Original Article

Polymorphisms in microRNA let-7 binding sites of the *HIF1AN* and *CLDN12* genes can predict pathologic complete response to taxane- and platinum-based neoadjuvant chemotherapy in breast cancer

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Background: Germline genetic polymorphisms in certain genes are associated with response to anthracycline- and taxane-based neoadjuvant chemotherapy in breast cancer (BC). Recent evidence has indicated that microRNA (miRNA) let-7 expression is associated with response to chemotherapeutics. This study aims to evaluate the potential role of let-7 miRNA-related single nucleotide polymorphisms (mirSNPs) in the prediction of pathologic complete response to taxane- and platinum-based neoadjuvant chemotherapy in locally advanced breast cancer (LABC).

Methods: We genotyped the SNPs that reside in and around miRNA let-7 binding sites of two target genes: hypoxia-inducible factor 1 subunit alpha inhibitor (HIF1AN) and claudin 12 (CLDN12). The distribution frequencies of the SNPs were genotyped in LABC patients who received taxane- and platinum-based neoadjuvant chemotherapy. Associations among tumour-relevant biomarkers, genotype and pathological complete response (pCR) were evaluated using Student's t-test for continuous variables and the chi-square or Fisher's exact tests for non-categorical variables. The modified odds ratios (ORs) with their 95% confidence intervals (CIs) were calculated by a multivariate logistic regression analysis to explore the association of genotype with pCR.

Results: For rs11292, which is located in the 3'-untranslated region (UTR) of HIF1AN, significant differences were detected in codominant, dominant and overdominant models between the patients who achieved pCR and those who did not (non-pCR) ($P<0.05$) in a multivariate analysis. For rs1017105, which is located in the 3'-UTR of CLDN12, significant differences were observed in the recessive model between the pCR and non-pCR patients with luminal-type BC.

Conclusions: Let-7-related mirSNPs could predict pathologic complete response to taxane- and platinum-based neoadjuvant chemotherapy in LABC, which suggests the potential role of variants of miRNA let-7-related gene networks as predictive markers in a clinical setting.

Keywords: Locally advanced breast cancer (LABC); miRNA let-7; miRNA-related single nucleotide polymorphisms (mirSNPs); neoadjuvant chemotherapy; pathologic complete response

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Introduction

MicroRNAs (miRNAs) are small, single-stranded molecules approximately 21 nucleotides in length (1). They are involved in the development and progression of malignant tumours, mainly through the post-transcriptional modulation of various physiological and pathological pathways (2-4). Several studies reported that miRNA may influence patient response to chemotherapy. The miRNA let-7 family and its role in oncogene regulation has also been studied. The let-7 family of miRNAs is involved in the suppression of several oncogenes (5-7), and let-7 is therefore considered a tumour suppressor miRNA. One study found that lower expression of let-7a miRNA might induce chemoresistance in breast cancer (BC) by enhancing cellular apoptosis (8), whereas other studies showed that let-7 expression is associated with response to chemotherapeutics (9-12).

Neoadjuvant chemotherapy is increasingly used in women with newly diagnosed early BC especially locally advanced breast cancer (LABC) (13,14). The advantage of neoadjuvant chemotherapy is that breast-conserving surgery can be performed more frequently (15). In addition, it offers the opportunity to directly assess a tumour's response to therapy. Pathological complete response (pCR) is defined as the absence of invasive cancer, and it has been demonstrated that pCR after neoadjuvant chemotherapy is associated with better long-term survival (16-18). Among the women who receive neoadjuvant chemotherapy, the clinical response rate is as high as 70%, but only approximately 15–30% of patients achieve pCR (13,14,19). Novel predictive biomarkers that can predict tumour response prior to chemotherapy are valuable for individualized treatment decisions and to maximize efficacy in cancer patients.

