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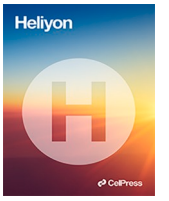

Research article

Oleanolic acid inhibits the tumor progression by regulating *Lactobacillus* through the cytokine-cytokine receptor interaction pathway in 4T1-induced mice breast cancer model

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ABSTRACT

The therapeutic mechanism of oleanolic acid (OA) in breast cancer has been widely reported, but little has been known about the combined effects of transcriptome and gut microbiome. In this study, the phenotypic effect of oleanolic acid on mice was tested at the end of the administration cycle, and RNA sequencing on murine tumor tissue and 16S-rRNA sequencing on intestinal contents were conducted to analyze gene expression profiles and microbial diversity between the control group and OA treated group using 4T1-induced mice breast cancer model. As a result, it has been confirmed that oleanolic acid would play a significant inhibitory effect on the development of breast tumors in mice. Based on the integrative analysis of the transcriptomic and metagenomic data, it was found that the abundance of *Lactobacillus* in the intestinal flora of mice significantly increased in the OA group. Moreover, the up-regulation of *Il10* had a significant effect on inhibiting the tumor progression, which played a role through cytokine-cytokine receptor interaction pathway.

1. Introduction

Oleanolic acid (OA) with a molecular formula of $C_{30}H_{48}O_3$ is a pentacyclic triterpenoid compound widely present in various plants and plays an important role in the treatment of many diseases [1,2]. Previous studies have shown that OA has excellent anti-inflammatory and liver protective effects [1,3–5]. In clinical practice, OA has been developed into various drug reagents to treat various types of hepatitis symptoms and can also be used to prevent liver damage caused by other drugs [6–9]. OA can exert antioxidant functions and eliminate free radicals that cause damage to the body [10,11]. In addition, many studies have shown that OA has a toxic effect on tumor cells, while causing almost no harm to normal cells [12–14]. OA has high efficiency and low toxicity pharmacological effects, and the growth and proliferation of many tumor cells are greatly affected under the therapeutic effect of OA. As a newly developed drug reagent for treating tumors, OA has enormous potential. By leveraging various anti-tumor mechanisms, it can affect the growth and proliferation of tumor cells, cause damage to them, and achieve the goal of treating malignant tumors. As an effective natural product for the treatment of breast cancer, it was shown that OA can induce apoptosis and autophagy in breast cancer

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cells [15]. In addition, OA may also play an important role in the treatment of brain metastases of breast cancer [16]. Moreover, it was also found that restoring gut microbial homeostasis is an emerging approach to treating breast cancer [17]. Recent studies have shown that certain bacteria in the gut can contribute to the progression of breast cancer [18], but certain probiotics can prevent or treat breast cancer by modulating gastrointestinal bacteria and the systemic immune system [19]. Nowadays, multi-omics analysis based on high-throughput sequencing technology has been widely applied to analyze the molecular mechanisms of natural product therapy for cancer [20].

Although there have been many reports on the therapeutic effects of OA in breast cancer, the mechanism of its anti-cancer effect has not been fully elucidated. Therefore, in this study, we integrated the methods of transcriptomics and intestinal microbiome for the first time and applied them to the mouse model of oleanolic acid anti breast cancer, in order to more systematically analyze the regulation of gene expression and the changes of flora in the process of OA treatment on the breast tumor progression.

2. Materials and methods

2.1. Materials

A total of 53 healthy BALB/c female mice, 4–6 weeks old, weight 18~20g, were purchased from Anhui Medical University. Luc 4T1 breast cancer cell line, DMEM complete medium were obtained from Bioindustry Company (Shanghai, China). Oleanolic acid (OA) with the purity of 98% and tamoxifen (TAM) were purchased from Meilun Biotechnology Co., Ltd. DMEM high-sugar medium and fetal bovine serum were purchased from BI company. RNA reverse transcription kit and qRT-PCR kit were purchased from Suzhou Nearshore Protein Technology Co., Ltd. Corn oil was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2. Cell culture and establishment of murine model

Mouse Luc 4T1 breast cancer cells were placed in a complete medium containing 90% DMEM, 10% fetal bovine serum (high-quality) and 1% penicillin-streptomycin, incubated and expanded in a 37 °C, 5% CO_2 incubator, and prepared into cell suspensions with a cell concentration of $1^{*}10^{7}$/ml for later use.

45 BALB/c mice were randomly selected for breast cancer cell injection and modeling. Seed 200 μl of the corresponding concentration of cell suspension subcutaneously under the flank wall of each mice, respectively.

2.3. Animal grouping

After the injection of 4T1 cells, the growth of tumors at the mice's living diet and inoculation site was observed every day, including the time of tumor appearance and tumor size. After inoculation of cells, nodules appeared about three days, and when the tumors grew to the size of rice grains, all the mice were randomly divided into 4 groups, 15 mice in the model group (CK), 15 mice in tamoxifen (TAM) group, 15 mice in oleanolic acid (OA) group, and 8 mice in the normal group (Blank). Mice are then subjected to daily gavage administration of 50 μl for 11 days, with corn oil as the drug solvent. Model group (CK): gavage 50 μl of drug solvent; Tamoxifen group (TAM): gavage 50 μl solution containing TAM 40 mg/kg; oleanolic acid group (OA): gavage 50 μl solution containing OA 40 mg/kg; Normal control group (Blank): gavage 50 μl of the drug solvent.

2.4. Phenotypic effects of OA on tumor-bearing mice

During the whole experimental cycle, the change of mouse weight and tumor volume was observed and recorded, and after the end of the experimental cycle, the multifunctional imager was used to observe the bioluminescence intensity of the mouse breast cancer tumor. The long diameter and short diameter were measured every day, and the calculation formula was as follows: tumor volume (mm^3) = ab [2]/2 (a is the long diameter, b is the short diameter), the tumor volume size was calculated. Cell apoptosis was tested through H&E staining and TUNEL immunostaining. The enzyme activity and MDA content of SOD were measured according to the operational requirements of the MDA kit and the operating instructions of the T-SOD kit, and the content of *Il-6* and *Tnf-α* in serum was determined by enzyme-linked immunosorbent assay (ELISA).

2.5. Analysis of microbial diversity

Under sterile conditions, the entire cecum of the OA group and CK group was removed, the required intestinal segment was cut with a sterile scalpel, the contents were dug up with a sterile scalpel and aliquoted into 2 mL EP tubes (sterile) or cryopreserved tubes (sterile), each tube had a tissue volume of 0.5–2g, each sample was aliquoted in 2–3 tubes for backup. Based on the Illumina platform of Personal Biotechnology (Shanghai, China), two groups of samples were analyzed for microbial diversity, and the raw data were preprocessed and analyzed by the conventional protocols.

2.6. Transcriptomic analysis

After the samples of murine tumor tissues from OA group and CK group were extracted, purified, and restocked, the libraries were sequenced using high-throughput sequencing based on the Illumina sequencing platform. The raw data was filtered, and the filtered

high-quality sequence data was compared to the reference genome of the species Mus_musculus.GRCm.38.83. Based on the alignment results, the expression of each gene is calculated. On this basis, the expression difference analysis, enrichment analysis, and cluster analysis of the samples were further analyzed.

