Changes of Three kinds of miRNAs, MALAT1 and PI3K/AKT/mTOR signaling pathway Protein Levels in Her-2 Positive Breast Cancer in neoadjuvant chemotherapy and their relationship with prognosis- -A small sample study report

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Research

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Abstract

Objective To investigate the roles of three kinds of miRNAs (MiR-99a, MiR-455 and MiR-143), Lnc MALAT1 and related proteins of PI3K/AKT/mTOR signaling pathway in neoadjuvant chemotherapy of patients with Her-2 positive breast cancer (BC) and analysed their related mechanisms.

Method The frozen tumor tissue and serum samples before and after neoadjuvant chemotherapy of 14 cases patients with Her-2 positive BC who received neoadjuvant chemotherapy were collected. The relative expressions level of MiR-99a, MiR-455, MiR-143, MALAT1 and related proteins of PI3K/AKT/mTOR signaling pathway in tumor tissue and a part of serum samples were detected by qRT-PCR or western blot.

Results MiR-99a level was negatively correlated with RFS and OS before and after chemotherapy. MiR-99a and MiR-455 levels after chemotherapy was negatively correlated with OS. The serum MALAT1 level was negatively correlated with RFS and OS in chemosensitivity group before and after chemotherapy. In chemotherapy resistance group, p-AKT level before chemotherapy was negatively correlated with T stage, p-mTOR level after chemotherapy was positively correlated with lymph node stage and negatively correlated with RFS and OS, PTEN level was negatively correlated with clinical TNM stage.

Conclusion Serum MALAT1 and MiR-99a, MiR-455, MiR-143, p-AKT, p-mTOR and PTEN of tumor tissues in Her-2 positive BC patients can be used as markers to judge neoadjuvant chemotherapy sensitivity in Her-2 positive BC. Chemotherapy resistance is may be related to PI3K/AKT/mTOR signaling pathway.

1. Introduction

Breast cancer (BC) is a highly heterogeneous malignant tumor, and the prognosis of different molecular subtypes of BC patients is quite different. BC has become the most common malignant tumor in women, and the incidence of BC is increasing ^[1]. Epidermal growth factor receptor 2 (Her-2)-positive BC is a subtype of BC, and 20–30% of BC patients have Her-2 overexpression. High expression of Her-2 is closely related to the occurrence, development, invasion and migration of BC ^[2, 3]. Her-2 positive BC patients are prone to metastasis and recurrence with poor prognosis. The median disease-free survival (DFS) and overall survival (OS) of patients are significantly shorter than those of Her-2 negative patients ^[4–7].

The neoadjuvant chemotherapy has become the important routine treatments for BC. It is often used to treat patients with locally advanced BC or BC patients who need to reduce the extent of surgical resection. Although the prognosis of patients with different types of BC has been greatly improved through the various comprehensive treatments, about 15% of patients still have recurrence and distant metastasis. Among of them, chemotherapy resistance to chemotherapeutic drugs is one of the main causes of recurrence and metastasis of BC. Due to the high heterogeneity and individual differences of BC, about 20–30% of neoadjuvant chemotherapy patients show resistance to chemotherapeutic drugs ^[8]. The chemotherapy resistance of neoadjuvant chemotherapy not only prevents patients from receiving local surgery after chemotherapy, delays their condition, but also reduces the breast-conserving rate of patients. Neoadjuvant chemotherapy can be used to predict the in vivo sensitivity and chemotherapy resistance to chemotherapeutic drugs for the part of sub-types of BC with poor prognosis, such as Her-2 positive BC. The high heterogeneity of BC is the main cause of chemotherapy resistance. If there is resistance to chemotherapy, it will seriously affect the prognosis and survival rate of patients.

At present, miRNA (MicroRNAs) and lncRNA (Long non-coding RNA) are involved in the development of Her-2-positive BC and play an important role in neoadjuvant chemotherapy. In the human genome, only about 2% of the genes encode proteins, while up to 80% of the genomes are transcribed into non-coding RNA. Transcriptional studies based on second-generation sequencing have shown that a large number of miRNAs and lncRNA are abnormally expressed in cancer cells, and participate in the advance and onset of cancer and the process of chemotherapy resistance [9].

MiRNAs may act on different pathways in various cancers and can serve as valuable sources for biomarker detections and optimal chemotherapeutic choices for BC patients [10–12]. Several studies have shown that some miRNAs are associated with prognosis in Her-2 positive BC [13, 14]. The circulating miRNAs can be as chemotherapeutic response predictors, prognosticators or potential therapeutic targets [15]. Gu X et al found that the relative expression level of miR-451 was lower in the neoadjuvant chemotherapy resistant BC patients, suggesting the circulating miR-451 may have a functional significance in predicting the resistance to neoadjuvant chemotherapy in BC patients [16]. lncRNA MALAT1 (Metastasis-associated Lung Adenocarcinoma Transcript 1) is a conservative lncRNA that is highly expressed in many cancers, including BC [17]. MALAT1 levels are reported to be overexpressed in the serum or tissues of most tumors, and the MALAT1 levels often affect the tumor size, tumor stage, lymph node metastasis, and distant diffusion. Therefore, MALAT1 can be used as a new biomarker for early diagnosis, assessment of severity, or prognostic judgement. There are few reports on the relationship between MALAT1 and chemotherapy resistance in malignant tumors, and no literature on BC has been reported. Li P et al's study showed that high MALAT1 level was associated with lower survival rate and bad response to oxaliplatin based chemotherapy in advanced colorectal cancer patients. Oxaliplatin-resistant colorectal cancer cells have high MALAT1 levels [18].

