A Methylation-Based Regulatory Network for MicroRNA 320a in Chemoresistant Breast Cancer

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ABSTRACT

We previously demonstrated that the overexpression of transient receptor potential channel C5 (TRPC5) and nuclear factor of activated T-cells isoform c3 (NFATC3) are essential for cancer chemoresistance, but how TRPC5 and NFATC3 are regulated was still unclear. In this study, microRNA 320a (miR-320a) was found to be down-regulated in chemoresistant cancer cells. MiR-320a directly targeted TRPC5 and NFATC3, and down-regulation of miR-320a triggered TRPC5 and NFATC3 overexpression. In chemoresistant cells, down-regulation of miR-320a was associated with regulation by methylation, which implicated promoter methylation of the miR-320a coding sequence. Furthermore, the transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1), which inhibited miR-320a expression, was activated in chemoresistant cancer cells; such activation was associated with hypomethylation of the ETS-1 promoter. Lastly, the down-regulation of miR-320a and high expression of TRPC5, NFATC3, and ETS-1 were verified in clinically chemoresistant samples. Low expression of MiR-320a was also found to be a significant unfavorable predictor for clinic outcome. In conclusion, miR-320a is a mediator of chemoresistance by targeting TRPC5 and NFATC3. Expression of miR-320a is regulated by methylation of its promoter and that of ETS-1.

Introduction

Chemoresistance is one of the main causes of failure in chemotherapy. One well-demonstrated mechanism for the acquisition of chemoresistance is the overexpression of drug transporters in cancer cells (Li et al., 2007; Liu et al., 2005). The P-glycoprotein (P-gp) encoded by the ABCB1 gene is typical of these. P-gp is frequently associated with drug-resistant phenotypes, so understanding its mechanism of overexpression may provide promising strategies for overcoming chemoresistance. In our previous study, we found that overexpression of the ABCB1 gene in chemoresistant cancer cells is controlled by TRPC5 (Ma et al., 2012), which can be used as a chemoresistance marker in breast cancers (Ma et al., 2014). TRPC5 is highly expressed in chemoresistant human breast cancer cells, induces the activation of nuclear factor of activated T-cells isoform c3 (NFATC3), and NFATC3 directly stimulates ABCC1 gene expression. However, it is not clear how the expression of TRPC5 and NFATC3 are regulated in chemoresistant cancer cells.

Alterations of tumor suppressors and proto-oncogenes can be mediated by abnormalities in the quality or quantity of microRNAs (miRNAs), single-stranded 19–25 nucleotide short RNAs that modulate gene expression at the posttranscriptional stage by targeting mRNAs, and the binding sites commonly occur within the 3’-untranslated region (UTR) of mRNAs. In chemoresistant cancer cells, miRNAs regulate features closely associated with chemoresistance, including apoptosis, chemotherapeutic agent response, DNA damage repair, cell cycle, stemness transition, and mesenchymal transition in cancer cells (Sarkar et al., 2010; Kutanzi et al., 2011).

Quantitative defects in miRNAs are mediated by several mechanisms, and recent studies have shown the involvement of methylation events (Saito and Jones, 2006). Human DNA methylation usually occurs at the CpG dinucleotide, and CpG islands (CpGis) are regions with a high density of these dinucleotides. Methylation at CpGis in promoter regions usually leads to gene suppression. Because the expression of miRNA is controlled by either the promoters of their host genes or separate promoters of their own, silencing or activating miRNAs...
by promoter hypermethylation or hypomethylation is emerging as a mechanism of producing quantitative miRNA defects. However, relatively little is known about the transcriptional regulation of miRNA genes. To date, among the >100 miRNAs associated with cancer, only a few studies have characterized their regulation by methylation (Datta et al., 2008; Furuta et al., 2010; Li et al., 2010; Alpini et al., 2011; He et al., 2011; Wong et al., 2011), and to the best of our knowledge, few of these miRNAs have been directly demonstrated to be associated with chemoresistance.