MiRNAs regulate gene expression by base pairing with sequences within the 3'-untranslated region (UTR), 5'-UTR, and coding sequence regions of target mRNAs (20). Several recent studies have emphasized that polymorphisms in miRNA binding sites are predictors of response to chemotherapy in patients with malignant tumours (21-24). Moreover, it has also been reported that miRNA let-7 regulates the *HIF1AN* (25,26) and *CLDN12* genes (27). Hypoxia-inducible factor 1-alpha inhibitor is a protein that is encoded by the *HIF1AN* gene. *HIF1AN* expression and differential cellular localization have been shown to be associated with shorter survival in BC (28). Claudins, which are the most important tight junction proteins, are also linked with cancer progression and metastasis. Claudin-12 belongs to a group of claudins and is encoded by *CLDN12* gene. In our previous study, we found that polymorphisms located in the *HIF1AN* and *CLDN12* genes were associated with BC susceptibility. Whether the polymorphisms located in the *HIF1AN* and *CLDN12* genes have a predictive role in response to neoadjuvant chemotherapy in LABC warrants further investigation.

In this study, we selected two let-7 miRNA-related single nucleotide polymorphisms (mirSNPs) (rs11292 located in the *HIF1AN* gene and rs1017105 located in the *CLDN12* gene). We hypothesized that genetic variations in miRNA let-7 binding sites located in the *HIF1AN* and *CLDN12* genes could predict response to taxane- and platinum-based neoadjuvant chemotherapy in BC. In this study, we identified the rs11292 and rs1017105 SNPs in participants from two prospective, phase II studies (29), in which patients received weekly paclitaxel and cisplatin as neoadjuvant chemotherapy.

Methods

Study population

From October 2013 until November 2016, 130 patients with LABC were recruited for this study. The inclusion criteria were females aged between 18 and 70 years with histologically confirmed and untreated large operable BC (T size ≥2 cm and N0–2). Patients with other malignancies were excluded. Other inclusion and exclusion criteria are described elsewhere (29). Patients enrolled in this study were participants from two open-label, prospective, phase II trials. In these two trials, patients were scheduled to receive an intravenous (i.v.) infusion of 80 mg/m^2 paclitaxel weekly on day 1 for 16 weeks and 25 mg/m^2 cisplatin on days 1, 8, and 15 every 28 days. Patients with human epidermal growth factor receptor 2 (HER2)-positive cancer could use concomitant trastuzumab on a weekly basis. Written informed consent was obtained from each subject. For each subject, a 5 mL peripheral blood sample was collected and stored at –80 ℃. The DNA was extracted from the peripheral blood samples of all subjects using a Qiagen DNA blood kit (Qiagen NV, Venlo, The Netherlands) according to the manufacturer's protocols. The study was approved by the ethics committee of Renji Hospital, Shanghai Jiao Tong University. This study was conducted in accordance with the Declaration of Helsinki. Detailed characteristics of the patients enrolled in this study are shown in *Table S1*.

Table 1 Candidate target genes and the corresponding SNPs

Target gene	SNP
hypoxia-inducible factor 1 subunit alpha inhibitor (*HIF1AN*)	rs11292
claudin 12 (*CLDN12*)	rs1017105

SNPs, single nucleotide polymorphisms.

Histopathologic response

The pCR was evaluated in the surgical specimens' post-chemotherapy and was defined as the absence of invasive cancer in the breast, with or without residual non-invasive intraductal carcinoma. All cases with residual invasive cancer of any size were classified as non-pCR.

Selection of SNPs in the HIF1AN and CLDN12 genes

First, we searched the miRBase (www.mirbase.org, Release 22), TargetScan (http://www.targetscan.org/, Release 7.2) and UCSC Genome Browser (http://genome.cse.ucsc.edu) databases to identify potential target genes of miRNA let-7. Second, we selected potential target genes of miRNA let-7 that are associated with malignant characteristics of BC, such as proliferation, angiogenesis, invasion, metastasis or inhibition of apoptosis. We screened the SNP loci that cover the extension of 2 kb at both sides of the let-7-binding sites in the target genes. We then screened for SNP loci in the binding sites of miRNA let-7 with low-binding free energy. *Table 1* lists the characteristics of the candidate loci.