We found that MiR-99a, MiR-455, MiR-143 and MALAT1 were associated with lymph node metastasis and prognosis in our previously experiment on Her-2 positive BC patients which has contributed to other magazines. In order to explore the role and mechanism of them in neoadjuvant chemotherapy of Her-2 positive BC, we further studied the three kind of miRNAs, MALAT1 and evaluated their correlation with clinical TNM stage, T stage, N stage of lymph node and survival time before and after neoadjuvant chemotherapy in Her-2 positive BC patients. We also detected the levels of related proteins of PI3K/AKT/mTOR signaling pathway (p-AKT, AKT, p-mTOR, mTOR and PTEN) in chemotherapy resistance groups, and explored the related mechanism of chemotherapy resistance. Because of the lack of experimental funds, we only carried out a small sample experiment. This study is the first to be reported.

2. Materials And Methods

2.1. Patients information We collected the frozen tissue samples (serum and tumor tissue) of 14 cases of patients with Her-2 positive BC who received neoadjuvant chemotherapy from June 2010 to May 2012 in our hospital sample bank, including 3 cases of stage IIB, 9 cases of stage IIIA and 2 cases of stage IIIC. The median age of 14 cases patients was 53.5 (38-65) years. All patients were confirmed as invasive ductal cancer by Mammotone biopsy before chemotherapy, and then received complete course of chemotherapy + targeted therapy. The chemotherapy regimens were EC*4→TH*4 (epirubicin+cyclophosphamide→docetaxel+herceptin). The therapeutic effects of neoadjuvant chemotherapy was evaluated by RECIST (version 1.1) after chemotherapy, which was divided into CR (complete response), PR (partial response), PD (progressive disease) and SD (stable disease). 10 cases of patients with CR or PR were enrolled in the chemosensitivity group, with a median age of 52 (38-65) years.

10 cases of chemosensitivity patients included 2 cases stage IIB, 6 cases stage IIIA and 2 cases stage IIIC. The efficacy of SD or PD was evaluated in the chemoresistance group. The median age of the chemotherapy resistance group was 56 (53-58) years old. 4 cases of chemoresistance patients including 1 cases stage IIB and 3 cases stage IIIA. Tumor inhibition rate after chemotherapy was evaluated in all patients and was calculated according to the change of the maximum diameter of the tumor before and after chemotherapy, it means Tumor inhibition rate = (Max diameter of pre-chemotherapy - Max diameter of post-chemotherapy)/ Max diameter of pre-chemotherapy x 100%. In addition, serum samples from 10 healthy persons were selected as healthy control group. The median age of the control group was 54.8 (39-69) years old.

2.2. Clinical monitoring indicators: The clinical TNM stage, tumor T stage, lymph node N stage, mortality rate, RFS (mom), OS (mon), and tumor inhibition rate were recorded. The deadline for follow-up was 31 December 2017. OS refers to the deadline for follow-up or the time of death during follow-up; RFS refers to the time of first recurrence and metastasis during follow-up.

2.3. Laboratory monitoring indicators: Quantitative RT-PCR was used to detect the relative expression level of three kinds of miRNAs and MALAT1 in tumor tissues of all BC patients, and also detect MALAT1 level in serum of healthy control group and all BC patients. Western blot was used to detect the relative expression levels of p-AKT, AKT, p-mTOR, mTOR and PTEN in 4 cases chemotherapy resistance patients. We studied the changes of the above molecular indicators levels before and after neoadjuvant chemotherapy in different groups, and compared the correlation between above molecular indicators and clinical monitoring indicators.

2.4 Experimental Detection Method of MiRNAs for Tumor Tissue

2.4.1 MiRNAs Extraction and Reverse Transcription: The main reagents are PureLink® miRNA Isolation Kit (Thermo Fisher, NO: K1570-01) and SuperScript™ III Reverse Transcriptase (Thermo fisher, NO: 18080085). See Table 1.

2.4.2 Main steps of qRT-PCR detection for Tumor Tissue: The main reagents are PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, NO: A25779).

(1) Design and synthesis of RT-PCR primers: Primer Premier 6.0 and Beacon designer 7.8 were used to design quantitative PCR primers, which were synthesized by Bioengineering (Shanghai) Co., Ltd. The sequence of primers was as follows in Table 2.

(2) The statistical analysis of Real-time PCR gene expression: each sample detection was repeated three times.

2.5 Detection of Serum MALAT1 by qRT-PCR: The reagent used for extracting miRNA is MicroNeasy Serum/Plasma Kit (Qiagen NO:217184), and the reagent used for reverse transcription experiment is SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen NO:11752-050). Relevant experiments are carried out according to relevant operational requirements. Real-Time PCR was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems NO: A25779). Primer Premier 6.0 and Beacon designer 7.8 were used to design quantitative PCR primers. Bioengineering Co., Ltd (Shanghai, China) was responsible for the synthesis of primers. The sequence of primers was as follows (See Table 3).

2.6 Detection of tissue MALAT1 by qRT-PCR: The reagents used for total RNA extraction are TRIzol® Plus RNA Purification Kit(Invitrogen NO:12183-555), RNase-Free DNase Set(Qiagen No. 79254), and the reagents used for reverse transcription experiment are SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen

NO:11752-050). Real-Time PCR was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems NO:4367659). Primer Premier 6.0 and Beacon designer 7.8 software were used to design quantitative PCR primers. Bioengineering Co., Ltd (Shanghai) was responsible for the synthesis of primers with the same primer sequence.

2.7 Western blot detection

2.7.1 Main detecting steps: We separated the tumor cells of drug resistant tumor tissue to do the Western blot detection. Total protein extraction kit (including Protease Inhibitor Cocktail) was used to extract total protein, and then BCA quantitative kit was used to quantify total protein detection. SDS-PAGE electrophoresis analysis, protein transfer membrane and transfer membrane sealing were performed according to relevant requirements.