Therefore, our study explored the dysregulation of miRNA by methylation in chemoresistance. Our previous study (Ma et al., 2012) found that decreased expression of microRNA 320a (miR-320a) regulated both TRPC5 and NFATC3 up-regulation (control). Luciferase activity was assessed with a Dual-Luciferase Reporter Assay System kit (Promega).

Materials and Methods

Cell Cultures. MCF-7/WT (wild-type) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and the colorectal adenocarcinoma cell line (HCT-8) and fluorouracil- and paclitaxel-resistant human breast cancer cells (MCF-7/ADM) were derived as previously described elsewhere (Ma et al., 2012) by treating MCF-7 cells with stepwise increasing concentrations of ADM and PTX over 8 months. All cells were cultured in RPMI supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 U/ml streptomycin.

Identification of MicroRNAs Targeting TRPC5 and NFATC3. To search for the miRNAs targeting TRPC5 and NFATC3, Targetscan and miRDB software (www.targetscan.org and www.mirdb.org) were applied together.

To verify the binding of miRNA-320a to TRPC5 and NFATC3, the 3′-UTR on mRNA; lowercase letters are the restriction enzyme recognition sequence. The expression of mRNA was analyzed by real-time PCR. RNAs were reverse-transcribed by Superscript II reverse transcriptase (Invitrogen). Real-time PCRs were performed using SYBR Green Supermix (Bio-Rad Laboratories) for 40 cycles of 95°C for 15 seconds and 60°C for 40 seconds. The primer pairs for TRPC5 were forward 5′-CCACAGCTACTCA-GATAAAGG-3′, reverse 5′-CGA AACAGCCCTTTACCUC-3′ (Liu et al., 2007); for NFATC3 were forward 5′-CTCACGGTCTGCTGGCTGACACG-3′, reverse 5′-CTGGTGAGGAGCAGCTAAGGGC-3′; and for ETS-1 were forward 5′-CTGCGGCTGAGTGAAGA-3′, reverse 5′-CCGTAAGATGTCCTCCCA-CAA-3′ (Chan et al., 2011).

Western Blot Analysis. Whole-cell lysates of treated cells were prepared and separated by 10% SDS-PAGE. The antibody binding was evaluated with an Odyssey imaging system (Li-Cor Bisciences, Lincoln, NE). Anti-TRPC5, NFATC3, and ETS-1 mAbs were obtained from Abcam (Cambridge, MA).

Construction of the TRPC5 Overexpression Vector. The 3′-UTR region of the TRPC5 gene was cloned into pcDNA3 plasmid and mutated with the Heff Mt Site-Directed Mutagenesis Kit (Yeasen, Shanghai, People’s Republic of China) to the site of miR-320a targeting. The coding sequence of the human TRPC5 gene was then cloned and fused with the mutated 3′-UTR region.

5′-Rapid Amplification of cDNA Ends Assay Analysis of the Transcriptional Start Site of miR-320a. Total RNA was extracted from MCF-7/WT cells with TRIzol (Invitrogen). Alkaline phosphatase treatment was used to remove the free 5′-phosphates and cap structures of RNA. The decapped RNA was ligated to RNA adapter oligonucleotide and reverse transcribed. Nested PCR was applied by Taq polymerase and reverse transcribed. Nested PCR was applied at 55°C for 30 seconds and 72°C for 2 minutes for 25 cycles; the primers were 5′-TATCATTTTTTCGCCCTCTC-3′ for the outer gene-specific primer and 5′-ACTCCGGGAGACCGGGAGG-3′ for the nested gene-specific primer.