SNP genotyping

SNP genotyping was performed at Shanghai Bene-gene Biotechnology Co., Ltd. (Shanghai, People's Republic of China) with the MassARRAY system (Sequenom, San Diego CA, USA) using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Detailed information on the primers is shown in *Table 2*.

Statistical analysis

Genotype distributions were tested for adherence to the Hardy-Weinberg equilibrium (HWE). Associations among tumour-relevant biomarkers, genotype and pCR were evaluated using Student's *t*-test for continuous variables and chi-square or Fisher's exact tests for non-categorical

Table 2 Detailed information on the primers used in the study

SNP	Primer 1	Primer 2	Extension of primer	Single-base primer
Rs11292	ACGTTGGATGGGAGGAGGCATGTTGAAAA	ACGTTGGATGGCACTGTGCTAAAGCTTTAC	R	ACGTTGGATGGCACTGTGCTAAAGCTTTAC
Rs1017105	ACGTTGGATGTCTTCTACCTCATGCCACTG	ACGTTGGATGGGTGATATTTAGAATGGTAG	F	ACGTTGGATGGGTGATATTTAGAATGGTAG

Table 3 Associations between genotypes of rs11292 and pCR after neoadjuvant chemotherapy in patients with locally advanced breast cancer

Model	Genotype	pCR	Non-pCR	OR (95% CI)[ψ]	P value
Codominant	TT	29 (27.6)	76 (72.4)	1.00	
	TC	10 (43.5)	13 (56.5)	4.46 (1.26–15.78)	0.020*
	CC	0 (0.0)	2 (100.0)	NA	NA
Dominant	TT	29 (27.6)	76 (72.4)	1.00	
	TC + CC	10 (40.0)	15 (60.0)	3.41 (1.02–11.33)	0.046*
Recessive	TT + TC	39 (30.5)	89 (69.5)	1.00	
	CC	0 (0.0)	2 (100.0)	NA	NA
Overdominant	TT + CC	29 (27.1)	78 (72.9)	1.00	
	TC	10 (43.5)	13 (56.5)	4.63 (1.31–16.33)	0.017*

[ψ], OR and 95% CI was analysed by logistic regression and adjusted by age, menstrual status, and ER, PR and HER2 status. The common genotype was used as a reference; *, P<0.05. pCR, pathological complete response; OR, odds ratios; CI, confidence intervals; NA, not available; ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth factor receptor 2.

variables. The modified odds ratios (ORs) with their 95% confidence intervals (CIs) were calculated by univariate and multivariate logistic regression analyses to explore the association of genotype with pCR. All statistical analyses were calculated by Stata 14.0 (StataCorp LP, College Station, TX, USA).

Results

Association of SNPs in the HIF1AN and CLDN12 genes with the histopathological characteristics of BC patients

Between 2013 and 2016, 130 patients with LABC were recruited for this study. The genotypic distribution, which was tested for deviation from HWE, showed no deviation for *HIF1AN* rs11292 or *CLDN12* rs1017105 (data not shown). The association of the rs11292 and rs1017105 genotypes with the clinicopathologic parameters of primary BC was noted. No significant correlation was observed between these two genotypes and clinicopathologic features, including age, menstrual status, or estrogen receptor (ER), progesterone receptor (PR) and HER2 status.