2.7.2 Incubation of first antibody: First antibody is dissolved in T-TBS (containing 3% skimmed milk powder or BSA) in a certain proportion and incubated overnight at 4 °C. Then T-TBS is rinsed for 5 min *4 times, see Table 4.

2.7.3 Second antibody incubation: Second antibody dissolves in T-TBS (containing 2% skimmed milk powder) at room temperature for 1 hour, then T-TBS is rinsed for 5 min *5 times, see Table 5.

2.7.4 Signal detection: Super Signal® West Dura Extended Duration Substrate was used, and follow the instructions to detect. To prepare 1 ml ECL, the transfer film was incubated at room temperature for 1 minute, then the redundant ECL reagent was removed, the fresh-keeping film was sealed, and X-ray film was placed in the dark box after exposure for 5-10 minutes.

2.7.5 Data analysis: Image J software was used to analyze the optical density of the bands. Each band was repeated three times. The relative expression of the target protein was expressed = {the objective protein (optical density)/internal parameter (optical density)} *10n, and the results were expressed as mean ± standard deviation.

2.8 Correlation analysis of experimental detection and clinical indicators: To evaluate the correlation of three miRNAs and MALAT1 levels in serum and tumor tissues before and after chemotherapy in different Her-2 positive group with clinical TNM stage, tumor T stage, lymph node stage, RFS, OS, and tumor inhibition rate (%). To evaluate the correlation between the levels of p-AKT, AKT, p-mTOR, mTOR and PTEN in tumor tissues of chemotherapy resistance groups with above clinical indicators.

2.9 Statistical methods: Paired T test was used to compare the different indicators of the same patient before and after chemotherapy. Pearson correlation analysis was used to analyze the correlation between the different indicators. Survival analysis (Mantel-Cox test) was used to compare the OS and RFS. P<0.05 was used to judge the statistical significance.

3 Results

3.1 Comparison of molecular indicators before and after chemotherapy between different groups: MiR-99a level after chemotherapy was significantly higher than that before chemotherapy (P<0.05). MiR-455 level in chemoresistance group was significantly lower than that before chemotherapy (P<0.01), and MiR-143 level in sensitive group was significantly higher than that before chemotherapy (P<0.01). There was no significant difference between OS and RFS in chemoresistance group and sensitive group (P>0.05). The relative expression level of MALAT1 in serum before and after chemotherapy was significantly higher than that in healthy control group (P<0.001). There was no significant difference in the MALAT1 level in tumor tissues before and after

chemotherapy ($P>0.05$). There was no significant difference in MALAT1 between chemosensitivity group and chemotherapy resistance group before and after chemotherapy ($P>0.05$). There was no significant difference in OS and RFS between chemotherapy resistance group and chemosensitivity group after chemotherapy ($P>0.05$). The MALAT1 level in chemosensitivity group before chemotherapy was significantly higher than that after chemotherapy ($P>0.05$). In chemotherapy resistance group, the relative expression levels of p-AKT and p-mTOR after chemotherapy were significantly higher than those before chemotherapy ($P<0.001$, $P<0.01$). But, PTEN was significantly lower than before chemotherapy ($P<0.05$). See Table 6-8 and Figure 1-7.

3.2 Correlation analysis between experimental and clinical indicators: The relative expression level of MiR-99a was negatively correlated with RFS ($R=-0.5514$) and OS ($R=-0.5554$) before and after chemotherapy ($P<0.05$). MiR-99a after chemotherapy was negatively correlated with OS ($R=-0.5663$, $P<0.05$). MiR-99a in sensitive group was negatively correlated with the tumor inhibition rate before chemotherapy ($R=-0.6412$, $P<0.05$), and MiR-455 was negatively correlated with OS after chemotherapy ($R=-0.7116$, $P<0.05$). MiR-99a was negatively correlated with RFS ($R=-0.9734$) and OS ($R=-0.9728$) before chemotherapy in chemoresistance patients ($P<0.05$). After chemotherapy, the relative expression level of MiR-99a was negatively correlated with RFS ($R=-0.9734$) and OS ($R=-0.9782$) ($P<0.01$). The relative expression of MALAT1 in serum and tumor tissue of 14 cases patients with Her-2 positive BC before and after chemotherapy was not correlated with clinical TNM stage, T stage, lymph node stage, RFS and OS ($P>0.05$). MALAT1 in serum was negatively correlated with RFS and OS in chemosensitivity group before and after chemotherapy ($R=-0.7842$, $R=-0.7713$ and $R=-0.6791$, $R=-0.6718$) ($P<0.01$). In chemotherapy resistance group, the relative expression level of p-AKT before chemotherapy was negatively correlated with T stage ($R=-0.9513$), AKT was positively correlated with OS ($R=0.9604$), mTOR was negatively correlated with lymph node stage ($R=-0.9784$), PTEN was positively correlated with RFS and OS ($R=0.9998$, $R=0.9977$) (all $P<0.05$). In chemotherapy resistance group after chemotherapy, the relative expression of p-mTOR was positively correlated with lymph node stage ($R=0.9563$, $P<0.05$) and negatively correlated with RFS and OS ($R=-0.9819$, $R=-0.9699$, $P<0.05$). The relative expression of PTEN was negatively correlated with clinical TNM stage ($R=-0.9656$, $P<0.05$).

4. Discussion

We tested three miRNAs, MALAT1 and some related proteins of PI3K/AKT/mTOR signaling pathway to find molecular markers that can predict neoadjuvant chemotherapy and prognosis of Her-2 positive BC patients. Because there was no project funding for this research, so we carried out a small sample study which is only an exploratory experiment.