2.7. Real-time PCR

The mRNA sequences of the relevant genes were consulted on the NCBI website, then primers were designed using Primer Premier 5 and synthesized by General Corporation, as shown in Table 1. Then, the reaction system was configured according to the kit instructions, and the reaction was carried out according to the set cycle program, with the housekeeper gene GAPDH as the reference and the relevant gene expression values were calculated.

3. Results and discussion

3.1. Analysis of the apparent effect of OA on murine breast tumor

According to the bioluminescence intensity observation of murine breast tumors with a multifunctional imager, it was found that there would be an obvious tumor fluorescence signal, and the larger the tumor, the stronger the fluorescence signal. The in vivo imaging results are consistent with the measurements (Fig. 1A and B). The group of OA treatment showed the smallest tumor volume, suggesting that OA is most effective in inhibiting breast tumor growth in mice. The result is consistent with our previous report [21].

Compared with the CK group and the TAM group, after 40 mg/kg OA treatment, we can observe that the growth and proliferation of breast tumors in mice are severely affected and the rate is significantly slowed down (Fig. 1C and D); The weight of the OA group and the model group showed an overall upward trend, but the weight of tumor tissue increased (Fig. 1E). It was shown that the administration of OA can promote the growth of immune organs (thymus), thereby enhancing the immunity of mice, thereby improving the anti-tumor ability of mice (Fig. 1F) [22]; From the perspective of tumor pathological structure, the effect of OA group is more significant, the arrangement of tumor cells is not regularly observed, the gap between tumor cells increases compared with CK group and TAM group, nucleoplasm shrinks, and apoptosis is more obvious (Fig. 1G). Apoptosis in murine breast tumor cells was detected by the TUNEL method of immunostaining (Fig. 1H). For the detection of T-SOD and MDA in serum, the administration of the drug OA can increase the enzyme activity of T-SOD and reduce the content of MDA (Fig. 1I and J). Based on the data of *Tnf-α* and *Il-6* levels in mouse serum by ELISA kits and the RT-qPCR of *Tnf-α* and *Il*-6 mRNA in tumor tissues, it was revealed that compared with the CK group, the content of *Tnf-α* and *Il-6* in serum showed a downward trend under OA treatment, which is consistent with the expression of *Tnf-α* and *Il*-6 mRNA in tumor tissues (Fig. 1K–N).

3.2. Microbial diversity analysis

To further study the effect of OA on the gut microbiome of mice, analysis of microbial diversity of the cecum was conducted in both OA group and CK group. It was found that the abundance of *Lactobacillus* in the gut microorganism increased in the mice of OA group.

The difference in intestinal flora structure between the OA group and CK group was evaluated by the box plot of the Alpha diversity index, and it was found that compared with the CK group, after the treatment intervention of oleanolic acid, the Chao 1 index, Observed_species index, Shannon index, Simpson index, Faith_pd index and Pielou_evenness index of the OA group decreased and the Goods_coverage increased. This indicates that the richness and diversity of microbial communities in the cecal contents decreases, indicating that gavage OA reduces the microbial diversity in the intestines of mice (Fig. 2).

Beta diversity refers to the dissimilarity of species composition or the rate of species replacement along the environmental gradient between different communities that vary along the environmental gradient. In the distance matrix vs. PCoA analysis, each point represents a sample, and different colored dots indicate different groupings (Fig. 3A). The closer the projection distance of the two points on the axis, the more similar the community composition of the two samples in the corresponding dimension. After OA treatment, the intestinal flora of the OA group changed significantly compared with the CK group. According to the results of hierarchical clustering analysis (Fig. 3B), it shows a stacked histogram of the top 10 genera in abundance. The *Lactobacillus* in the intestines

Table 1
Sequences of targets primers.

Gene symbol	Forward primer	Reverse primer
Myb	GCTGAAGAAGCTGGTGGAAC	CAACGCTTCGGACCATATTT
Hc	GCTAGCCTTCACACCTCCAG	CAGGGTGAAGGTCACCAAGT
Gdf10	ACCCCTGCAAGACAATGAAC	AAGTCCAGCACCTGAGAGGA
Id4	GAGACTCACCCTGCTTTGCT	AGAATGCTGTCACCCTGCTT
Il10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
Bmp2	TGGAAGTGGCCCATTTAGAG	TGACGCTTTTCTCGTTTGTG
Clu	CAGTTCCCAGACGTGGATTT	TGAGGTGTTGAGCATCTTCG
Cx3cr1	TTCATCACCGTCATCAGCAT	GATGCGGAAGTAGCAAAAGC
Bdkrb1	AGCGCCTAACCATAGCAGAA	AACAGGTTGGCCTTGATGAC
Ccn2	CAAAGCAGCTGCAAATACCA	GGCCAAATGTGTCTTCCAGT

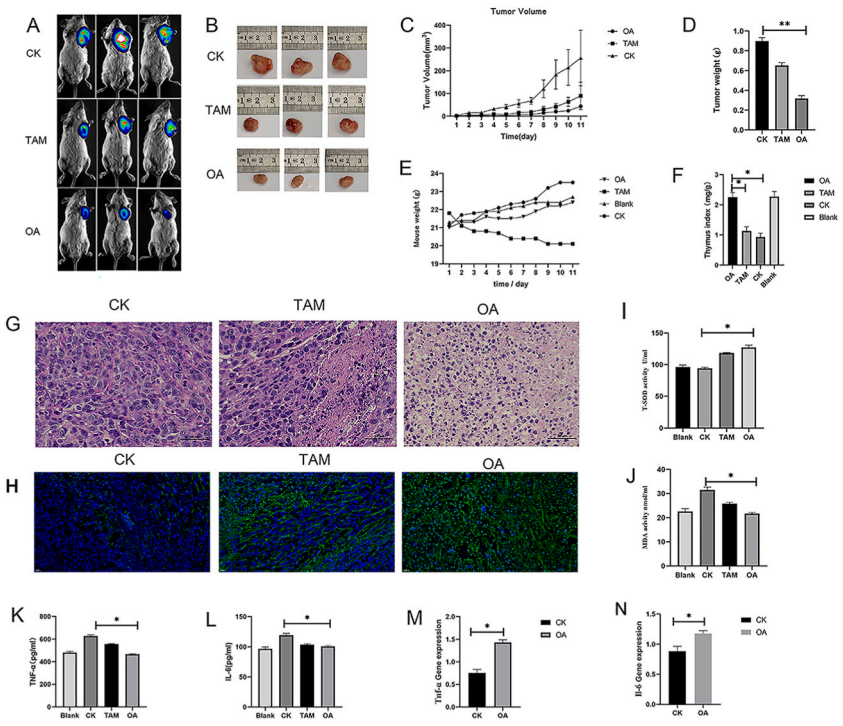

Fig. 1. Phenotypic effects of OA treatment on murine breast tumor A. Murine tumor luminescence imaging map; B. Murine solid tumor photo; C. Trends in tumor volume changes in mice; D. Comparison of tumor weights in mice; E. Changes in mouse body weight during the experimental cycle; F. Analysis of thymus index in each group; G. H&E staining of mouse breast tumor tissue in CK, TAM and OA group; H. TUNEL staining of mouse breast tumor tissue in CK, TAM and OA group; I. T-SOD enzyme activity in mouse serum; J. MDA content in mouse serum; K. The concentration of Tnf-α in mouse serum; L. The concentration of Il-6 in mouse serum; M. The expression of gene Tnf-α in tumor tissue; N. The expression of gene Il-6 in tumor tissue.

of mice in the OA group changed significantly compared with the CK group.

