medicines for patients susceptible to tumor recurrence and/or have a high risk of metastasis. The possible applications of mushroom extracts in cancer prevention have garnered heightened interest in recent years [13,14].

Research has demonstrated that an extract derived from cultured *Lentinula edodes* mycelia exhibits inhibitory effects on several forms of cancer, including breast cancer, as observed in both animal models and clinical investigations[15–18]. There is a growing body of evidence indicating that the compound referred to as AHCC has the potential to regulate the growth of cancer stem cells by the anti-inflammatory and epigenetic processes. It was reported that AHCC is capable on increasing tumor suppressor *miR-335*, thus avoiding immune system evasion seen in cancer cells behavior [19,20].

Moreover, numerous studies related to AHCC's effects on immune cells modulation have reported its effects on increased natural killer cell activity, [21] decrease tumor formation [22], enhanced T cell proliferation [23], altered cytokine release via altered T cell activity[22,24,25], anti-inflammatory, and anti-oxidant properties [26–28]. Furthermore, a significant body of evidences derived from clinical investigations proved safety, feasibility and efficacy of this supplement in healthy volunteers [29,30] as well as patients with malignancies [31,32]. Evidence also indicate that AHCC regulate immune response by sensitizing the intestinal epithelium, that is facilitated through recognition of non-pathogenic food-associated molecular patterns (FAMPS) by *TLR4* and *TLR2* receptors [33].

In addition to the AHCC's potential impacts on immune cells, emerging evidences noted that the AHCC's may also play a role in reprogramming tumor microenvironment [34]. This includes impacting on miRNAs, small non-coding RNAs that regulate genes by posttranscriptional regulation of gene expression, involved in self-renewal, progression, and metastasis [34,35]. They can be categorized based on their function, into two distinct groups: oncomiRs that promote tumor formation and progression. Studies shown that oncomiRs are differentially expressed in cancer cells and the tumor microenvironment [36], whereas miRsupps that induce apoptosis and inhibit proliferation, invasion and growth, tumor growth [37].

We have previously showed that AHCC could target CSCs via epigenetic reprogramming and altering some key micro-RNAs and reduce functionally enriched stem cells growth and sphere formation [19]. The aim of this study was to analyze the impact of AHCC on modulation of MSCs by indirectly modulating sphere formation in sensitive and chemoresistance breast cancer cell lines. The auspicious features of MSCs which came from their regenerative abilities and plasticity's into different cell lineage, make them as an good candidate to better understand the dynamic of interaction between MSCs- CSCs and tumor environment [38,39]. A glance at the clinical trial database shows that more than ten thousand trials have been registered using MSCs as the therapeutic agent in different clinical applications [40–42]. However, the application of these cells in cancer treatment remains controversial. While there is lot of evidence highlighting the potential roles of MSC in cancer progression and pathogenesis, emerging data reveals some beneficial effects on cancer treatment. Regarding tumor environment, MSCs can exert mutually exclusive effects on

surrounding cells including cancer cells. In some cases, naïve MSCs showed tumor suppression through the activity of *Wnt* signaling inhibitor protein Dickkopf-related protein 1 (DKK1) [43–45]. This consequently leads in *c-Myc* and *Cyclin-D2* downregulation [46]. Moreover, it has been showed that naïve MSCs can inhibit angiogenesis and promote the apoptosis of vascular endothelial cells [47]. On the other hand, reports confirming the adverse effect of these cells on tumor cells by up-regulating Akt/mTOR signaling pathway, enhancing angiogenesis and promoting metastasis [48,49]. MSCs communicate with cancer or non-cancer cells in tumor microenvironment by releasing EVs. EVs are nano-size, membranous particles which can play an important role as a bio-vehicle generated from original cells to the recipient cells and consequently changing the fate and physiology of the recipient cells [50]. The unique characteristics of the EVs have created a paradigm shift for their usage as therapeutic agent, [51,52]. EVs derived from unmodified MSCs have demonstrated significant potential in various experimental and clinical studies and showed promising therapeutic effects [53,54].