4.1 MiR-99a

The roles of miR-99a in BC has been reported differently, one view is considered miR-99a to be a tumor suppressor and another view is considered miR-99a to be a cancer-promoting factor. Existing research data indicated that serum miR-99a expression was downregulated in BC patients, this downregulation was associated with poor prognosis, suggesting that serum miR-99a could function as a tumor suppressor in BC^[19]. According to reports, miR-99a has been reported as a tumor suppressor gene in various cancers in humans. However, only limited information about the function of miR-99a in human BCs is available. Hu Y et al investigated the expression of miR-99a in BC tissue specimens and its antitumor activity in BC cells. They identified that the expression of miR-99a was significantly reduced in four kind of BC cell lines. More importantly, we found downregulation of miR-99a in BC specimens from ten different patients^[20]. On the other hand, miR-99a was noticed to significantly inhibit cell proliferation and migration in triple-negative BC cells^[21]. Another microarray study suggested that expression level

of miR-99a was relatively low in BC tissues with lymph node metastasis ^[22]. In our study, the dynamic changes of miR-99a before and after neoadjuvant chemotherapy suggest that it may be a tumor suppressor.

4.2 MiR-143

MiR-143 has important physiological functions in BC cell proliferation, apoptosis, invasion, metastasis, angiogenesis and cell division. Its differential expression may be a molecular marker for clinical diagnosis and prognosis of BC. The role of miR-143 in BC is still controversial. Some literature suggests that miR-143 is high or low expression in BC ^[23-25]. Currently, more studies consider miR-143 as a tumor suppressor gene ^[26]. There's a study has shown that miR-143 is highly expressed in MCF-7 cells. Baradaran et al have found that miR-143 can inhibit the proliferation, invasion and migration of BC cells MDA-MB-468 ^[27]. Xia C et al results showed that the expression of miR-143-3p is significantly downregulated in BC cells. Upregulation of miR-143-3p inhibited the proliferation and migration of BC cells. Conversely, inhibition of miR-143-3p promoted the proliferation of cancer cells ^[28]. In our study, the dynamic changes of miR-143 before and after neoadjuvant chemotherapy suggest that it may be a tumor suppressor.

4.3 MiR-455-3p

The patients with infiltrating carcinoma or lymph node metastasis had a lower serum level of miR-455-3p than patients with the carcinoma in situ or patients without lymph node metastasis ^[29]. Wang B et al explored the effect of miR-455 on cell proliferation of BC, and found that miR-455 was downregulated in BC tissues and cells. Its overexpression inhibited cell proliferation, whereas its knockdown promoted cell proliferation of BC ^[30]. qRT-PCR analysis confirmed that the expression of miR-455-3p in TNBC cell lines MDA-MB-231 and MDA-MB-468 was higher than that in HR positive BC cell line MCF-7 ($P < 0.01$) ^[31].

Our study found that the relative expression level of MiR-99a after chemotherapy was significantly higher than that before chemotherapy. MiR-99a were negatively correlated with OS before and after chemotherapy. MiR-99a in the chemotherapy resistance group was negatively correlated with RFS and OS. MiR-455 after chemotherapy was negatively correlated with OS. MiR-455 in the chemotherapy resistance group was significantly lower than that before chemotherapy. MiR-143 in the chemotherapy sensitive was significantly higher than that before chemotherapy. In conclusion, three kinds of miRNAs in Her-2 positive BC tissues may become new kinds of molecular marker for predicting chemotherapy resistance of BC, and even become new kinds of potential molecular target. It is of great significance to further explore the molecular mechanisms by which three miRNAs regulate chemoresistance in BC.

4.4 MALAT1 and PI3K/AKT/mTOR Signaling Pathway

MALAT1 is a typical lncRNA that is markedly up-regulated in BC. However, current understanding of the involvement of MALAT1 in BC development and prognosis remains unclear. In the Zheng L's study, the expression of MALAT1 in clinical samples of BC tissues was found to be significantly up-regulated that was consistent with the result based on the dataset of the Cancer Genome Atlas (TCGA) at cBioportal. A negative correlation between overall survival and the expression of MALAT1 was statistically significant in the group of diagnosis age below 60 or in the group of infiltrating ductal carcinoma analyzed by TCGA database, which declared that MALAT1 might be a potentially useful prognostic factor ^[32]. Miao Y et al's research showed that an elevated MALAT1 expression in BC tissue was significantly associated with lymph metastasis and adverse 5-year disease-free survival (DFS). They

concluded that upregulation of MALAT1 plays an important role in BC development, serum MALAT1 level may be a potential tumor marker for BC diagnosis^[33]. MALAT1 level was positively correlated with lymph node condition, estrogen receptor and tumor stage indicating its prognostic value. It can be used as a marker for diagnosis of BC^[34]. Jadaliha M reported that assessment of the prognostic significance of MALAT1 in BC patients reveal elevated MALAT1 expression is associated with decreased disease-specific survival in ER negative, lymph node negative patients of the Her-2 and TNBC (triple negative breast cancer) molecular subtypes. They propose that MALAT1 can be used as a prognostic marker^[35]. Wang Z et al found that BC patients with high expression of MALAT1 has a twofold increase in risk of relapse ($p = 0.0083$) compared to those with low expression. High level of MALAT1 is associated with BC relapse^[36]. Our previous studies have shown that MALAT1 is the core signal molecule to promote the development and metastasis of Her-2-positive BC. MALAT1 is positively correlated with the numbers of lymph node metastasis of Her-2-positive BC. Knocking down the expression of MALAT1 in Her-2-positive BC cell lines can inhibit the proliferation and migration of BC cells^[37].