The PCR products were separated on 3% agarose gel, inserted into pUC19-T plasmid, and transfected into the DH5α Ericheria coli system on LB plates (100 μg/ml ampicillin with IPTG and X-Gal). Three E. coli colonies were selected randomly. Then plasmids were extracted, digested by EcoRI/HindIII, and separated on agarose gel for 3% agarose gel for 3′-UTR and 5′-UTR. The expression of mRNA was analyzed by real-time PCR. The purified PCR products were then cloned into the DH5α E. coli system and amplified on LB plates (100 μg/ml ampicillin). Two successfully transfected E. coli colonies were selected randomly, and the insertion of PCR product was verified by KpnI/XhoI digestion following by 1% agarose gel separation. The purified PCR product was then transferred into the pGL3 basic plasmid (Promega), transfected into the DH5α E. coli system, and amplified on LB plates (100 μg/ml ampicillin). Seven plasmids with correct insertion of the promoter region was then transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen), and the
Luciferase activity was assessed with a Dual-Luciferase Reporter Assay System kit (Promega).

Hypomethylation of miR-320a. MCF-7/ADM cells were treated with 5 μM 5-aza-2’-deoxycytidine (DAC; Sigma-Aldrich, St. Louis, MO) for 48 hours, then the RNA or protein was extracted for qRT-PCR or Western blot.

ETS-1 Targeting of the miR-320a Promoter. The promoter and 5’-UTR region were cloned together into pGL3 basic plasmid as described earlier with these primers: forward 5’-gggctgacctGTGTGACACCTGAATT-CACCTGATTCCCT-3’, reverse 5’-cgtctggcgcGGGCTGAATATAACTGT-TGGG-3’. The cloned region was verified by sequencing and blasting, the pGL3 plasmid with correct insertion of the promoter region was later transfected together with pcDNA3 plasmid overexpressing ETS-1 into HEK293 cells with Lipofectamine 2000 (Invitrogen), and the luciferase activity was assayed by the Dual-Luciferase Reporter Assay System kit (Promega).

Bisulfite Sequencing PCR of miR-320a and ETS-1 Promoters. Cpg plot online software (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) was used to analyze CpgIs on promoters. Total DNA was extracted (Wizard genomic DNA purification kit; Promega) and bisulfite converted by an Epitext Bisulfite Kit (Qiagen, Valencia, CA). The DNA was then amplified by PCR at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 40 seconds for 30 cycles with these primers for miR-320a: forward 5’-TGGATTGAGGGGTTYG-3’, reverse 5’-ACCCCTCAACCCYATTAAT-3’; ETS-1 outer, forward 5’-ATGTTGTAAGGAAAGG-3’, reverse 5’-ATTGTCCTCTTCTTTC-3’; and ETS-1 nested, forward 5’-GCGGTTTAYATTTGTT-3’, 5’-TCTCTCCTCTCCGTC-3’ (because CpG sites are unavoidable in a primer, de-generate bases Y represent C and T in forward primers, and R represent G and A in reverse primers). The PCR products were cloned into pDM19-T plasmid (Takara Biotechnology, Dalian, China) and sequenced.

Chromatin Immunoprecipitation Assay. The binding sites of ETS-1 on miR-320a were predicted by Jaspar online software (jaspar.genereg.net/); three sites (Supplemental File 4, S1 to S3) showed the highest probability of binding, which was later confirmed using a chromatin immunoprecipitation assay kit (Beyotime, Shanghai, People’s Republic of China). Briefly, MCF-7/ADM cells in T75 disks were fixed with 1% formaldehyde for 10 minutes at 37°C followed by neutralization with 125 mM glycine. The cells were then lysed in ice on SDs lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO). Then the lysate was sonicated on ice (10-second pulse, 60-second break, seven times, 10% amplitude; Sonics & Materials, Newton, CT) to break the genome into 200–1000-bp fragments. We added 2 μg of primary antibody (ETS-1, Santa Cruz Biotechnology, Santa Cruz, CA; or preimmune IgG as negative control) to 2 ml of Protein G–pretreated lysis buffer to allow precipitation of ETS-1–associated genomic DNA fragments by overnight rotation at 4°C, then washed with different buffers. The DNA-protein cross-links were reversed, and the chromatin DNA was purified and subjected to PCR analysis. The primer pairs for S1 were forward 5’-GGGCTTCTGCGCCAGTTGCTC-3’, reverse 5’-AAAGGCTCTCACCCTGGGTGTC-3’; S2 forward 5’-TGATTTTTCGGCCCTTCTACACC-3’, reverse 5’-ATTGTCCTCTCAGCGGCGATG-3’; and S3 forward 5’-GGTGAATACCTGTGGGAGCTCC-3’, reverse 5’-CAAACTCCTGCAACGGCGACC-3’. The PCR products were cloned into pBluescript KS+ (Stratagene, CA) and sequenced.