Of relevance for breast cancer is the bipartite relationship of MSC-derived EVs on the cancer development and progression. Tumor homing capacity of EVs make these vesicles as the attractive agents for potential tumor-targeted delivery vehicles [55]. Several studies reported the anti-proliferative effects of MSC-derived EVs against ovarian, bladder, hepatoma and sarcoma tumor cells [56–58]. Furthermore, it has been reported that MSC-derived EVs transport different kinds of proteins, metabolites and miRNAs to the cancer cells and alter the behavior and fate of the target cells through releasing of their contents [59–61]. Therefore, MSC-derived EV has been proposed as a promising alternative for MSCs for tackle with various kinds of cancers including breast cancer [61,62].

The present study was conducted to evaluate the epigenetic anti-proliferative effects of AHCC-treated MSCs derived EVs on MCF-7 (wild type) and MCF-7 /DOX cell lines.

Therefore, we evaluated the effect of conditioned media of MSCs exposed to AHCC on cancer stem cells and the differentially expressed miRNA profile, including oncogenic (*miR-155*) and tumor suppressor miRNAs (*miR-34a*, *miR-Let7*, *miR-200c*).

2. Materials and Methods

2.1. Chemical and Reagent

AHCC® (lot S26-0530-2; Amino Up Co., Ltd., 363-32 Shin-ei, Kiyota-ku, Sapporo, Japan) is a standardized extract of cultured *Lentinula edodes* mycelia, provided by Amino Up Co., Ltd. (Sapporo, Japan). The extract was prepared in Dulbecco's modified Eagle's (DMEM) provided from Gibco, Grand Island, NY, USA for adherent cell culture and DMEM-F12 (Invitrogen), for Sphere Culture, at the 40mg/mL concentration and filtered through a 0.22 µm Millex-GV filter.

2.2. Cell Culture

Human MSCs cultured on T75 tissue culture flask in DMEM, supplemented with 10%FBS and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified chamber with 5%. The medium was refreshed every other day, and Cells were observed daily. Serial passages were done when confluent monolayer formed.

The MCF7wt was purchased from American Type Cell Collection (ATCC; Chicago, IL). MCF7/DOX (kindly gifted from Dr. Amado M. Parissenti). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) containing Fetal bovine serum (FBS) (10%, v/v) (Sigma-Aldrich, Oakville, ON, Canada), penicillin/streptomycin (0.05 mg/mL) at 37° C in a humidified atmosphere with 5% CO₂ inside CO₂ incubator.

2.3. Spheroid Culture and Growth for Initial Assessment of AHCC

Adherent cells were detached by trypsin and single cells were counted using Countess (Invitrogen). Single cells were plated in ultralow attachment 96-well plates (Corning, NY, USA) at 0.3×10^5 cells/0.2 ml/well in DMEMF12 (Invitrogen), supplemented with 10 ng/ml EGF, 20 ng/ml bFGF, 5 μ g/ml insulin, 1 mM sodium pyruvate, 0.5 μ g/ml hydrocortisone, and penicillin/streptomycin (0.05 mg/mL). Cells were treated with 4 mg/ml AHCC and grown in as non-adherent conditions were counted after 2-3 days. For sphere culture from the cell lines (MCF-7, MCF-7/Dox), 0.3×10^5 freshly trypsinated detached single cells were suspended and seeded in any wells of the 6-well ultra-low attached flask (Corning, NY, USA) in serum free DMEN-F12(1:1) supplemented with 250 mM L-glutamine (Sigma Aldrich, Oakville, ON), 100mM Sodium pyruvate, 10 mg/mL insulin (ThermoFisher), 20 μ g/mL bFGF (ThermoFisher), 20 μ g/mL EGF (ThermoFisher), 1000x streptomycin-penicillin (Sigma Aldrich) and 100 μ g/mL hydrocortisone (Sigma Aldrich), in accordance with our previous published experiments. Spheres were cultured in two groups: the first group were supplemented with the media as the control and the second were exposed to 4 mg/ml AHCC as the treatment group. Both groups were incubated in a humidified incubator (5% CO₂ at 37 °C).