This study showed that the relative expression of MALAT1 in serum of sensitive patients before and after chemotherapy was negatively correlated with RFS and OS, which further indicated that MALAT1 might be a positive regulator of BC proliferation and metastasis. In this study, we found that the mechanism of Her-2 positive BC chemotherapy resistance may be related to PI3K/AKT/mTOR resistance signaling pathway.

The mechanism of chemotherapy resistance in cancer cells is very complex, involving genomic variation and changes in various signaling pathways. Among of them, PI3K/AKT/mTOR signaling pathway has been studied. The PI3K family is divided into three types. The most common PI3K (IA) is a heterodimer composed of regulatory subunit p85 and catalytic subunit p110, which catalyzes the formation of second messenger phosphoinositide triphosphate (PIP3) from phosphatidylinositol diphosphate (PIP2). PIP3 promotes phosphorylation of AKT, while phosphorylated AKT indirectly activates downstream mTOR complex. The mTOR complex family is a classical downstream effector of PI3K signaling pathway. PTEN is an important negative regulator of this pathway. PTEN inhibits the phosphorylation of AKT by dephosphorylating PIP3. PI3K/AKT/mTOR signaling pathway is widely involved in the survival, proliferation and metastasis of cancer cells. On the other hand, a large number of studies have shown that this pathway also plays an important role in the chemotherapy resistance of tumors. Mutation of PI3K gene, amplification of Akt gene and inactivation of PTEN are the main causes of abnormal activation of PI3K signaling pathway in BC cells. Among of them, PTEN gene inactivation is the most common, about 40-50% of BC cells can detect PTEN inactivation^[38].

In BC, the activation of PI3K/AKT/mTOR signaling pathway is closely related to the chemotherapy resistance of endocrine therapy drugs, targeted drugs and chemotherapy drugs. Previous studies have shown that trastuzumab can reactivate PI3K/AKT/mTOR signaling pathway by reducing the stability of PTEN in the treatment of Her-2-positive BC targeting drugs, and promote the survival and chemotherapy resistance of BC cells^[39]. Jin Y et al reported that MALAT1 can induce EMT via PI3K/AKT pathway^[40]. Wang C et al^[41] reported that the MALAT1 effects on cholangiocarcinoma cells might be through activating the PI3K/AKT signaling pathway. Unfortunately, because there were only four cases in the chemotherapy resistance group, we found that there is no correlation between MALAT1 level and drug-resistant pathway proteins.

This study showed that PI3K/AKT/mTOR signaling pathway was significantly activated in tumor tissues of chemotherapy resistance group after neoadjuvant chemotherapy compared with chemosensitivity. In chemotherapy resistance group, the relative expression levels of p-AKT and p-mTOR after chemotherapy were

significantly higher than that before chemotherapy, but PTEN after chemotherapy was lower than that before chemotherapy.

The expression of MALAT1 in BC was positively correlated with the activation of PI3K/AKT/mTOR signaling pathway^[42]. This study showed that in chemotherapy resistance group before chemotherapy the relative expression level of p-AKT was negatively correlated with T stage, AKT was positively correlated with OS, mTOR was negatively correlated with lymph node stage, PTEN was positively correlated with RFS and OS. The relative expression level of p-mTOR in chemotherapy resistance group after chemotherapy was positively correlated with lymph node stage, negatively correlated with RFS and OS, and negatively correlated with PTEN. These results suggest that MALAT1 may play an important role in the development of chemotherapy resistance in Her-2 positive BC by inducing the decline of PTEN protein and activation of PI3K/AKT/mTOR signaling pathway.

This study also showed that there was no significant difference in OS and RFS between chemotherapy resistance group and chemosensitivity group after chemotherapy, this result really surprised us. It may be caused small sample study. If there is funding in the future, we plan to increase the number of samples and carry on more in-depth research. Although there are few samples in this study, there are still many findings which can provide ideas for our future research.

In conclusion, Serum MALAT1 and p-AKT, p-mTOR and PTEN of tumor tissue can be used as markers to judge neoadjuvant chemotherapy sensitivity in Her-2 positive BC. Chemotherapy resistance is related to PI3K/AKT/mTOR signaling pathway. Serum MALAT1 may become a new molecular marker for predicting chemotherapeutic resistance in BC, and even a potential new molecular target. It is of great significance to further explore the molecular mechanism of MALAT1 regulating chemoresistance in BC cells.