Results

Targeting of TRPC5 and NFATC3 by miR-320a. Based on our previous findings that TRPC5 is critical for the induction of chemoresistance by activating NFATC3 and thus triggering P-gp expression (Ma et al., 2012), we predicted the potential miRNAs targeting TRPC5 and TRPC5-related pathways by applying online TargetScan and miRDB software together. We selected miR-320a because it was predicted to target both TRPC5 and NFATC3 (Fig. 1A). The luciferase reporter assay was then used to verify this prediction. We found that miR-320a suppressed the expression of luciferase by targeting the 3’-UTRs of the TRPC5 and NFATC3 mRNAs, and mutation of the 3’-UTRs destroyed the homology with miR-320a at its target site (Fig. 1B).

The theoretical binding sites predicted by the online software were not totally supported in a real biologic system. Indeed, the interaction between miR-320a and NFATC3 was not affected by mutation in the third binding site, so this predicted site of miR-320a binding does not exist. Therefore, these data suggest that TRPC5 and NFATC3 are targeted by miR-320a.

The interactions between miR-320a and its targets were then confirmed in chemoresistant MCF-7/ADM cells, which express high levels of TRPC5 and NFATC3 compared with MCF-7/WT cells (Ma et al., 2012) (Fig. 1C). MCF-7/ADM cells were transfected with an miR-320a mimic designed to overexpress the endogenous miRNA, and the expression of TRPC5 and NFATC3 were analyzed. When the mimic was introduced into MCF-7/ADM cells, this forced expression of miR-320a induced down-regulation of TRPC5 and NFATC3 at both the mRNA and protein levels (Fig. 1, C and D).

MicroRNA-320a Modulates Chemoresistance. To define the role of miR-320a in chemoresistance, we performed qRT-PCR, which showed that miR-320a expression was significantly decreased in MCF-7/ADM cells compared with their parental control MCF-7/WT cells (Fig. 2A). This suggests that reduction of
miR-320a contributes to the high expression of TRPC5 and NFATC3 in MCF-7/ADM cells.

Previously, we had demonstrated that suppression of the high TRPC5 and NFATC3 activity reduces the chemoresistance of MCF-7/ADM cells (Ma et al., 2012). Therefore, we hypothesized that overexpression of miR-320a, which downregulated TRPC5 and NFATC3, may also play a role in reducing chemoresistance. To test this hypothesis, MCF-7/ADM cells were transfected with the miR-320a mimic. We found that overexpression of miR-320a sensitized the cells to ADM; the half-maximal inhibitory concentration (IC50) of ADM decreased in MCF-7/ADM cells when miR-320a was overexpressed (Fig. 2B). Furthermore, a TRPC5 overexpression vector was constructed by fusing the coding sequence of TRPC5 with its 3'UTR in pcDNA3 vector, in which the 3'UTR was mutated so that the miR-320a could not target the mRNA of TRPC5. When the MCF-7/ADM cells were transfected with the empty vector, the miR-320a mimic decreased their chemoresistance (Fig. 2C, first and second bars), suggesting that the empty vector does not influence the chemoresistant feature.

In addition, overexpression of TRPC5 increased the chemoresistance slightly compared with the empty vector (Fig. 2C, first and third bars), suggesting that the chemoresistance cannot increase indefinitely with the overexpression of TRPC5, probably because distribution of TRPC5 in the plasma membrane is already overloaded in MCF-7/ADM cells. When the miR-320a mimic was transfected into the cells with mutated TRPC5, the chemoresistance could no longer be decreased (Fig. 2C, third and fourth bars).