2.4. Purification Characterization and Modification of EVs

The passages 3-8 of MSCs were cultured in up to 70% cell confluency to isolate EVs. The FBS-enriched medium was replaced with MSC basal medium supplemented with EV-free FBS for the control group. The MSCs were treated with EV-free FBS enriched media plus 3 mg/ml AHCC as the experimental group. Forty-eight hours post-incubation with EV-free medium, the conditioned medium (CM) of each group was collected and stored at -80 °C to purify EVs. Both groups' condition media were collected 48 h post-incubation with EV-free FBS CM and EV-free FBS CM+AHCC and store at -80 °C. To purify EVs, we used the exoEasy Maxi kit (Qiagen 76204). The isolated EVs have been homogenized, pooled, and aliquoted to be used for the experiments. A sample of EVs was used for protein content assay and nanoparticle tracking analysis (NTA). The protein concentration of EVs was measured using a BCA protein assay kit (Pierce, ThermoFisher).

2.5. Sphere Culture and Administration of Modified EVs

For EV administration in sphere culture, both cell lines cultivated in the same condition as mentioned above. Spheres were cultured in two groups: the first group treated with MSC-derived EVs as the control and the second were exposed to AHCC treated MSC-EVs as the treatment group. Immediately after seeding single cells. Both groups were incubated in a humidified incubator (5% CO₂ at 37 °C).

2.6. In-Vitro Assessment of the Immunostimulatory Effect of AHCC on Breast Cancer Cell Lines

Three samples of MCF-7 wild type and MCF-7/DOX cells were grown in a 6-well plate at a concentration of 0.3×10^6 cells/ml in DMEM complete medium. The cells were then exposed to concentrations of 4 mg/ml AHCC for 24 hours as we previously established. The control groups for each cell line were also grown in an equal volume of medium. Cells were harvested for micro-RNA profiling in next steps.

2.7. Quantitative RT-PCR for MicroRNA Profiling

RNAs were extracted from three samples of adherent and sphere cultures from both groups (control group and AHCC exposed sphere group) using Trizol protocol, miRNeasy mini kit according to manufacturer's protocol (Qiagen, Toronto, ON). The quality and quantity of extracted RNA were assessed by Nano-drop (Nanodrop Technologies, Wilmington, DE, USA).

Then, complementary DNA (cDNA) was synthesized from 2ng/ μ l of 1:60 diluted of the total RNA using reverse transcription miRCURY LNA RT (Qiagen, USA). The quantitative RT-PCR was

carried out with the Syber-Green (Qiagen, Toronto, Canada) in accordance with the RT-PCR system and standard PCR conditions in a CFX96 machine (Bio-Rad). Primers for *miR-155*, *miR-34a*, *let-7a*, and *miR-200c* were obtained from the miRCURY LNA miRNA PCR assay (Qiagen, Toronto, Canada) as described in previous studies. These were used to assess changes in expression levels relative to the internal control *SNORD65* (mmu) [63–66].

2.8. Statistical Analysis

Statistical analysis of miRNA expression was performed using GraphPad Prism software (version 5). Data was presented as mean ± SEM from at least three independent experiments. We utilized unpaired t test to evaluate the statistical significance of differences between groups. Differences were statistically significant when *value* < 0.05.

3. Results

3.1. Regulatory Effects of AHCC on Tumor Suppressor miRNA and Oncogenic miRNA Expression in Breast Cancer Cell Lines

In this investigation, we assessed the expression of miRNA in cultures obtained from wild-type MCF-7 and MCF-7/DOX cell lines. First, these cell cultures were subjected to treatment with AHCC at a dose of 4 mg/mL for a duration of 24 hours. A distinct expression pattern of microRNAs (miRNAs), namely *miR-155*, *miR-34a*, *miR-200c*, and *miR-Let7a*, was detected in the experimental group compared to the control group. It is worth mentioning that the expression of *miR-155* was dramatically reduced in the MCF-7 cells treated with AHCC in comparison to the control group, as illustrated in Figure 1a.