Declarations

Ethics approval and consent to participate: The Ethics Committee of Zhejiang Cancer Hospital was secured for our research reported, and all authors abided the related rules of Ethics Committee when this study began. All authors abided the ethics in this clinical study. The Ethics Committee of Zhejiang Cancer Hospital approved to publish this paper. The research involving human subjects, human material, and human data have been performed in accordance with the Declaration of Helsinki and have been approved by an appropriate ethics committee of Zhejiang Cancer Hospital.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*. 2018;68(6):394-424.
2. Jordan NV, Bardia A, Wittner BS, Benes C, Ligorio M, Zheng Y, Yu M, Sundaresan TK, Licausi JA, Desai R. HER2 expression identifies dynamic functional states within circulating breast cancer cells. *Nature*. 2016;537(7618):102-106.
3. Disis ML, Wallace DR, Gooley TA, Dang Y, Slota M, Lu H, Coveler AL, Childs JS, Higgins DM, Fintak PA, dela Rosa C, Tietje K, Link J, Waisman J, Salazar LG. Concurrent trastuzumab and HER2/neu-specific vaccination in patients with metastatic breast cancer. *J Clin Oncol*. 2009;27(28):4685-4692.
4. Cameron D, Piccart-Gebhart MJ, Gelber RD, Procter M, Goldhirsch A, de Azambuja E, Castro G, Jr., Untch M, Smith I, Gianni L, Baselga J, Al-Sakaff N, Lauer S, McFadden E, Leyland-Jones B, Bell R, Dowsett M, Jackisch C. 11 years' follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive early breast cancer: final analysis of the HERceptin Adjuvant (HERA) trial. *Lancet*. 2017;389(10075):1195-1205.
5. Tong ZJ, Shi NY, Zhang ZJ, Yuan XD, Hong XMJBR. Expression and prognostic value of Her-2/neu in primary breast cancer with sentinel lymph node metastasis. *Bioscience Reports*. 2017;37(4):BSR 20170121.
6. Hu Y, Qiu Y, Yague E, Ji W, Liu J, Zhang J. miRNA-205 targets VEGFA and FGF2 and regulates resistance to chemotherapeutics in breast cancer. *Cell Death Dis*. 2016;7(6): e2291.
7. Yi Y, Durkeshwari A, Hoon LL, Perumal SR, Shanmugam MK, Prem KA, Gautam S, Lobie PE, Lim LHKJO. ANXA1 inhibits miR-196a in a negative feedback loop through NF-kB and c-Myc to reduce breast cancer proliferation. *Oncotarget*. 2016;7(19):27007-27020.
8. Teshome M1, Hunt KK2. Neoadjuvant therapy in the treatment of breast cancer. *Surg Oncol Clin N Am*. 2014;23(3):505-23.
9. Li XJ, Zha QB, Ren ZJ, Tang JH, Yao YF. Mechanisms of breast cancer resistance to anthracyclines or taxanes: an overview of the proposed roles of noncoding RNA. *Curr Opin Oncol*. 2015;27(6):457-65.
10. Teoh SL, Das S. The Role of miRNAs in Diagnosis, Prognosis, Metastasis and Resistant Cases in Breast Cancer. *Curr Pharm Des*. 2017;23(12):1845-1859.
11. Lou W, Liu J, Ding B, Xu L, Fan W. Identification of chemoresistance-associated miRNAs in breast cancer. *Cancer Manag Res*. 2018;10:4747-4757.
12. Chen X, Lu P, Wang DD, Yang SJ, Wu Y, Shen HY, Zhong SL, Zhao JH, Tang JH. The role of miRNAs in drug resistance and prognosis of breast cancer formalin-fixed paraffin-embedded tissues. *Gene*. 2016;595(2):221-226.
13. Li H, Liu J, Chen J, Wang H, Yang L, Chen F, Fan S, Wang J, Shao B, Yin D, Zeng M, Li M, Li J, Su F, Liu Q, Yao H, Su S, Song E. A serum microRNA signature predicts trastuzumab benefit in HER2-positive metastatic breast

- cancer patients. *Nat Commun.* 2018;9(1):1614.
14. Lu X, Ma J, Chu J, Shao Q, Zhang Y, Lu G, Li J, Huang X, Li W, Li Y, Ling Y, Zhao T. MiR-129-5p Sensitizes the Response of Her-2 Positive Breast Cancer to Trastuzumab by Reducing Rps6. *Cell Physiol Biochem.* 2017;44(6):2346-2356.
 15. Casey MC, Sweeney KJ, Brown JA, Kerin MJ. Exploring circulating micro-RNA in the neoadjuvant treatment of breast cancer. *Int J Cancer.* 2016;139(1):12-22.
 16. Gu X, Xue JQ, Han SJ, Qian SY, Zhang WH. Circulating microRNA-451 as a predictor of resistance to neoadjuvant chemotherapy in breast cancer. *Cancer Biomark.* 2016;16(3):395-403.
 17. Lei L, Chen J, Huang J, Lu J, Pei S, Ding S, Kang L, Xiao R, Zeng Q. Functions and regulatory mechanisms of metastasis-associated lung adenocarcinoma transcript 1. *J Cell Physiol.* 2018;234(1):134-151.
 18. Li P, Zhang X, Wang H, Wang L, Liu T, Du L, Yang Y, Wang C. MALAT1 Is Associated with Poor Response to Oxaliplatin-Based Chemotherapy in Colorectal Cancer Patients and Promotes Chemoresistance through EZH2. *Mol Cancer Ther.* 2017;16(4):739-751.
 19. Li J, Song ZJ, Wang YY, Yin Y, Liu Y, Nan X. Low levels of serum miR-99a is a predictor of poor prognosis in breast cancer. *Genet Mol Res.* 2016 Aug 26;15(3). doi: 10.4238/gmr.15038338.
 20. Hu Y, Zhu Q, Tang L. MiR-99a antitumor activity in human breast cancer cells through targeting of mTOR expression. *PLoS One.* 2014 Mar 17;9(3): e92099.
 21. Xia M, Li H, Wang JJ, Zeng HJ, Wang SH. MiR-99a suppress proliferation, migration and invasion through regulating insulin-like growth factor 1 receptor in breast cancer. *Eur Rev Med Pharmacol Sci.* 2016. 20(9): 1755-63.
 22. Wang X, Li Y, Qi W, Zhang N, Sun M, Huo Q, Cai C, Lv S, Yang Q. MicroRNA-99a inhibits tumor aggressive phenotypes through regulating HOXA1 in breast cancer cells. *Oncotarget.* 2015; 6(32): 32737-47.
 23. Li D, Hu J, Song H, Xu H, Wu C, Zhao B, Xie D, Wu T, Zhao J, Fang L. miR-143-3p targeting LIM domain kinase 1 suppresses the progression of triple-negative breast cancer cells. *Am J Transl Res* 2017;9(5): 2276-2285.
 24. Zheng T, Zhang X, Wang Y, Yu X. Predicting associations between miRNAs and target genes in breast cancer by bioinformatics analyses. *Oncol Lett* 2016;12(2):1067-1073.
 25. Yan X, Chen X, Liang H, Deng T, Chen W, Zhang S, Liu M, Gao X, Liu Y, Zhao C, Wang X, Wang N, Li J, Liu R, Zen K, Zhang CY1, Liu B, Ba Y. miR-143 and miR-145 synergistically regulate ERBB3 to suppress cell proliferation and invasion in breast cancer. *Mol Cancer.* 2014; 13:220.
 26. Shi Y, Ye P, Long X. Differential Expression Profiles of the Transcriptome in Breast Cancer Cell Lines Revealed by Next Generation Sequencing. *Cell Physiol Biochem.* 2017;44(2):804-816.
 27. Tavanafar F, Safaralizadeh R, Hosseinpour-Feizi MA, Mansoori B, Shanehbandi D, Mohammadi A, Baradaran B. Restoration of miR-143 expression could inhibit migration and growth of MDA-MB-468 cells through down-regulating the expression of invasion-related factors. *Retour Au Numéro.* 2017, 91:920-924.
 28. Xia C, Yang Y, Kong F, Kong Q, Shan C. MiR-143-3p inhibits the proliferation, cell migration and invasion of human breast cancer cells by modulating the expression of MAPK7. *Biochimie.* 2018; 147:98-104.
 29. Guo J, Liu C, Wang W, Liu Y, He H, Chen C, Xiang R4, Luo Y. Identification of serum miR-1915-3p and miR-455-3p as biomarkers for breast cancer. *PLoS One.* 2018 Jul 26; 13(7):e0200716.
 30. Wang B, Zou A, Ma L, Chen X, Wang L, Zeng X, Tan T. miR-455 inhibits breast cancer cell proliferation through targeting CDK14. *Eur J Pharmacol.* 2017; 15; 807:138-143.