In order to further compare the differences in species composition between samples and to show the trend of species abundance distribution in each sample, heat maps can be used for species composition analysis. We plotted the heatmap by default using the abundance data of the top 20 genera of average abundance (Fig. 3C). Manhattan plots showed that compared with CK, *Lactobacillus* is an increased probiotic in the OA group, which indicates that OA mainly affects the maturation of immune cells and their products in the intestine by increasing the abundance of *Lactobacillus*, thereby exerting anti-tumor effects, which in turn exerts a significant inhibitory effect on breast cancer (Fig. 3D) [23–25]. *Lactobacillus* can also modulate the immune response by inducing the production of *Il10* [26,27]. The antitumor effects of *Il10* are mainly by inducing a protective antitumor immune response mediated by NK cells or $CD8^+$ T cells [28,29], which can also inhibit angiogenesis [30,31].

3.3. Transcriptome sequencing analysis of breast cancer genes in mice treated with OA

In order to study the effect of OA on breast cancer in mice at a deeper level, a transcriptome analysis of tumor tissues in the OA group and CK group was performed. In this study, the conditions for screening differentially expressed genes were: the cutoff with $|\log2FoldChange| > 1$ and the significance level of p value < 0.05. The differentially expressed genes analysis and hierarchical clustering between the OA group and the CK group samples were carried out by transcriptomics analysis software. As a result, there were 111 upregulated genes and 81 downregulated genes to be significantly identified by comparing the gene expression profile of the OA group with the data of the CK group (Fig. 4A and B). For example, *Ccn2, Cldn4, Clu, Gabrp* and *Cdh23* were the downregulated genes, and *Nptx1, Bdkrb1, Elf5, Igfbp5, Myb* and *Alx4* were the upregulated genes.

Cellular communication network factor 2 (*Ccn2*) promotes bone metastasis in breast cancer [32]. Claudin 4 (*Cldn4*) is a key member of the tight junction protein that contributes to cell migration and invasion in breast cancer cells and is involved in breast cancer cell migration and invasion through a novel PAK4-CEBPB-CLDN4 axis [33]. Clusterin (*Clu*) is an extracellular chaperone functionally implicated in DNA repair, cell cycle regulation, apoptotic cell death, and tumorigenesis, and has been reported to have potent anti-apoptotic activity, and *Clu* may play a role in tumorigenesis and progression in human breast cancer [34]. Cadherin related 23 (*Cdh23*) mediates heterotypic cell adhesion between breast cancer epithelial cells and fibroblasts and may play a role in the early stages of breast cancer metastasis [35]. Gamma-aminobutyric acid type A receptor subunit pi (*Gabrp*) stimulates breast cancer cell migration by activating extracellular regulatory kinase 1/2 (ERK1/2) [36].

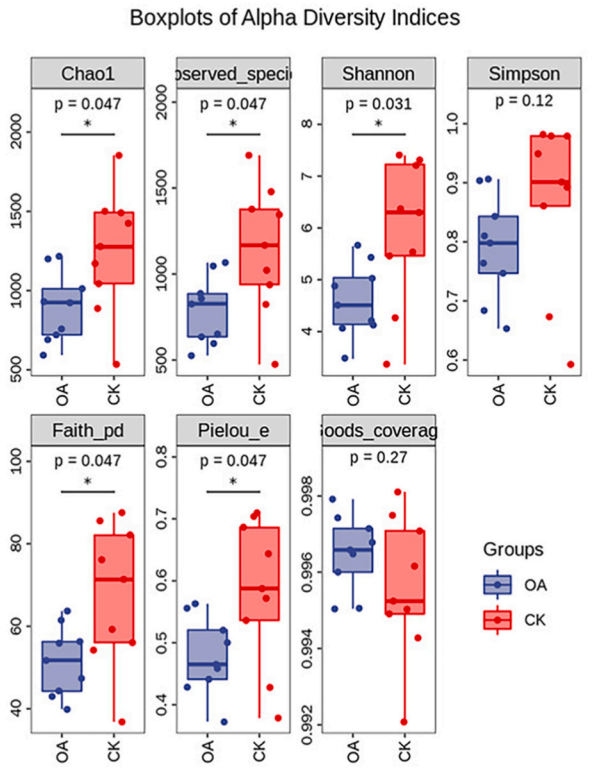

Fig. 2. Alpha diversity analysis of 16srRNA of mouse gut microbiota.

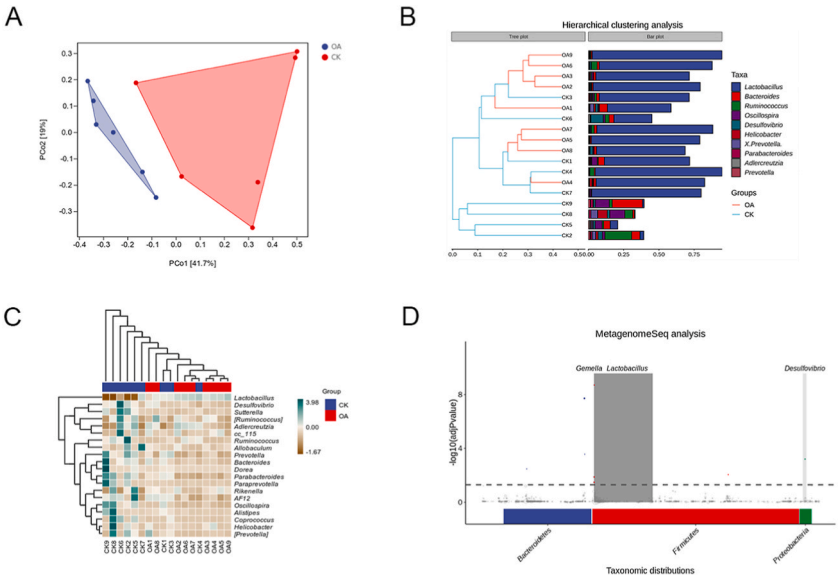

Fig. 3. Beta diversity analysis of 16srRNA of mouse gut microbiota at the genus level related to the OA treatment in breast tumor mice A. Primary coordinate analysis; B. Hierarchical clustering analysis of beta diversity; C. Heat map analysis of species composition; D. Manhattan map of species composition.