In contrast, there was a significant increase in the expression levels of tumor-suppressive microRNAs, including *miR-34a*, *miR-Let7a*, and *miR-200c*. the differentially expressed miRNAs were also analyzed in MCF7/DOX cells treated with AHCC with similar pattern expression (Figure 1b).

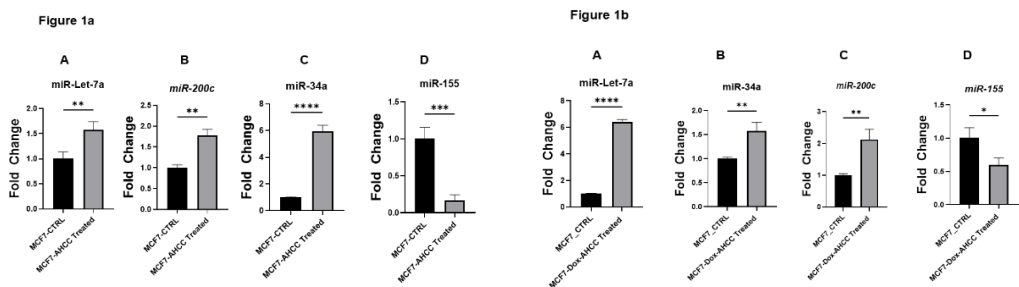


Figure 1. 1a- AHCC induces tumor-suppressive miRNA expression (*miR-Let-7a*, *miR-200c*, and *miR-34a*) and reduces oncogenic *miR-155* in wild-type MCF-7 cells.

Quantitative RTPCR assessments of miRNA expression in wild-type MCF-7 following treatment with 4 ug/mL AHCC. Panels A, B and C demonstrate the significant upregulation of the tumor-suppressive miRNAs; *miR-Let7a*, *miR-200c* and *miR-34a* respectively, whereas Pan D shows the downregulation of the oncogenic *miR-155* under the same conditions. Figure 1b- AHCC enhances tumor-suppressive miRNAs and downregulates *miR-155* in MCF-7/DOX cells. Comparative expression analysis of miRNAs in MCF-7/DOX cell lines chronologically following treatment with 4 ug/mL dosage of AHCC. Panel A shows higher expression levels of *Let7a* miRNA, Panel B, shows significant higher expression of *miR34-a* as compared with the control group, and C depicted the increased levels of, *miR-200c*, panel D statistical graph show the expression of *miR - 155* which significantly got down.

3.2. Purification and Characterization of EVs

The results of the BCA assay demonstrated a significant increase in the protein content of EVs formed from MSCs treated with AHCC. The protein concentration in the EVs from AHCC-treated MSCs was four times higher compared to EVs from untreated MSCs, with values of 0.66 µg/µL and 0.167 µg/µL, respectively. The observed increase was additionally supported by the utilization of nanoparticle tracking analysis (NTA), a technique that quantifies light scatters from EVs. The results of NTA demonstrated that EVs derived from MSCs treated with AHCC exhibited a nearly two-fold rise in particle count compared to EVs derived from untreated MSCs as presented in Figure 2 (1.2×10¹⁰ particles/mL for AHCC-treated EVs versus 5.7×10⁹ particles/mL for untreated MSC-derived EVs).

