Suppression of MD2 inhibits breast cancer in vitro and in vivo

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Abstract

Background: To explore the effects of the intervening measure targeting myeloid differentiation 2 (MD2) on breast cancer progression in vitro and in vivo.

Methods: The expression of MD2 in normal breast cells (Hs 578Bst) and three kinds of breast carcinoma cell lines (MCF-7, MDA-MB-231s and 4T1) were detected by western blot. MTT assay was used to detect the proliferation of 4T1 cells treated by L6H21, cell migration and invasion was measured by wound healing assay and transwell matrigel invasion assay, respectively. In addition, to further study the role of MD2 in tumor progression, we assessed the effects of inhibition of MD2 on the progression of xenograft tumors in vivo.

Results: The expression of MD2 is much higher in MDA-MB-231s and 4T1 cells than that in normal breast cells (Hs 578Bst) or MCF-7 cells (P<0.05). In vitro, suppression of MD2 by L6H21 has a significant inhibition of proliferation, migration and invasion in 4T1 cells in dose-dependent manner. In vivo, L6H21 pretreatment significantly improved survival of 4T1-bearing mice (P<0.05). Additionally, we also observed that there was none of the mice died from the toxic of 10 mg·kg⁻¹ L6H21 in 60 days.

Conclusion: Overall, this work indicates that suppression of MD2 shows progression inhibition in vitro and significantly prolong survival in vivo. These findings provide the potential experimental evidence for using MD2 as a therapeutic target of breast carcinoma.

Key words: Breast neoplasms, myeloid differentiation 2 (MD2), proliferation, migration, invasion.
Introduction

Breast cancer is one of the most common cancers affecting women worldwide[1]. Despite intensive efforts and remarkable advances in the management of breast cancer, the physiological conditions that lead to tumorigenesis including breast cancer are not well understood and distant metastasis are still occurred in part of patients after treatments. It was reported that over 90% of the deaths of cancer patients are caused by metastasis[2]. Therefore, Finding new modalities that treat the local and systemic components of the disease has become increasingly important.

Toll like receptors (TLRs), which are essential components of innate immune system and serve as major contributor to chronic inflammation[3], have garnered an extraordinary amount of interest in cancer research because of their role in tumor progression[4-7]. Among TLRs, TLR4 has already been linked to tumors including breast cancer and has been implicated in tumor cell invasion, survival, and metastasis[8-11].

MD2, well known as an accessory protein of TLR4, plays an essential role in activation of TLR4 signaling pathway in inflammatory response[12]. However, whether MD2 has a similar effect on the progression of breast cancer is still poorly understood and there was rare evidence indicating the correlation of MD2 and tumor progression. Previously, our cooperators synthesized a new chalcone derivative, (E)-2,3-dimethoxy-4′-methoxychalcone (L6H21), which identified MD2 as its molecular target. In addition, they demonstrated that L6H21 shows excellent inhibition of the TLR4-mediated inflammatory response and septic injury both in vitro and in vivo[13]. Therefore, in order to investigate whether L6H21 has antitumor effects, in this study, we first detected the
expression of MD2 in four kinds of cell lines by western blot assay. Next, we aimed to observe and confirm that the effects of L6H21 on 4T1 cells proliferation, migration and invasion in vitro. Additionally, model of transplanted tumor on BALB/c nude mouse were used to study the anticancer effect of L6H21 in vivo.

Materials and methods

Cell culture

Normal breast cells (Hs 578Bst) and breast cancer cell lines MCF-7, MDA-MB-231s and 4T1 were obtained from ATCC and grown according to ATCC recommended culture conditions. The cells were cultured in DMEM (Gibco) or RPMI 1640 medium (Gibco) with 10% fetal bovine serum (FBS) (Gibco), and then incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

Western blot

Cell protein samples (50μg) were subjected to 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories). After being blocked in blocking buffer (5% milk intris-buffered saline containing 0.05% Tween 20) for 1.5 h at room temperature, membranes were incubated with different primary antibodies overnight at 4°C. The membranes then were washed in TBS-T and reacted with secondary horseradish peroxidase-conjugated antibody (Santa Cruz, CA, USA;1:5000) for 1-2 h at room temperature. Blots were then visualized using enhanced chemiluminescence reagents (Bio-Rad Laboratories). The density of the immunoreactive bands was analysed using Image J software (NIH, Bethesda, MD, USA).
**MTT assay**