Neuronal pentraxin 1 (*Nptx1*) down-regulates breast cancer cell cycle progression through Wnt/β-catenin signaling [37]. Expression of bradykinin receptor, beta 1 (*Bdkrb1*) is associated with a good prognosis in women with estrogen receptor (ER)-negative breast tumors [38]. E74-like factor 5 (*Elf5*) is an alkaline transcription factor that plays a key role in breast tissue and gland development. *Elf5* inhibits the migration and invasion of breast cancer cells by regulating CD24 expression [39]. Insulin-like growth factor

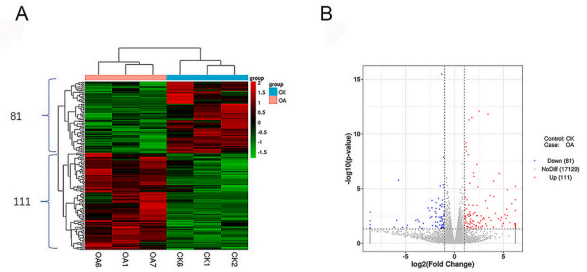

Fig. 4. Analysis of differentially expressed genes of OA treatment on breast tumor in mice A. Heatmap of differentially expressed genes; B. Volcano map of differentially expressed genes.

binding protein 5 (*Igfbp5*) inhibits the growth of human breast cancer cells in vitro and in vivo, and *Igfbp5* is a potent growth inhibitor and pro-apoptotic agent in human breast cancer cells by regulating cell cycle regulation and apoptosis mediators [40]. Functional studies have shown that ectopic expression of aristaless-like homeobox 4 (*Alx4*) in breast cancer cells inhibits cell proliferation and metastasis in vitro and in vivo. Mechanistic studies have found that *Alx4* inhibits breast cancer progression by interfering with the Wnt/β-catenin pathway and exerts its anti-tumor function [41]. High expression of myeloblastosis oncogene (*Myb*) is associated with a favorable prognosis and may act as a tumor suppressor in breast cancer, both inhibiting tumor cell metastasis and inhibiting breast cancer progression through immunomodulatory effects [42,43].

Furthermore, the gene ontology (GO) enrichment analysis of differentially expressed genes was performed, the top 10 GO terms of molecular function (MF), biological process (BP) and cell component (CC) with the most significance level were selected in each GO classification (Fig. 5A and B). Among which, the most important functional enrichment category is the intercellular space, plasma membrane, cytosol, hydrolases, cell death, the immune system and its response to stimuli.

Based on the results of the KEGG enrichment analysis of differentially expressed genes, the top 20 pathways were identified (Fig. 6A and B and Table 2). As a result, the most significantly associated pathways responding to OA treatment of murine breast tumors were mainly enriched in the Hippo signaling pathway, TGF beta signaling pathway, Complement and coagulation cascades, and Cytokine-cytokine receptor interaction. Among the 192 differentially expressed genes significantly identified by RNA-seq, six upregulated genes including *Myb*, *Hc, Gdf10, Id4, Bdkrb1* and *Il10* as well as four downregulated genes including *Bmp2, Ccn2, Clu* and *Cx3cr1* were involved in the above enriched functions (Fig. 7A and B). The expression regulation patterns of these genes have been validated by qPCR, except for the *Clu* gene (Fig. 7C and D).

Expression of *Bdkrb1* is associated with a good prognosis in women with estrogen receptor (ER)-negative breast tumors [38]. Upregulation of *Gdf10* effectively inhibits breast cancer metastasis [44,45]. *Il10* tumor size reduction as well as prolonged mouse survival had a significant effect. *Il10* (interleukin-10) has direct antitumor and antimacrophage toxicity in vitro, enhancing its effectiveness in mediating antitumor immune responses. It can also mediate the immune response to tumors and inhibit tumor cell metastasis [46,47], and *Id4* is a member of the protein Id family (*Id1-Id4*), and *Id4* acts as a tumor suppressor to exert anti-tumor effects [48,49]. *Hc* promotes tumor regression by interfering with the cell cycle in mouse breast cancer and plays a role in tumor immunity [50,51]. The *Bmp2* gene is associated with proliferation and migration in breast cancer [52,53], overexpression of *Clu* in breast cancer

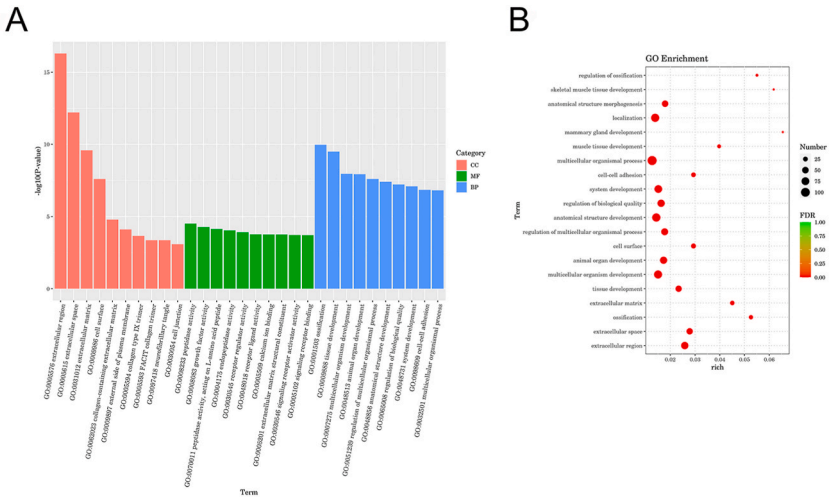

Fig. 5. Functional GO enrichment analysis of differentially expressed genes A. Histogram of enriched GO terms; B. Bubble chart of enriched GO terms.

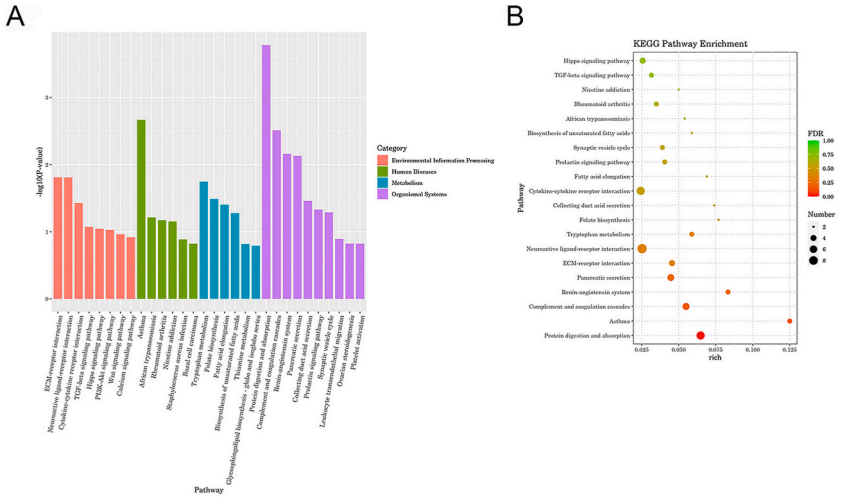

Fig. 6. Functional KEGG enrichment analysis of differentially expressed genes A. Histogram of enriched KEGG pathways; B. Bubble chart of enriched KEGG pathways.

Table 2
Significantly associated pathways and involved genes.