Figure 2

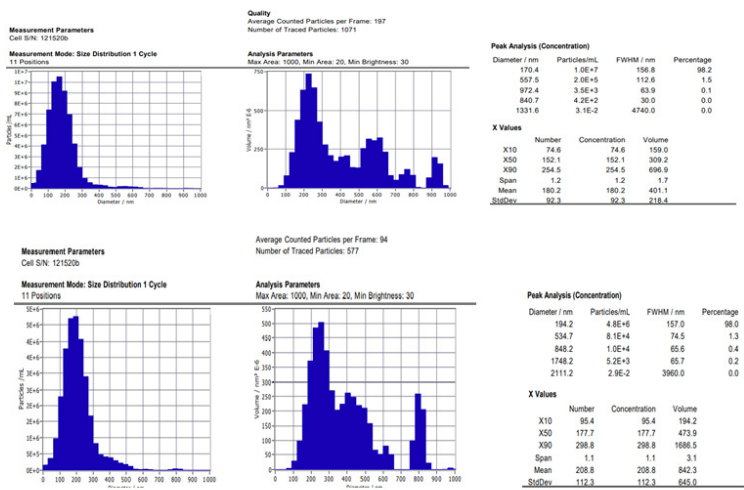


Figure 2. 2-Nanoparticle tracking analysis of EV concentrations from AHCC-treated versus untreated MSCs. Panel A shows the Concentration measurement results of (MSCs) treated with AHCC EVs: 1.2E+10 Particles / mL. Panel B shows MSCs that were not treated with AHCC produced EVs with a concentration of 5.7E+9 particles/mL.

3.3. Inhibitory Effects of AHCC-Treated MSC-Derived EVs on Colony Formation and Sphere Growth in MCF7 and MCF7-Dox Cells

The impact of EVs derived from MSCs on the colony-forming abilities in MCF7 and MCF7-Dox cells was also assessed. The control group consisted of EVs obtained from untreated MSCs, whereas the experimental group consisted of EVs derived from MSCs treated with AHCC. The findings of the study demonstrated that the presence of AHCC-treated MSC-derived EVs had a suppressive effect on colony formation and sphere growth. This observation was made during a 24-hour timeframe. Subsequent inquiries were undertaken to unravel the underlying processes responsible for the observed inhibitory impact, focusing on the examination of miRNA profiles.

3.3. Modified EVs Delivered Their Cargo and Changed the Expression Level of Target miRNAs

Using the sphere culture model, we investigated the effects of AHCC-modified MSC-derived EVs on miRNA expression in MCF7 WT and MCF7-Dox cell cultures. Naïve MSC-derived EVs were used as the control in our experimental design. Different quantities of EV proteins were utilized (5 µg/ml, 10 µg/ml, 15 µg/ml) to conduct miRNA profiling. The results revealed that there was differential expression observed for all the targeted miRNAs in both cell lines when they were subjected to non-attached growing circumstances. Significantly, the administration of 10 µg/ml AHCC-modified MSC-derived EVs resulted in a substantial upregulation of miR-34a, miR-200c, and

miR-let7a expression levels inside the MCF7-Dox sphere cultures, in contrast to the MCF7-Dox sphere cultures treated with untreated MSC-derived EVs. Although the decrease in miR-155 expression did not reach statistical significance in the MCF7-Dox spheres, a discernible downward trend was detected at a dose of 10 µg/ml (Figure 3a). In contrast, a notable decrease in the expression of miR-155 was observed at a concentration of 10 µg/ml in the MCF7 spheres that were treated with extracellular vesicles produced from AHCC-modified MSCs, compared to the control group. Moreover, notable elevations in the expression of miR-200c and miR-34a were detected in MCF7- WT spheres (Figure 3b).