Cell proliferation was determined using the MTT method. Briefly, 48 h after treatment, the MCF-7, MDA-MB-231s and 4T1 cells were seeded into 96-well culture plates (BD Biosciences, Franklin Lakes, NJ, USA) and incubated overnight at 37°C in 5% CO₂. Cell proliferation was assessed at 24, 48, 72, 96 and 120 h following addition of 0.5 mg/ml MTT (Sigma, USA) solution. After a 4-h incubation, the reaction was stopped by addition of 150 µl DMSO (Sigma). After 10 min of agitation (100 rpm), the optical density (OD) at 490 nm was determined with microplate reader (BioTek). Each sample was tested with six replicates. All experiments were performed in biological triplicate.

**Wound migration assay**

The 4T1 cells were grown in six-well plates to a confluent monolayer and subsequently wounded with sterile pipette tips. The wounded monolayers were then incubated with lipopolysaccharide (LPS) (Sigma-Aldrich) and corresponding protein as indicated in Figure(3A) for 48 hours. The wound area was measured under microscope. The percentage of wound healing rate was estimated as follows: Wound healing rate % = [1-(wound width at 48 hours/wound width at 0 hour)] ×100%. ×100 microscopic fields under microscope.

**Transwell Matrigel invasion assay**

Tumor cell invasion was performed using Transwell system (Millipore) with 8 µm-pore polycarbonate filter membrane. The upper chamber was covered with Matrigel (Sigma-Aldrich)
and incubated at 4°C overnight. The upper chamber was then seeded with 1×10^4 4T1 cells incubated with LPS and corresponding protein as indicated in Figure(3B) and inserted into the lower chamber containing complete medium. After incubation at 37°C in 5% CO₂ for 24 hours, the cells on the interior of upper chamber were removed, and the polycarbonate membranes were stained with 0.1% crystal violet (BASO) for 10 minutes. The number of migrating cells was counted in eight randomly selected ×400 microscopic fields under microscope.

**Xenograft assays in nude mice**

To evaluate in vivo tumorigenesis, breast carcinoma xenografting mouse model was used. Male BALB/c mice weighing 18-22g were obtained from the Wenzhou Medical University Animal Centre and prepared for tumor implantation. All experimental procedures involving animals were performed in accordance with animal protocols approved by the Institutional Animal Use and Care Committee of Wenzhou Medical University and performed according to the institutional ethical guidelines for animal experiment. The mice were randomly divided into four groups (n=8 per group). 4T1-bearing mice were injected with 3×10^5 4T1 cells (i.v. through the tail vein) 3 days after being treated with L6H21 (at 10 mg·kg⁻¹ or 5 mg·kg⁻¹) and saline by intragastric administration, respectively. To investigate the toxicity of L6H21, mice of the fourth group were only treated with L6H21 at 10 mg·kg⁻¹ but without inoculation of 4T1 cells. Each group of mice were then intragastrically administrated per day until death or day 60. The survival curve was made to analyse the survival rate.

**Statistical analysis**
For all data, statistical analysis was performed in SPSS 22.0 for Windows (SPSS Inc.). All data are shown as means±SDs. The statistical significance between groups was obtained by Student’s t-test or one-way ANOVA test and the significance level was set at P < 0.05.

Results

MD2 expression in four kinds of cell lines

In our experiment, we first used western blot assay to examined the expression of MD2 in normal breast cells (Hs 578Bst) and three kinds of breast carcinoma cell lines (MCF-7, MDA-MB-231s and 4T1). The logarithmic phase of these cells were cracked and the total protein was extracted. MD2 protein expression was then detected by western blot. As shown in Figure 1, MD2 expressed in each kinds of cell lines, of these, MD2 is highly expressed in highly malignant MDA-MB-231s and 4T1 cells.

Figure 1 MD2 is highly expressed in MDA-MB-231s and 4T1 cells (p < 0.05 compared to that expressed in Hs 578Bst cells and MCF-7 cells)

L6H21 inhibition of breast cancer cells proliferation

To explore the role of MD2 in breast cancer cells proliferation, we assessed the effects of inhibition of MD2 by L6H21 in 4T1 cells by MTT assay. As shown in Figure 2, L6H21
significantly inhibited the proliferation of 4T1 cells in dose-dependent manner (IC$_{50}$=10.41 μM), which has a similar inhibition effect in each group by curcumin, respectively.