Pathway	Level	Up genes	Down genes
Hippo signaling pathway	Signal transduction	*Areg*、*Wnt4*	*Bmp2*、*Ccn2*
TGFbeta signaling pathway	Signal transduction	*Id4*	*Bmp2*
Complement and coagulation cascades	Immune system	*Hc*、*Bdkrb1*、*Fgg*、*Fgb*	*Clu*
Cytokine-cytokine receptor interaction	Signaling molecules and effects	*Il10*、*Prlr*、*Il1rl1*、*Gdf10*、*Il17rb*	*Cx3cr1*、*Bmp2*

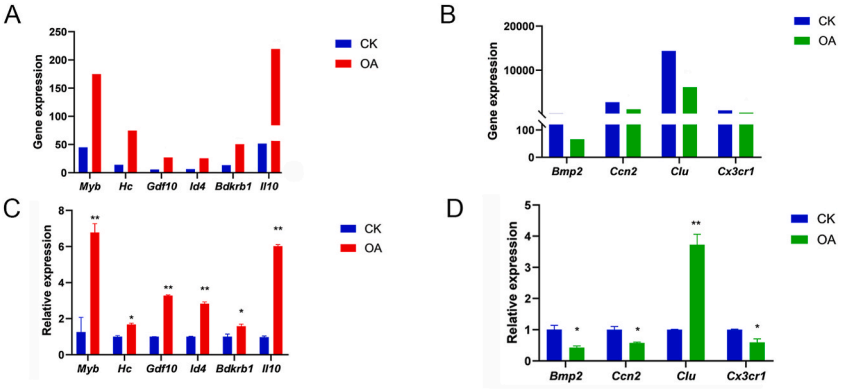

Fig. 7. Validation of significantly associated genes with OA treatment on breast tumor mice A. Mean expression levels of identified up-regulated gene targets tested by RNA-seq; B. Mean expression levels of identified down-regulated gene targets by RNA-seq; C. Relative expression of up-regulated genes by real-time PCR test; D. Relative expression of down-regulated genes by real-time PCR test.
* represents $p < 0.05$; ** represents $p < 0.01$.

is associated with lymphatic metastasis [54], and *Ccn2* promotes bone metastasis in breast cancer [32]. *Cx3cr1* induces the spread of primary tumor metastasis and promotes breast cancer through the *trans*-activation of the EGF pathway [55].

The Hippo signaling pathway and TGF beta signaling pathway are both associated with promoting the progression of breast cancer [56,57], the *Bmp2* gene in these two pathways is associated with the proliferation and migration of breast cancer [52,53], and *Ccn2* in the Hippo signaling pathway promotes bone metastasis in breast cancer [32], and *Bmp2* was analyzed compared with the model group under OA treatment The downregulation of the *Ccn2* gene may block the Hippo signaling pathway and TGFbeta signaling pathway, thereby inhibiting the proliferation and migration of breast cancer.

Activation of the coagulation cascade favors the spread of breast cancer metastasis [58], and *Clu* overexpression in breast cancer in the complement and coagulation cascades pathway is associated with lymphatic metastasis [54], however, under OA therapy, *Clu*

downregulation may block the coagulation cascade pathway, thereby affecting the progression of breast cancer. As part of the complement system, *Hc* responds when the body is injured and tissue cells are damaged, which is an important part of the host. *Hc* has been shown to promote tumor regression by interfering with the cell cycle in mouse breast cancer and to play a role in tumor immunity [50,51].

Cytokine-cytokine receptor interaction, involved in innate and adaptive inflammatory host defense, cell death, and other processes, after OA treatment, compared with the CK group, *Gdf10, Il10* gene upregulation, *Gdf10* expressed protein has been shown to promote nerve repair after stroke, and may act as a tumor suppressor. Edward regulation of *Gdf10* effectively inhibits breast cancer metastasis [44,45]. *Il10* had a significant effect on tumor volume reduction as well as prolonging mouse survival. *Il10* has direct antitumor and anti-macrophage toxicity in vitro, enhancing its effectiveness in mediating anti-tumor immune responses. It can also mediate the immune response to tumors and inhibit tumor cell metastasis [46,47], and *Il10* can also inhibit tumor-induced angiogenesis and enhance the production of tumor toxic molecules, which leads to tumor regression in some clinical models [59,60]. *Cx3cr1* induces the spread of primary tumor metastasis and promotes breast cancer through *trans*-activation of the EGF pathway [55], Since the *Cx3cr1* gene in the cytokine-cytokine receptor interaction pathway was downregulated under OA treatment, *Cx3cr1* inhibited its progression in mouse breast cancer.

4. Conclusion

In conclusion, OA may inhibit the development of breast cancer by regulating lactic acid bacteria through the cytokine-cytokine receptor interaction pathway (Fig. 8). Compared to CK, *Lactobacillus* is an increased probiotic in the OA group, which can induce $CD4^+$ T cells to proliferate, differentiate into Thr cells and produce *Il10*, macrophages can also secrete *Il10*-producing *Il10*, which has a direct antiproliferative effect on tumor cells of breast cancer, possibly because of their ability to increase the host antitumor response [26,61]. *Il10* is involved in anti-tumor immunity in the cytokine-cytokine receptor interaction pathway, and the antitumor effect of *Il10* is mainly by inducing a protective anti-tumor immune response mediated by NK cells or $CD8^+$ T cells [28,29], and can also inhibit angiogenesis [30,31].

Myb and *Hc* can not only inhibit tumor cell metastasis, but also inhibit breast cancer progression through an immunomodulatory effect [42,51]. *Gdf10, Id4, and Bdkrb1* genes are mostly related to the function of inhibiting breast cancer proliferation, promoting apoptosis, and inhibiting breast cancer migration and invasion [38,44,48]. *Bmp2, Ccn2, Cx3cr1* and other genes are reported to function in promoting cancer cell proliferation and metastasis, inhibiting apoptosis [32,52,54,55]. And through the Hippo signaling pathway, TGF beta signaling pathway, Complement and coagulation cascades, these genes can affect the progress of breast cancer.

Tumor necrosis factor-α (*Tnf-α*) and interleukin-6 (*Il-6*) in the OA group were significantly reduced in serum, RNA was extracted from tumor tissues in the CK group and OA group, and real-time PCR was performed, which was consistent with the trend of *Tnf-α* and *Il-6* results in serum. Previous results have shown that *Tnf-α* has a tumor-promoting effect in the process of breast cancer progression and metastasis, and promotes breast cancer cell migration [62,63], and interleukin-6 (*Il-6*) is a pleiotropic cytokine with obvious tumor-promoting and tumor suppressive effects. And it can trigger the migration and invasion of malignancy in breast cancer cells by activating YAP signaling [64,65]. Due to the significant reduction of *Tnf-α* and *Il-6* under the treatment of OA, this reflects that OA inhibits the migration and invasion of breast cancer by reducing *Tnf-α* and *Il-6*.

In this study, it was explored that after OA treatment, the abundance of *Lactobacillus* was increased to affect the maturation of immune cells and their products in the intestine, thereby exerting anti-tumor effects, and then exerting obvious inhibitory effects on breast cancer, but the relationship between inflammatory factors and immunology needs to be further explored. OA has a wide range of biological activities, including but not limited to anti-cancer, hypolipidemic, anti diabetes, anti osteoporosis, anti-inflammatory, immune regulation and other effects, so it has great potential for drug development. In our study, the practical use of OA in the treatment of 4T1-induced mice breast cancer model was reported, and its regulatory mechanism was preliminarily revealed. However, the limitations of the experimental results still exist. Firstly, this study mainly focuses on 4T1 cells, and other reliable breast cancer cell models are still needed to confirm the clinical potential of OA in the treatment of breast cancer. Secondly, although the significant therapeutic effect of OA was confirmed in the mouse model in this experiment, the clear key target proteins and regulatory pathway information of the drug's action are still unclear. Finally, the interaction between OA treatment and the *lactic acid bacteria* function during tumor progression needs further clarification. The future supplementation of these experimental contents will help to comprehensively evaluate the potential therapeutic effects and safety of OA.