On one hand, this result suggested that the mutated TRPC5 retains its original activity in mediating chemoresistance; when the endogenous TRPC5 is targeted by the miR-320a mimic, the TRPC5 with a mutated 3'UTR from the overexpression vector could act as a substitute. On the other hand, it suggested that
miR-320a modulates chemoresistance via the TRPC5 signaling pathway. Therefore, these data suggest that down-regulation of miR-320a is involved in the development of chemoresistance.

**Down-Regulation of miR-320a Is Associated with Methylation in Its Promoter Region.** We next studied the mechanism of miR-320a down-regulation in chemoresistant MCF-7/ADM cells. Because miRNAs are transcribed from the genome by RNA polymerase II (Lee et al., 2004) and controlled by promoter elements that show great similarity to protein-coding genes (Rodriguez et al., 2004; Saito and Jones, 2006), the expression of miRNAs can be controlled by methylation of their promoters of the host gene or their own promoters. We found that no protein-coding genes overlapped with the coding sequence of miR-320a (miRBASE database), so this miRNA might have its own promoter. Therefore, the promoter region of miR-320a was identified by locating the transcriptional start site (TSS) with 5'-rapid amplification of cDNA ends (5'-RACE, Supplemental Fig. 1A). The 5'-RACE product was cloned into pUC19-T plasmid and transfected into the DH5α E. coli system for amplification and DNA sequencing. Three positively transfected E. coli colonies were selected randomly, and the amplified pUC19-T was cut with restriction enzymes. The second colony (lane 3 in Supplemental Fig. 1B) was chosen because two clear bands were generated after enzyme cutting of pUC19-T within the colony (lane 4 in Supplemental Fig. 1B); one band was the empty pUC19-T plasmid, and the other was the 5'-RACE product.

The enzyme-cutting results of the second colony not only suggested a successful 5'-RACE assay that generated a product of ~1000 bp, but also indicated that the pUC19-T plasmid was correctly cloned with the 5'-RACE product, which was necessary for reliable DNA sequencing in the subsequent processes. The 5'-RACE products that were cloned within the pUC19-T plasmid were then sequenced and blasted with the human genome (Supplemental File 1, Supplemental Tables S1.1 and S1.2). The TSS was then identified as the nucleobase guanine at 965 bp upstream of the miR-320a coding sequence. According to the UCSC database (GRCh37/hg19), the TSS was at chr8: 22,103,522 (Supplemental Fig. 1A and Supplemental File 1).

We then analyzed the promoter activity around the TSS and identified a region ~2035 bp from the TSS to the TSS itself (chr8: 22,103,522–5,556) as a putative promoter region (Supplemental File 2). This region was later cloned into the upstream of the luciferase gene of the pGL3 basic plasmid (Supplemental Fig. 2), a plasmid lacking promoter and enhancer sequences, thus allowing the study of putative regulatory sequences. Analysis of the luciferase activity induced by the promoter showed that insertion of the region from ~2035 bp to the TSS induced strong luciferase expression (Fig. 3A), indicating that this region displays promoter activity.

The methylation status of miR-320a was then determined. According to the UCSC database, several CpGis regions cover the whole promoter region and coding sequence of miR-320a. As methylation at both the promoter and 5'-UTR region (Brenet et al., 2011) inhibits gene expression, the methylation status of both the promoter and the 5'-UTR region (TSS to coding sequence) was analyzed. CpG plot software identified three CpGis regions (Supplemental File 2). Methylation of these regions was then analyzed by bisulfite sequencing PCR (BSP). The results showed that the promoter methylation was significantly increased in ADM cells (Fig. 3B, a and b), however, there was no significant methylation in the 5'-UTR (Fig. 3B, c), suggesting that down-regulation of miR-320a is regulated by methylation of its promoter.