Relationship of the SNPs in the HIF1AN and CLDN12 genes with response to neoadjuvant chemotherapy

Thirty-six of 130 (27.7%) patients achieved pCR after neoadjuvant chemotherapy. The pCR rate was 27.6% in patients with the *HIF1AN* rs11292 TT genotype and 43.5% in patients with the TC genotype. In the univariate analysis, no significant differences were detected in the rs11292 genotype between the pCR and non-pCR patients. In the multivariate analysis, significant differences were detected in the codominant, dominant and overdominant models of rs11292 between these two groups (P<0.05), which suggests that the rs11292 genotype is associated with response to neoadjuvant chemotherapy. The genotype distributions in the pCR and non-pCR patients are shown in *Table 3*. In the codominant model, women carrying the TC (OR =4.46, 95% CI: 1.26–15.78, P=0.02) genotype of *HIF1AN* rs11292 had a higher pCR rate than those carrying the TT genotype. In the dominant model, women carrying the TC + CC (OR =3.41, 95% CI: 1.02–11.33, P=0.046) genotypes of *HIF1AN* rs11292 had a higher pCR rate than those carrying the TT genotype. In the overdominant model, women carrying the TC (OR =4.63, 95% CI: 1.31–16.33, P=0.017) genotype of *HIF1AN* rs11292 had a higher pCR rate than those carrying the TT + CC genotypes (*Table 3*).

The pCR rate was 18.8% in patients with the CLDN12 rs1017105 CC genotype, 9.8% in patients with the CT genotype and 28.6% in patients with the TT genotype. In the univariate and multivariate analyses, no significant differences were detected in the genotypes of rs1017105 between the pCR and non-pCR patients. Further analysis revealed significant differences in rs1017105 between the pCR and non-pCR patients with luminal-type BC (*Table 4*). The genotype distributions in the pCR and non-pCR patients with luminal-type BC are shown in *Table 4*. In the recessive model, women carrying the TT (OR

Table 4 Associations between genotypes of rs1017105 and pCR after neoadjuvant chemotherapy in patients with luminal-type locally advanced breast cancer

Model	Genotype	pCR	Non-pCR	OR (95% CI)[ψ]	P value
Codominant	CC	9 (18.8)	39 (81.2)	1.00	
	CT	4 (9.8)	37 (90.2)	1.44 (0.43–4.87)	0.556
	TT	2 (28.6)	5 (71.4)	2.33 (0.95–5.67)	0.063
Dominant	CC	9 (18.8)	39 (81.2)	1.00	
	CT + TT	6 (12.5)	42 (87.5)	1.65 (0.54–5.00)	0.378
Recessive	CC + CT	13 (14.6)	76 (85.4)	1.00	
	TT	2 (28.6)	5 (71.4)	5.80 (1.02–23.49)	0.014*
Overdominant	CC + TT	11 (20.0)	44 (80.0)	1.00	
	CT	4 (9.8)	37 (90.2)	1.26 (0.39–4.01)	0.698

[ψ], OR and 95% CI was analysed by logistic regression and adjusted by age, menstrual status, and ER, PR and HER2 status. The common genotype was used as a reference; *, P<0.05. pCR, pathological complete response; OR, odds ratios; CI, confidence intervals; NA, not available; ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth factor receptor 2.

=5.80, 95% CI: 1.02–23.49, P=0.014) genotype of *CLDN12* rs1017105 had a higher pCR rate than those carrying the CC + CT genotypes (*Table 4*), which suggests that the genotypes of rs1017105 are associated with response to neoadjuvant chemotherapy.

Discussion

To our knowledge, this is the first study that has investigated mirSNPs that potentially affect miRNA let-7 binding to the *HIF1AN* and *CLDN12* genes and that has revealed their impact on pathologic response to taxane- and platinum-based neoadjuvant chemotherapy in BC. In this translational study, we found for the first time that SNPs in miRNA let-7-related genes (rs11292 located in the *HIF1AN* gene and rs1017105 located in the *CLDN12* gene) could predict pCR to taxane- and platinum-based neoadjuvant chemotherapy in LABC. In the neoadjuvant setting, it is important to identify non-responders to avoid ineffective treatments and to provide alternative therapies such as surgery or switching to a non-cross resistant chemotherapy regimen.