Ethical approval

Animal experiments were approved by the Institutional Animal Care and Use Committee of Anhui University (IACUC(AHU)-2022-041).

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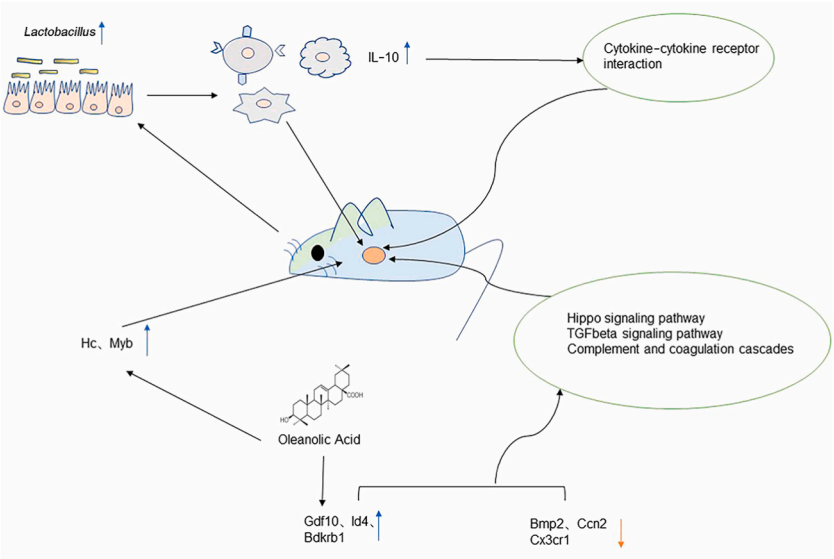

Fig. 8. Regulatory mechanism of gut microbiota and transcriptomics of oleanolic acid inhibiting the breast tumor progression in mice.

Data availability statement

The RNA-seq data generated in this study were submitted to the Gene Expression Omnibus (GEO) repository with the accession number GSE231570. The gut microbiomics data that support the findings of this study are available in SRA database under the reference number PRJNA954529.

CRediT authorship contribution statement

Kan He: Writing – original draft, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Xia Meng:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis. **Jinxing Su:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis. **Shangquan Jiang:** Writing – review & editing, Visualization, Validation, Formal analysis. **Min Chu:** Writing – review & editing, Validation, Formal analysis. **Bei Huang:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] J. Pollier, A. Goossens, Oleanolic acid, Phytochemistry 77 (2012) 10–15.
[2] J.M. Castellano, S. Ramos-Romero, J.S. Perona, Oleanolic acid: extraction, characterization and biological activity, Nutrients (2022) 14.
[3] K.A. Kim, J.S. Lee, H.J. Park, J.W. Kim, C.J. Kim, I.S. Shim, N.J. Kim, S.M. Han, S. Lim, Inhibition of cytochrome P450 activities by oleanolic acid and ursolic acid in human liver microsomes, Life Sci. 74 (2004) 2769–2779.
[4] X. Wang, R. Liu, W. Zhang, X. Zhang, N. Liao, Z. Wang, W. Li, X. Qin, C. Hai, Oleanolic acid improves hepatic insulin resistance via antioxidant, hypolipidemic and anti-inflammatory effects, Mol. Cell. Endocrinol. 376 (2013) 70–80.
[5] C. Xue, H. Lv, Y. Li, N. Dong, Y. Wang, J. Zhou, B. Shi, A. Shan, Oleanolic acid reshapes the gut microbiota and alters immune-related gene expression of intestinal epithelial cells, J. Sci. Food Agric. 102 (2022) 764–773.
[6] N. Sun, D. Li, X. Chen, P. Wu, Y.J. Lu, N. Hou, W.H. Chen, W.L. Wong, New applications of oleanolic acid and its derivatives as cardioprotective agents: a review of their therapeutic perspectives, Curr Pharm Des 25 (2019) 3740–3750.
[7] W. Wang, L. Wu, J. Li, J. Ji, K. Chen, Q. Yu, S. Li, J. Feng, T. Liu, J. Zhang, J. Chen, Y. Zhou, Y. Mao, F. Wang, W. Dai, X. Fan, C. Guo, J. Wu, Alleviation of hepatic ischemia reperfusion injury by oleanolic acid pretreating via reducing HMGB1 release and inhibiting apoptosis and autophagy, Mediat. Inflamm. 2019 (2019) 3240713.
[8] R. Jayasuriya, U. Dhamodharan, D. Ali, K. Ganesan, B. Xu, K.M. Ramkumar, Targeting Nrf2/Keap 1 signaling pathway by bioactive natural agents: possible therapeutic strategy to combat liver disease, Phytomedicine 92 (2021) 153755.
[9] J. Liu, J. Liu, C. Meng, Q. Gu, C. Huang, F. Liu, C. Xia, NRF2 and FXR dual signaling pathways cooperatively regulate the effects of oleanolic acid on cholestatic liver injury, Phytomedicine 108 (2023) 154529.
[10] X. Wang, X.L. Ye, R. Liu, H.L. Chen, H. Bai, X. Liang, X.D. Zhang, Z. Wang, W.L. Li, C.X. Hai, Antioxidant activities of oleanolic acid in vitro: possible role of Nrf2 and MAP kinases, Chem. Biol. Interact. 184 (2010) 328–337.
[11] S.J. Tsai, M.C. Yin, Antioxidative and anti-inflammatory protection of oleanolic acid and ursolic acid in PC12 cells, J. Food Sci. 73 (2008) H174–H178.
[12] J. Wiemann, L. Heller, R. Csuk, Targeting cancer cells with oleanolic and ursolic acid derived hydroxamates, Bioorg Med Chem Lett 26 (2016) 907–909.

[13] J.S. Woo, E.S. Yoo, S.H. Kim, J.H. Lee, S.H. Han, S.H. Jung, G.H. Jung, J.Y. Jung, Anticancer effects of oleanolic acid on human melanoma cells, Chem. Biol. Interact. 347 (2021) 109619.

[14] Y. Hua, Z. Zhang, J. Li, Q. Li, S. Hu, J. Li, M. Sun, Z. Cai, Oleanolic acid derivative Dex-OA has potent anti-tumor and anti-metastatic activity on osteosarcoma cells in vitro and in vivo, Invest. N. Drugs 29 (2011) 258–265.

[15] L. Gao, Y. Wang, Z. Xu, X. Li, J. Wu, S. Liu, P. Chu, Z. Sun, B. Sun, Y. Lin, J. Peng, G. Han, S. Wang, Z. Tang, SZC017, a novel oleanolic acid derivative, induces apoptosis and autophagy in human breast cancer cells, Apoptosis 20 (2015) 1636–1650.

[16] Y. Bao, S. Zhang, Z. Chen, A.T. Chen, J. Ma, G. Deng, W. Xu, J. Zhou, Z.Q. Yu, G. Yao, J. Chen, Synergistic chemotherapy for breast cancer and breast cancer brain metastases via paclitaxel-loaded oleanolic acid nanoparticles, Mol. Pharm. 17 (2020) 1343–1351.

[17] Z.P. Feng, H.Y. Xin, Z.W. Zhang, C.G. Liu, Z. Yang, H. You, H.W. Xin, Gut microbiota homeostasis restoration may become a novel therapy for breast cancer, Invest. N. Drugs 39 (2021) 871–878.