31. Li Z, Meng Q, Pan A, Wu X, Cui J, Wang Y, Li L. MicroRNA-455-3p promotes invasion and migration in triple negative breast cancer by targeting tumor suppressor EI24.Oncotarget. 2017;8(12):19455-19466.
32. Zheng L, Zhang Y, Fu Y, Gong H, Guo J, Wu K, Jia Q, Ding X. Long non-coding RNA MALAT1 regulates BLCAP mRNA expression through binding to miR-339-5p and promotes poor prognosis in breast cancer.Biosci Rep. 2019; Feb 15;39(2).
33. Miao Y, Fan R, Chen L, Qian H.Clinical Significance of Long Non-coding RNA MALAT1 Expression in Tissue and Serum of Breast Cancer.Ann Clin Lab Sci. 2016;46(4):418-24.
34. Zidan HE, Karam RA, El-Seifi OS, Abd Elrahman TM.Circulating long non-coding RNA MALAT1 expression as molecular biomarker in Egyptian patients with breast cancer.Cancer Genet. 2018; 220:32-37.
35. Jadaliha M, Zong X, Malakar P, Ray T, Singh DK, Freier SM, Jensen T, Prasanth SG, Karni R, Ray PS, Prasanth KV.Functional and prognostic significance of long non-coding RNA MALAT1 as a metastasis driver in ER negative lymph node negative breast cancer.Oncotarget. 2016;7(26):40418-40436.
36. Wang Z, Katsaros D, Biglia N, Shen Y, Fu Y, Loo LWM, Jia W, Obata Y, Yu H.High expression of long non-coding RNA MALAT1 in breast cancer is associated with poor relapse-free survival.Breast Cancer Res Treat. 2018;171(2):261-271.
37. Xiping Z, Bo C, Shifeng Y, Feijiang Y, Hongjian Y, Qihui C, Binbin T.Roles of MALAT1 in development and migration of triple negative and Her-2 positive breast cancer.Oncotarget.2018;9(2):2255-2267.
38. Morrow PK, Wulf GM, Ensor J, Booser DJ, Moore JA, Flores PR, Xiong Y, Zhang S, Krop IE, Winer EP, Kindelberger DW, Coviello J, Sahin AA, Nuñez R, Hortobagyi GN, Yu D, Esteva FJ.Phase I/II study of trastuzumab in combination with everolimus (RAD001) in patients with HER2-overexpressing metastatic breast cancer who progressed on trastuzumab-based therapy.J Clin Oncol.2011;29(23):3126-32.
39. Gallardo A, Lerma E, Escuin D, Tibau A, Muñoz J, Ojeda B, Barnadas A, Adrover E, Sánchez-Tejada L, Giner D, Ortiz-Martínez F, Peiró G.Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. Br J Cancer.2012;106(8):1367-73
40. Jin Y, Feng SJ, Qiu S, Shao N, Zheng JH. LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. Eur Rev Med Pharmacol Sci.2017;21(14):3176-3184.
41. Wang C, Mao ZP, Wang L, Wu GH, Zhang FH, Wang DY, Shi JL.Long non-coding RNA MALAT1 promotes cholangiocarcinoma cell proliferation and invasion by activating PI3K/Akt pathway.Neoplasma.2017;64(5):725-731.
42. Xu S, Sui S, Zhang J, Bai N, Shi Q, Zhang G, Gao S, You Z, Zhan C, Liu F, Pang D3.Downregulation of long noncoding RNA MALAT1 induces epithelial-to-mesenchymal transition via the PI3K-AKT pathway in breast cancer.Int J Clin Exp Pathol.2015;8(5):4881-91.