In this study, our data showed that *HIF1AN* rs11292, a let-7 mirSNP, could predict pathologic complete response to taxane- and platinum-based neoadjuvant chemotherapy in BC. Saridaki *et al.* found that a let-7 miRNA binding site polymorphism in KRAS predicts improved outcome in patients with metastatic colorectal cancer who are treated with salvage cetuximab/panitumumab monotherapy (21). The impact of miRSNPs in the *HIF1AN* gene on the efficacy of neoadjuvant chemotherapy has not previously tested. Zheng *et al.* found that body fat possibly plays an important role in the development of BC and that this risk might be modified by specific genotypes of some potential genes including the *HIF1AN* gene (30). The results of our study and others are consistent, which suggests that genetic variations in miRNA let-7 binding sites in the *HIF1AN* gene may modulate the signalling response and the maintenance of genomic stability; this ultimately affects the efficacy of chemotherapy.

We demonstrated that *CLDN12* rs1017105 could predict pathologic complete response to taxane- and platinum-based neoadjuvant chemotherapy in women with luminal-type BC. Hansen *et al.* hypothesized that the characterization of taxane resistance-associated molecular alterations and the identification and validation of putative predictive markers will have the potential to result in more effective use of taxanes, with improved survival and reduced toxicities and costs. In their study, the use of human BC cell lines grown *in vitro* allowed for the investigation of genomic alterations during multiple steps in the process towards a docetaxel-resistant phenotype. Additionally, they showed that MCF-7 cells acquired several copy number gains in chromosome 7, including in the *ABCB1*, *ABCB4* and *CLDN12* genes (31). Yang *et al.* investigated the relationship between claudins and IL-18. They showed that exogenous IL-18 enhanced BC cell migration and suppressed the expression of the

tight junction proteins claudin-1, claudin-3, claudin-4 and claudin-12 in MCF-7 cells (32). The knockdown of claudin-3, claudin-4, and claudin-12 increased BC cell migration with maximal effects observed in claudin-12 siRNA-transfected cells. Their results suggest that IL-18 is an important factor for the induction of BC cell migration through downregulation of claudin-12. Taken together, these results align with our findings. Therefore, SNPs of miRNA let-7-related gene networks may serve as predictive biomarkers of taxane- and platinum-based neoadjuvant chemotherapy in luminal-type BC.

The present study has some limitations. The sample size of the patient cohort was small. Our findings indicated that SNPs of let-7-related genes (rs11292 located in the *HIF1AN* gene and rs1017105 located in the *CLDN12* gene) were associated with response to neoadjuvant chemotherapy and had a potential role as predictive markers in a clinical setting. However, we admit that we did not investigate the underlying molecular mechanisms.

In conclusion, the results of the present study showed that miRNA let-7 binding site genetic variants located in the *HIF1AN* and *CLDN12* genes could predict pCR to taxane- and platinum-based neoadjuvant chemotherapy in LABC. However, further work is needed to clarify the underlying molecular mechanisms.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was conducted in accordance with the Declaration of Helsinki. Informed consent for participation in the study or use of their tissue was obtained from all participants. Each patient provided written informed consent for the publication of any associated data and accompanying images.

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Supplementary

Table S1 Patient characteristics of the study

Variables	Value (n=130) (%)
Age at randomization	
Median	53.5
Range	29–59
Menstrual status	
Premenopausal	55 (42.3)
Postmenopausal	72 (55.4)
Unknown	3 (2.3)
ER	
Positive	97 (74.6)
Negative	33 (25.4)
PR	
Positive	114 (87.7)
Negative	16 (12.3)
HER2 status	
Positive	53 (40.8)
Negative	77 (59.2)
Pathological response	
pCR	36 (27.7)
Non-pCR	94 (72.3)

ER, estrogen receptor; PR, progesterone receptor; HER2, epidermal growth factor receptor 2; pCR, pathological complete response.