[18] J.R. Lakritz, T. Poutahidis, S. Mirabal, B.J. Varian, T. Levkovich, Y.M. Ibrahim, J.M. Ward, E.C. Teng, B. Fisher, N. Parry, S. Lesage, N. Alberg, S. Gourishetti, J. G. Fox, Z. Ge, S.E. Erdman, Gut bacteria require neutrophils to promote mammary tumorigenesis, Oncotarget 6 (2015) 9387–9396.

[19] L. Mendoza, Potential effect of probiotics in the treatment of breast cancer, Onco Rev. 13 (2019) 422.

[20] X. Liu, J. Zhao, L. Xue, T. Zhao, W. Ding, Y. Han, H. Ye, A comparison of transcriptome analysis methods with reference genome, BMC Genom. 23 (2022) 232.

[21] Z. Liang, R. Pan, X. Meng, J. Su, Y. Guo, G. Wei, Z. Zhang, K. He, Transcriptome study of oleanolic acid in the inhibition of breast tumor growth based on high-throughput sequencing, Aging (Albany NY) 13 (2021) 22883–22897.

[22] X. Zhou, G. Xu, B. Shen, [Effect of freeze-dried sea cucumber powder of eastern sea on tumor and immune index of S180-bearing mouse], Wei Sheng Yan Jiu 37 (2008) 30–32.

[23] F. Aragón, S. Carino, G. Perdigón, A. de Moreno de LeBlanc, The administration of milk fermented by the probiotic *Lactobacillus casei* CRL 431 exerts an immunomodulatory effect against a breast tumour in a mouse model, Immunobiology 219 (2014) 457–464.

[24] M.H. Yazdi, M. Mahdavi, N. Setayesh, M. Esfandyar, A.R. Shahverdi, Selenium nanoparticle-enriched *Lactobacillus brevis* causes more efficient immune responses in vivo and reduces the liver metastasis in metastatic form of mouse breast cancer, Daru 21 (2013) 33.

[25] M. Rachid, C. Matar, J. Duarte, G. Perdigon, Effect of milk fermented with a *Lactobacillus helveticus* R389(+) proteolytic strain on the immune system and on the growth of 4T1 breast cancer cells in mice, FEMS Immunol. Med. Microbiol. 47 (2006) 242–253.

[26] J.E. Kim, A. Sharma, G. Sharma, S.Y. Lee, H.S. Shin, D. Rudra, S.H. Im, *Lactobacillus pentosus* modulates immune response by inducing IL-10 producing Tr1 cells, Immune Netw 19 (2019) e39.

[27] D. Zheng, Z. Wang, L. Sui, Y. Xu, L. Wang, X. Qiao, W. Cui, Y. Jiang, H. Zhou, L. Tang, Y. Li, *Lactobacillus johnsonii* activates porcine monocyte derived dendritic cells maturation to modulate Th cellular immune response, Cytokine 144 (2021) 155581.

[28] N. Kundu, T.L. Beaty, M.J. Jackson, A.M. Fulton, Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer, J Natl Cancer Inst 88 (1996) 536–541.

[29] R. Dorsey, N. Kundu, Q. Yang, C.S. Tannenbaum, H. Sun, T.A. Hamilton, A.M. Fulton, Immunotherapy with interleukin-10 depends on the CXC chemokines inducible protein-10 and monokine induced by IFN-gamma, Cancer Res. 62 (2002) 2606–2610.

[30] M.E. Stearns, J. Rhim, M. Wang, Interleukin 10 (IL-10) inhibition of primary human prostate cell-induced angiogenesis: IL-10 stimulation of tissue inhibitor of metalloproteinase-1 and inhibition of matrix metalloproteinase (MMP)-2/MMP-9 secretion, Clin. Cancer Res. 5 (1999) 189–196.

[31] S. Huang, K. Xie, C.D. Bucana, S.E. Ullrich, M. Bar-Eli, Interleukin 10 suppresses tumor growth and metastasis of human melanoma cells: potential inhibition of angiogenesis, Clin. Cancer Res. 2 (1996) 1969–1979.

[32] T. Shimo, S. Kubota, N. Yoshioka, S. Ibaragi, S. Isowa, T. Eguchi, A. Sasaki, M. Takigawa, Pathogenic role of connective tissue growth factor (CTGF/CCN2) in osteolytic metastasis of breast cancer, J. Bone Miner. Res. 21 (2006) 1045–1059.

[33] F. Wang, Y. Gao, L. Tang, K. Ning, N. Geng, H. Zhang, Y. Li, Y. Li, F. Liu, F. Li, A novel PAK4-CEBPB-CLDN4 axis involving in breast cancer cell migration and invasion, Biochem. Biophys. Res. Commun. 511 (2019) 404–408.

[34] M. Redondo, E. Villar, J. Torres-Muñoz, T. Tellez, M. Morell, C.K. Petito, Overexpression of clusterin in human breast carcinoma, Am. J. Pathol. 157 (2000) 393–399.

[35] M. Apostolopoulou, L. Ligon, Cadherin-23 mediates heterotypic cell-cell adhesion between breast cancer epithelial cells and fibroblasts, PLoS One 7 (2012) e33289.

[36] G.M. Sizemore, S.T. Sizemore, D.D. Seachrist, R.A. Keri, GABA(A) receptor pi (GABRP) stimulates basal-like breast cancer cell migration through activation of extracellular-regulated kinase 1/2 (ERK1/2), J. Biol. Chem. 289 (2014) 24102–24113.

[37] R.Y. Ye, X.Y. Kuang, N. Shao, S.M. Wang, Y. Lin, Downregulation of NPTX1 induces cell cycle progression through Wnt/β-catenin signaling in breast cancer, J. Biol. Regul. Homeost. Agents 35 (2021) 1177–1183.

[38] S. Esseghir, J.S. Reis-Filho, A. Kennedy, M. James, M.J. O'Hare, R. Jeffery, R. Poulsom, C.M. Isacke, Identification of transmembrane proteins as potential prognostic markers and therapeutic targets in breast cancer by a screen for signal sequence encoding transcripts, J. Pathol. 210 (2006) 420–430.

[39] X. Qu, Q. Li, S. Tu, X. Yang, W. Wen, ELF5 inhibits the proliferation and invasion of breast cancer cells by regulating CD24, Mol. Biol. Rep. 48 (2021) 5023–5032.

[40] A.J. Butt, K.A. Dickson, F. McDougall, R.C. Baxter, Insulin-like growth factor-binding protein-5 inhibits the growth of human breast cancer cells in vitro and in vivo, J. Biol. Chem. 278 (2003) 29676–29685.

[41] J. Yang, F. Han, W. Liu, H. Chen, X. Hao, X. Jiang, L. Yin, Y. Huang, J. Cao, H. Zhang, J. Liu, ALX4, an epigenetically down regulated tumor suppressor, inhibits breast cancer progression by interfering Wnt/β-catenin pathway, J. Exp. Clin. Cancer Res. 36 (2017) 170.

[42] L. Knopfová, E. Biglieri, N. Volodko, M. Masařík, M. Hermanová, J.F. Glaus Garzón, M. Dúcka, T. Kučírková, K. Souček, J. Šmarda, P. Beneš, L. Borsig, Transcription factor c-Myb inhibits breast cancer lung metastasis by suppression of tumor cell seeding, Oncogene 37 (2018) 1020–1030.