Figure 2 MTT assay showed that L6H21(10μM, 30μM and 100μM) significantly inhibited the proliferation of 4T1 cells in dose-dependent manner.

L6H21 inhibition of breast cancer cells migration and invasion

To study the effect of MD2 in cell migration, we adopted a scratch wound model in the presence of mitomycin C which inhibited proliferation. In these conditions, migration was significantly decreased in L6H21 group (10μM) and anti-MD2 group (1μg/mL) as compared to the control group. Next, we performed transwell invasion assay to investigate the effect of L6H21 in 4T1 cell invasion further. As shown in Figure 5, there were significant differences of effect of invasion in control group, 5μM L6H21 group and 10μM L6H21 group. Moreover, there was almost no cell observed in 10μM L6H21 group as well as in 1μg/ml anti-MD2 group. These results indicate that L6H21 has a significant inhibition of migration and
invasion in 4T1 cell concentration-dependently.

Figure 3 L6H21 inhibition of 4T1 cells migration and invasion. (A) L6H21 significantly inhibited the migration of 4T1 cells. The images (100×) were obtained by microscope. (B) L6H21 significantly inhibited the invasion of 4T1 cells (400×). In 5μM L6H21 group, there were a few migrating cells observed compared to that of control group. While in 10μM L6H21 group and in 1μg/ml anti-MD2 group, there was almost no migrating cell observed.

**L6H21 suppresses tumor progression in the nude mice**

To further investigate the role of MD2 in tumor progression, we assessed the effects of inhibition of MD2 on the progression of xenograft tumors *in vivo*. Figure 4 shows the survival rate of 4T1-bearing mice (treated with saline, 5mg·kg⁻¹ and 10 mg·kg⁻¹ L6H21,
respectively) and toxic control mice (L6H21 treated but without inoculation of 4T1 cells). As expected, the 5 mg·kg⁻¹ L6H21- or 10 mg·kg⁻¹ L6H21-treated mice survived significantly (p <0.05) longer than the saline-treated mice. The mean survival times of 4T1-bearing mice treated with saline, 5 mg·kg⁻¹ and 10 mg·kg⁻¹ L6H21 were 23.8±4.8, 30.9±8.9, 40.4±12.6 days, respectively. In addition, after treated for 60 days, there were still two mice (25%) survive in 4T1-bearing mice treated with 10 mg·kg⁻¹ L6H21. Interestingly, we also observed that there was none of the mice died from the toxic of 10 mg·kg⁻¹ L6H21 in 60 days. These data indicated that as an inhibitor of MD2, L6H21, could prolong survival efficiently with reliable security.

![Graph showing survival rates](image)

**Figure 4** L6H21 enhanced survival in nude mice. Male BALB/c mice were randomly divided into four groups (n=8 per group). 4T1-bearing mice were injected with 3 × 10⁵ 4T1 cells (i.v. through the tail vein) 3 days after being treated with L6H21 (at 10 mg·kg⁻¹ or 5 mg·kg⁻¹) and saline by intragastric administration, respectively, and the mice of the fourth group were only treated with L6H21 at 10 mg·kg⁻¹ but without inoculation of 4T1 cells. Each group of mice were then intragastrically administrated per day until death or day 60. The survival curve was made to analyse the survival rate.
Statistical analysis

For all data, statistical analysis was performed in SPSS 22.0 for Windows (SPSS Inc.). All data are shown as means±SDs. The statistical significance between groups was obtained by Student’s t-test or one-way ANOVA test and the significance level was set at P < 0.05.

Discussion

Diverse studies have shown that TLR4 is associated with tumor development and progression. In breast cancer, TLR4 activation has been linked to both cancer inhibition and growth[10,14-16]. Huan Yang et al reported that TLR4 expressed higher levels than any other TLRs and knockdown of TLR4 could actively inhibit proliferation and survival of human breast cancer cells MDA-MB-231. Functional analyses of ribonucleic acid interference (RNAi) against TLR4 revealed this successfully inhibited the growth and proliferation of MDA-MB-231 cells and resulted in a significant (P<0.05) reduction of inflammatory cytokines[11]. In other work, 4T1 cells challenged with lipopolysaccharide induced tumor growth and metastasis, by increasing angiogenesis, vascular permeability, and tumor invasion[17-18]. A total of 74 breast carcinomas were collected from patients to study the clinical relevance of TLRs in breast cancer. Tumors with high TLR4 expression, in mononuclear cells were found to have a higher probability of metastasis[19]. These studies suggest TLR4 involvement in breast cancer progression.