Tables

Table 1. RNA reverse transcription stem ring primer sequence

Gene	Genbank Accession	Primer sequence (5'→3')
hsa-miR-99a-5p	MIMAT0000097	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCACAAG
hsa-miR-143-3p	MIMAT0000435	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGAGCTA
hsa-miR-455-5p	MIMAT0003150	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACCGATGT
SNORD48 (Internal reference)	NR_002745.1	GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACGGTCAG

Table 2. Real-Time PCR Primers and Conditions

Gene	Forward Primer and Universal Primer (5' to 3')	Annealing (°C)
hsa-miR-99a-5p	GCGAACCCGTAAGATCCGAT	60
hsa-miR-143-3p	CGCGTGAGATGAAGCACTGT	60
hsa-miR-455-5p	CGCGTATGTGCCTTTGGACT	60
SNORD48(Internal reference)	GATGATGACCCAGGTAAGTCT	60
Universal reverse primers(micro-R)	AGTGCAGGGTCCGAGGTATT	60

Table 3. Real-Time P CR Primers and Conditions

(Same sequence of serum and tissue)

Gene	Genbank Accession	Primer Sequences (5' to 3')	Size(bp)	Annealing (°C)
Human GAPDH	NM-002046.5	CCATGACAACCTTTGGTATCGTGGAA GGCCATCACGCCACAGTTTC	107	60
Human MALAT1	FJ209305.1	GAAGAGGCAATGTCCATCTCAAA CTCAATCCACTTGATCCCAACTCA	142	60

Table 4. Relevant information of first antibody

Name of the first antibody	Brand and item number	Dilution degree	Molecular weight(kDa)
Akt (Pan)	CST 4691	1:1000	60
P-Akt (Ser473)	CST 4060	1:1500	60
mTOR	CST 2983	1:1000	289
p-mTOR (Ser2448)	CST 5536	1:1000	289
PTEN	CST 9188	1:1000	54
β-actin (C4) (Internal reference)	Santa Cruz SC-47778	1:1500	43

Table 5. Information on second antibody

Name of the second antibody	Brand and item number	Dilution degree
Goat anti-Mouse IgG(H+L) Secondary antibody	Thermo Pierce, item number:31160	1:5000
Goat anti-Rabbit IgG(H+L) Secondary antibody	Thermo Pierce, item number:31210	1:5000

Table 6. Relative expression level of three miRNAs in tumor tissue of different groups

Groups	Treatment duration	miR-99	miR-455	miR-143
chemosensitive group	Before chemotherapy	1.443±0.811	0.949±0.672	1.532±0.802
	After chemotherapy	1.621±0.727	1.095±0.704	1.815±1.017
chemoresistant group	Before chemotherapy	2.048±1.246	1.323±0.374	1.300±0.758
	After chemotherapy	2.580±1.538	0.88±0.381**	1.045±0.606**

Compare to before chemotherapy, **P<0.01

Table 7 Relative expression level of MALAT1 in different groups

Groups	Treatment duration	serum MALAT1	MALAT1 in tumor tissue
Health control group	-	1.235±0.320	-
chemosensitive group	Before chemotherapy	4.896±1.455	1.632±0.494
	After chemotherapy	4.251±1.141*	1.468±0.489
chemoresistant group	Before chemotherapy	4.975±1.578	1.230±0.536
	After chemotherapy	5.513±2.071	1.333±0.627

Compare to before chemotherapy, *P<0.05

Table 8. Relative expression level of different PI3K pathway indicators of tumor tissue in chemoresistant group

Groups	p-AKT	AKT	p-mTOR	mTOR	PTEN
Before chemotherapy	3.785±1.766	20.165±0.858	3.635±0.412	15.918±0.203	12.830±1.300
After chemotherapy	11.220±2.493***	20.895±0.469	10.340±1.610**	16.348±0.398	9.890±0.896*

Compare to before chemotherapy, *P<0.05, **P<0.01, ***P<0.001

Figures

Figure 1

Comparison of expression levels of three miRNAs before and after chemotherapy in all patients

Figure 2

Comparison of expression levels of three miRNAs before and after chemotherapy in sensitive and resistant groups

Figure 3

OS and RFS comparison between chemotherapy resistance and chemosensitivity groups Legend: There was no significant difference in OS and RFS between chemotherapy resistance group and chemosensitivity group ($P>0.05$).

Figure 4

Relative expression level of MALAT1 in all patients Note: compared to health control, $***P<0.001$; NS means no significant Legend: The level of serum MALAT1 expression in all patients before and after chemotherapy was

significantly higher than that in healthy control group ($P < 0.001$). There was no significant difference in the expression of MALAT1 in tumor tissue before and after chemotherapy ($P > 0.05$). (A: serum; B: tumor tissue)

Figure 5

Relative expression level of MALAT1 in chemosensitive and chemoresistant patient groups before and after chemotherapy Note: compared to before chemotherapy, $*P < 0.05$; NS means no significant Legend: There was no significant difference in MALAT1 expression in tumor tissue between chemosensitivity group and chemotherapy resistance group before and after chemotherapy ($P > 0.05$). MALAT1 in the sensitive group was significantly lower than that before chemotherapy ($P < 0.05$). There was no significant difference in the expression level of MALAT1 in the chemotherapy resistance group before and after chemotherapy ($P > 0.05$). There was no significant difference in the expression level of MALAT1 between the chemotherapy resistance and the chemosensitivity group before and after chemotherapy ($P > 0.05$). (A: serum; B: tumor tissue)

Figure 6

Expression of PI3K/AKT/mTOR pathway related proteins in chemotherapy resistance group Note: compared to before chemotherapy, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ Legend: The expression of p-AKT and p-MTOR after chemotherapy was significantly higher than that before chemotherapy ($P < 0.001$, $P < 0.01$), and PTEN before chemotherapy was significantly lower than that after chemotherapy ($P < 0.05$). There was no significant difference between the other groups ($P > 0.05$).

Figure 7

Expression levels of PI3K pathway related proteins by Western blot detection