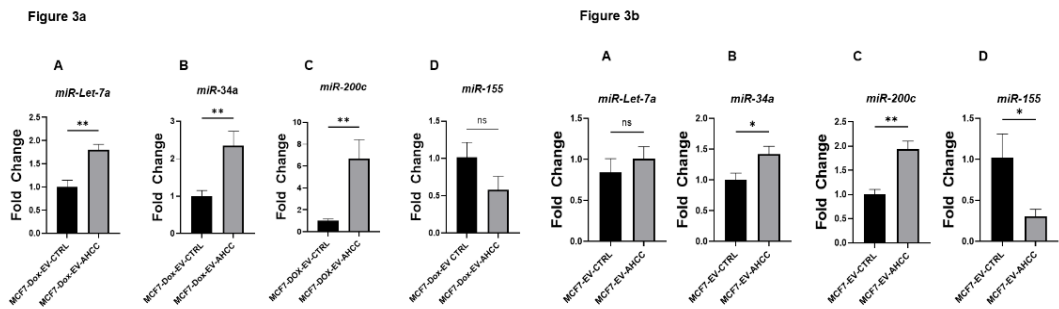


Figure 3. **Figure 3a.** AHCC-treated MSC-derived EVs upregulate tumor-suppressive miRNAs in MCF-7/DOX spheres. Quantitative RTPCR assessments of miRNA expression in MCF-7/DOX spheres, following treatment with AHCC-treated MSC-derived EVs and non-treated MSC-derived EVs as control group. Panels A, B and C demonstrate the significant upregulation of the tumor-suppressive miRNAs such as miR-34a, miR-Let7a, and miR-200c respectively. Panel D No significant difference was observed between the control and AHCC treated MSC-derived EVs treatment groups. **Figure 3b.** Effects of AHCC-treated MSC-derived EVs on miRNA expression in MCF-7 cell spheres. Quantitative RT-PCR analysis of miRNA expression in wild-type MCF-7 cell sphere after treatment with AHCC-treated MSCs derived EVs and Naïve MSCs derived EVs as control group. A The expressions level of Let-7a represented no significant difference vs control. B, Significant upregulation of miR-34a. C shows the expression levels of tumor-suppressive miRNAs which are significantly upregulated after 24 hours of exposure to AHCC-treated MSC-derived EVs. Panel D illustrates the reduction of the oncogenic miR-155 in the same sphere culture at the same condition.

4. Discussion

In the continuous battle against cancer, primary, secondary and tertiary prevention and integrative medicine have emerged as crucial components. There is a growing trend among breast cancer patients to include evidence-based alternative and complementary medicine into their treatment protocols. In recent times, the extract of Cultured Lentinula edodes Mycelia has garnered considerable attention as as evidence-based supported component in the field of preventative medicine. A significant proportion of breast cancer patients are embracing its utilization due to its potential to regulate the immune system and function as a chemo-preventive agent [67,68]. The extract is particularly enriched with the acetylated form of the α-1,4 glucan and other bioactive compounds released following the process of culture or fermentation [67,69]. Of interest, we have previously shown that it can influence CSC via modulating miR-335 [19].

miRNAs play important functions in gene regulation expression in normal and disease conditions [70]. Several studies have shown that dysregulation in miRNA expression results in tumor development through mechanisms involved in gene expression manipulation, defects in miRNA biogenesis, and dysregulated cell microenvironment [71]. MicroRNAs may act as either tumor suppressors or oncogenes [72]. In this study, four miRNAs were targeted: oncogenes miR-155, and tumor suppressor categories: miR-34a, miR-200c, and miR-Let7a.

miR-155 is an oncomir known to act as an oncogene by inhibiting anti-tumoral activity of p53 [63]. miR-155 is significantly upregulated in breast cancer and positively correlated with high tumor grade, progressive stage and metastases and it is inversely correlated with overall and disease-free

survival [73]. miR-155 plays an important role in the immunopathogenesis of breast cancer. Expression of miR-155 is also promoted by factors that induce tumor inflammation, like interleukin IL6 and interferon IFN- γ [74]. AHCC was able to downregulate this oncomiR in CSC of chemo-resistance cell line. The results showed that modified EVs can effectively decrease oncomiR-155. The miR-34a is a tumor suppressor linked to cancer progression prevention [64]. Therefore, down-regulation of miR-34a expression correlates with cancer progression. For instance, aberrant CpG methylation of miR-34a promoter showed a significant reduction its expression in several cancers [75]. A growing body of evidence has pointed to the role of mir-34a in cell cycle, apoptosis, and mitotic signaling [76] via the regulation of several genes involved in stemness phenotype maintenance, chemo-resistance, and metastasis [75,77]. In this regard, it has been shown that oncogenic pathways (Wnt, Notch) and transcription factors such as c-Myc, Oct4, and Klf4 downregulate miR-34a expression in many types of cancers [78–80]. Moreover, computational prediction and some experimental studies represent c-Myc, as a target for miR-34a [81]. In the current study, treating tumourspheres with AHCC led to significant upregulation of miR-34a in MCF-7 and MCF-7/DOX spheres as depicted in figures (1,2,4,5).