Although TLR4 plays an essential part in breast cancer progression, the role of its accessory protein, MD2, known for several years as an essential co-factor for TLR4 signaling, has not
yet been clarified. One study highlighted that MD2 was overexpressed in highly invasive colorectal cancer cells (SW837), in poorly differentiated, moderately invasive colorectal cancer cells (HT-29), and in well-differentiated but non-invasive colorectal cancer cells (Caco-2)[17]. Another study reported that serum amyloid A3, a major component of acute inflammation, binds to MD2 and activates the MyD88-dependent TLR4/MD2 pathway and thus facilitates lung metastasis[18]. Therefore, MD2 could be related to the degree of differentiation, proliferation, and migration capacity of cancers. However, there was few research focus on the relationship between the MD2 expression and the progression of breast cancer.

In the first stage of our study, we detected the expression of MD2 in several cell lines. As expected, the western blot assay showed MD2 is highly expressed in MDA-MB-231s (the estrogen receptor-negative, progestrone receptor-negative and HER2 negative -human breast cancer cells) and 4T1 cells (spontaneously metastasizing mammary adenocarcinoma). Our preliminary results may probably indicate that MD2 is higher expressed in highly malignant cell lines than that in normal breast cells (Hs 578Bst) or MCF-7 cells (P<0.05).

In order to investigate the role of MD2 in breast cancer progression, we next utilized L6H21, a new MD2 inhibitor, for down-regulating the expression of the MD2. And we performed experiments to observe the change of biological behavior of 4T1 cells by using L6H21. MTT assay was performed and the results showed that L6H21 significantly inhibited the proliferation of 4T1 cells in dose-dependent manner, especially in 100μM group. Moreover,
scratch wound model and transwell invasion assay demonstrated that L6H21 has a significant inhibition of migration and invasion in 4T1 cell. These results indicated that suppression of MD2 could effective repress tumor cell proliferation, migration and invasion \textit{in vitro}, in part consistent with the findings of Virginie Grondin \textit{et al} which was experimented with HT-29 cell[20]. To further determine whether MD2 regulates tumor progression \textit{in vivo}, we used tumor xenografts by inoculating 4T1 cells in L6H21-treated or saline-treated nude mice. And we provided evidence that MD2 suppression by L6H21 prolonged survival in nude mice with hypotoxicity.

\textbf{Conclusion}

In conclusion, our study indicates that 4T1 cells treated with L6H21 show progression inhibition \textit{in vitro}. Moreover, L6H21 can significantly prolong survival \textit{in vivo}. To our knowledge, this is the first report to describe the significance of MD2 expression to breast caner cells \textit{in vitro} and \textit{in vivo}. Although the precise molecular mechanisms behind the altered expression of MD2 in breast cancer remain poorly understood, our data suggest that MD2 may be a promising candidate as a potential therapeutic target for breast cancer intervention.

\textbf{Abbreviations}

MD2: myeloid differentiation 2

TLRs: Toll like receptors
L6H21: (E)-2,3-dimethoxy-4′-methoxychalcone

FBS: fetal bovine serum

OD: the optical density

LPS: lipopolysaccharide

RNAi: ribonucleic acid interference

Declarations

Ethics approval and consent to participate:
The authors are accountable for all aspects of the work in ensuring that questions related to
the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Consent for publication:
Not applicable

Availability of data and materials:
The datasets used and/or analysed during the current study are available from the
corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions:

GL G and SR Z designed the study. SR Z and WD F conducted this experiment and RM M helped to design study model. WD F, SR Z, QD H, JW G, JY Z and KK L collected data. SR Z, WD F, RM M and QD H did the statistical analyses and prepared figures. SR Z, WD F and GL G reviewed the results and interpreted data. SR Z and WD F wrote the manuscript. All authors have made an intellectual contribution to the manuscript and approved the submission. WD F and SR Z are co-first authors.

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