[43] S. Gautam, J. Fioravanti, W. Zhu, J.B. Le Gall, P. Brohawn, N.E. Lacey, J. Hu, J.D. Hocker, N.V. Hawk, V. Kapoor, W.G. Telford, D. Gurusamy, Z. Yu, A. Bhandoola, H.H. Xue, R. Roychoudhuri, B.W. Higgs, N.P. Restifo, T.P. Bender, Y. Ji, L. Gattinoni, The transcription factor c-Myb regulates CD8(+) T cell stemness and antitumor immunity, Nat. Immunol. 20 (2019) 337–349.

[44] T. Zhou, L. Yu, J. Huang, X. Zhao, Y. Li, Y. Hu, Y. Lei, GDF10 inhibits proliferation and epithelial-mesenchymal transition in triple-negative breast cancer via upregulation of Smad 7, Aging (Albany NY) 11 (2019) 3298–3314.

[45] M. Tandon, K. Gokul, S.A. Ali, Z. Chen, J. Lian, G.S. Stein, J. Pratap, Runx 2 mediates epigenetic silencing of the bone morphogenetic protein-3B (BMP-3B/GDF10) in lung cancer cells, Mol. Cancer 11 (2012) 27.

[46] L.M. Zheng, D.M. Ojcius, F. Garaud, C. Roth, E. Maxwell, Z. Li, H. Rong, J. Chen, X.Y. Wang, J.J. Catino, I. King, Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism, J. Exp. Med. 184 (1996) 579–584.

[47] R.M. Berman, T. Suzuki, H. Tahara, P.D. Robbins, S.K. Narula, M.T. Lotze, Systemic administration of cellular IL-10 induces an effective, specific, and long-lived immune response against established tumors in mice, J. Immunol. 157 (1996) 231–238.

[48] S. Yu, Y. Zhou, L. Niu, Y. Qiao, Y. Yan, Mesenchymal stem cell-derived exosome mir-342-3p inhibits metastasis and chemo-resistance of breast cancer through regulating ID4, Genes Genomics 44 (2022) 539–550.

[49] P. de Candia, M. Akram, R. Benezra, E. Brogi, Id4 messenger RNA and estrogen receptor expression: inverse correlation in human normal breast epithelium and carcinoma, Hum. Pathol. 37 (2006) 1032–1041.

[50] H.D. Manthey, T.M. Woodruff, S.M. Taylor, P.N. Monk, Complement component 5a (C5a), Int. J. Biochem. Cell Biol. 41 (2009) 2114–2117.

[51] D.Y. Kim, C.B. Martin, S.N. Lee, B.K. Martin, Expression of complement protein C5a in a murine mammary cancer model: tumor regression by interference with the cell cycle, Cancer Immunol. Immunother. 54 (2005) 1026–1037.

[52] H. Jin, J. Pi, X. Huang, F. Huang, W. Shao, S. Li, Y. Chen, J. Cai, BMP2 promotes migration and invasion of breast cancer cells via cytoskeletal reorganization and adhesion decrease: an AFM investigation, Appl. Microbiol. Biotechnol. 93 (2012) 1715–1723.

[53] J.H. Clement, M. Raida, J. Sänger, R. Bicknell, J. Liu, A. Naumann, A. Geyer, A. Waldau, P. Hortschansky, A. Schmidt, K. Höffken, S. Wölft, A.L. Harris, Bone morphogenetic protein 2 (BMP-2) induces in vitro invasion and in vivo hormone independent growth of breast carcinoma cells, Int. J. Oncol. 27 (2005) 401–407.
[54] J. Li, L. Jia, P. Zhao, Y. Jiang, S. Zhong, D. Chen, Stable knockdown of clusterin by vectorbased RNA interference in a human breast cancer cell line inhibits tumour cell invasion and metastasis, J. Int. Med. Res. 40 (2012) 545–555.
[55] M. Tardáguila, E. Mira, M.A. García-Cabezas, A.M. Feijoo, M. Quintela-Fandino, I. Azcoitia, S.A. Lira, S. Mañes, CX3CL1 promotes breast cancer via transactivation of the EGF pathway, Cancer Res. 73 (2013) 4461–4473.
[56] P. Shi, J. Feng, C. Chen, Hippo pathway in mammary gland development and breast cancer, Acta Biochim. Biophys. Sin. 47 (2015) 53–59.
[57] T. Imamura, A. Hikita, Y. Inoue, The roles of TGF-β signaling in carcinogenesis and breast cancer metastasis, Breast Cancer 19 (2012) 118–124.
[58] M. Smeda, M. Stojak, K. Przyborowski, M. Sternak, J. Suraj-Prazmowska, K. Kus, K. Derszniak, A. Jasztal, A. Kij, A. Kurpinska, A. Kieronska-Rudek, K. Wojnar-Lason, E. Buczek, T. Mohaissen, S. Chlopicki, Direct thrombin inhibitor dabigatran compromises pulmonary endothelial integrity in a murine model of breast cancer metastasis to the lungs; the role of platelets and inflammation-associated haemostasis, Front. Pharmacol. 13 (2022) 834472.
[59] K. Asadullah, W. Sterry, H.D. Volk, Interleukin-10 therapy–review of a new approach, Pharmacol. Rev. 55 (2003) 241–269.
[60] L. Cervenak, L. Morbidelli, D. Donati, S. Donnini, T. Kambayashi, J.L. Wilson, H. Axelson, E. Castaños-Velez, H.G. Ljunggren, R.D. Malefyt, H.J. Granger, M. Ziche, M.T. Bejarano, Abolished angiogenicity and tumorigenicity of Burkitt lymphoma by interleukin-10, Blood 96 (2000) 2568–2573.
[61] A. Aquino-López, V.V. Senyukov, Z. Vlasic, E.S. Kleinerman, D.A. Lee, Interferon gamma induces changes in natural killer (NK) cell ligand expression and alters NK cell-mediated lysis of pediatric cancer cell lines, Front. Immunol. 8 (2017) 391.
[62] D. Cruceriu, O. Baldasici, O. Balacescu, I. Berindan-Neagoe, The dual role of tumor necrosis factor-alpha (TNF-α) in breast cancer: molecular insights and therapeutic approaches, Cell. Oncol. 43 (2020) 1–18.
[63] D. Wolczyk, M. Zaremba-Czogalla, A. Hryniewicz-Jankowska, R. Tabola, K. Grabowski, A.F. Sikorski, K. Augoff, TNF-α promotes breast cancer cell migration and enhances the concentration of membrane-associated proteases in lipid rafts, Cell. Oncol. 39 (2016) 353–363.
[64] C.O. Abana, B.S. Bingham, J.H. Cho, A.J. Graves, T. Koyama, R.T. Pilarski, A.B. Chakravarthy, F. Xia, IL-6 variant is associated with metastasis in breast cancer patients, PLoS One 12 (2017) e0181725.
[65] L. Hou, S. Xie, G. Li, B. Xiong, Y. Gao, X. Zhao, J. Hu, S. Deng, J. Jiang, IL-6 triggers the migration and invasion of oestrogen receptor-negative breast cancer cells via regulation of Hippo pathways, Basic Clin. Pharmacol. Toxicol. 123 (2018) 549–557.