The let-7 family is a family of miRNAs first studied [82]. Down-regulation of let-7 expression has shown to have a prognostic effect on lung cancer patients' survival after surgery [82]. Many members of the let-7 family are considered as the tumor suppressor; however, let-7a is the most studied miRNA, with impact on oncogenic c-Myc [83]. Our data indicates that AHCC upregulates let-7 expression in a dose-dependent manner and affects sphere formation effectively in both wild and chemo resistant cell lines cell lines (1,2,4,5).

The miR-200c is a member of the miRNAs family, which has shown to be a tumor suppressor in some cancers, including breast cancer [65]. It regulates epithelial-mesenchymal transition (EMT) via ZEB1/2 downregulation and E-cadherin upregulation [66]. There is also evidence that miR-200c is a novel regulator of TLR4 and HMBG-1 signaling and plays essential roles in cell cycle control, proliferation, invasion, apoptosis, and metastasis in different cancer types [84]. We assessed the expression of miR-200c in MCF-7, and MCF-7/ Dox treated with AHCC after 24 hours. Our data confirms the AHCC effect on miR-200c expression, as shown in figures 3-6.

AHCC is recognized for its system modulation and preventive effects against cancer. Our study emphasizes its influence on oncomiRs and tumor suppressing miRNAs like miR 155, miR 34a, miR-let7a and miR 200c. It shows that AHCC reduces the expression of oncomiR miR-155, which hinders the tumor suppressor p53 and is linked to tumor stages and spread. Moreover, AHCC boosts the levels of tumor suppressing miRNAs such as miR-34a, miR-let7a and miR-200c which play roles in impeding cancer advancement, epithelial mesenchymal transition and inhibiting cancer growth.

In summary, the research highlights the impact of AHCC in reducing chemoresistance and modulating miRNAs in breast cancer. Through the MSC derived EVs.

5. Future Investigation

Exploring how AHCC interacts with pathways related to cancer progression or inhibition; Future investigations should aim at unraveling the connections between AHCC and other cancer-related pathways, like p53 signaling, Wnt/ β catenin, Notch and Hedgehog.

Investigating the impact of AHCC on regulation in breast cancer cells involves studying how AHCC influences DNA methylation/demethylation patterns and histone modifications on transcription factors. Additionally exploring how AHCC affects the characteristics of CSCs includes examining its influence on CSC maintenance, differentiation and self-renewal abilities in relation, to related transcription factors. In-vivo modeling of AHCC treatment: Exploit animal models to assess the therapeutic efficacy of AHCC, on tumor growth and metastasis prevention. Evaluate the potential synergic effect with check point inhibitors like PD1/PDL1 and CTLA4 to obtain the best results of immunotherapy.

Author Contributions: Conceptualization, Farzaneh Sharifzad and Chantal Matar; methodology, Farzaneh Sharifzad; validation, Farzaneh Sharifzad, Hamed Yasavoli, Roqia Shahbazi, and Dylen Burger; formal analysis, Farzaneh Sharifzad; investigation, Farzaneh Sharifzad, Hamed Yasavoli, Roqia Shahbazi, and Dylen Burger; NTA analysis and related experiments, Farzaneh Sharifzad and Nawal Alsadi; writing-original draft preparation, Farzaneh Sharifzad and Nawal Alsadi; writing-review and editing, Chantal Matar; supervision, Chantal Matar; project administration, Farzaneh Sharifzad. All authors have read and agreed to the published version of the manuscript. Chantal Matar is the corresponding author. All authors have read and agreed to the published version of the manuscript.”

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Abbreviations

The following abbreviations are used in this manuscript:

EVs	Extracellular vehicles
MSCs	Mesenchymal Stromal/Stem Cells
BCSCs	Breast cancer stem cells
FAMPS	Food-associated molecular patterns

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