We then used DAC to hypomethylate DNA, and this abolished most of the promoter methylation of miR-320a (Supplemental Fig. 3). The expression of miR-320a, TRPC5, and NFATC3 were then examined; when miR-320a was hypomethylated, its expression increased significantly (Fig. 3C) while the expression of TRPC5 and NFATC3 decreased (Fig. 3D).

**Down-Regulation of miR-320a Is Associated with ETS-1 Hypermethylation in Chemoresistant Cells.** A recent report showed that miR-320a expression is suppressed...
by the proto-oncogenic transcription factor ETS-1 (v-ets erythroblastosis virus E26 oncogene homolog 1) (Tang et al., 2012). Based on that work, we analyzed the activity of ETS-1 in chemoresistant cells. First, consistent with the previous work, we confirmed that the promoter and 5' UTR region of miR-320a were able to induce luciferase activity in pGL3 plasmid. However, overexpression of ETS-1 significantly suppressed the luciferase activity (Fig. 4A). Then we analyzed the ETS-1 expression in MCF-7 cells and found significantly increased expression in chemoresistant MCF-7/ADM cells compared with MCF-7/WT cells (Fig. 4, B–D). The increased ETS-1 was found to aggregate within the nucleus (Fig. 4D), where most of the transcription factors reside. Then, we analyzed the binding activity of ETS-1 on the promoter and 5' UTR of miR-320a by chromatin immunoprecipitation assay in MCF-7/ADM cells. Three theoretical binding sites were found by Jaspar online software (Supplemental File 4), and we confirmed that site 2 (S2) was the real binding site in MCF-7/ADM cells because the ETS-1 antibody was able to pull down the DNA sequence in S2, while preimmune IgG antibody (negative control) failed to pull down this part of the DNA (Fig. 4E). On the other hand, sites 1 and 3 (S1 and S3) did not bind to ETS-1 in MCF-7/ADM cells, because although the PCR product showed the correct sizes of S1 and S3 when the binding sites were pulled down by ETS-1 antibody, the negative control also showed the same bands at the same sizes, indicating that these results are false positives. Taken together, these results suggest that ETS-1 is elevated and activated to regulate miR-320a in MCF-7/ADM cells.

We then studied the methylation of ETS-1 using BSP in chemoresistant cells. As shown by the CpG plot, a CpGis(230 bp) was found immediately upstream of the ETS-1 coding sequence (Supplemental File 3). Because this CpGis contained relatively more bases, nested BSP was applied to enhance the quality of the PCR. We found that the promoter of ETS-1 was highly methylated in MCF-7/WT cells, but strong demethylation occurred in MCF-7/ADM cells (Fig. 4F). Therefore, these data suggest that the overexpression of ETS-1 is regulated by promoter hypomethylation in MCF-7/ADM cells.

**Effect of MiR-320a in Chemoresistance of Other Cancer Cells.** We then tested our findings in different chemoresistant...
cell lines. PTX-resistant MCF-7 cells (MCF-7/PTX) and 5-FU–resistant HCT-8 cells (HCT-8/5-FU) were tested for miR-320a, TRPC5, and NFATC3 expression. The miR-320a expression was significantly decreased, but TRPC5 and NFATC3 were increased in both MCF-7/PTX and HCT-8/5-FU cells compared with their parental controls (MCF-7/WT and HCT-8/WT) (Fig. 5 A, a–c). At the same time, restoring the activity of miR-320a markedly decreased the chemoresistance in MCF-7/PTX cells (Fig. 5B) but only slightly decreased in HCT-8/5-FU cells.

**Low miR-320a Expression Is Associated with Clinical Chemoresistance and Predicts Outcomes of Breast Cancers.** The activity of miR-320a was then tested in clinical samples of breast tumors from patients receiving anthracycline-taxane–based neoadjuvant chemotherapy. Tumors with a reduction in size <10% in response to this therapy were considered to be chemoresistant (termed nonresponders). Postchemotherapy samples from nonresponders and responders were compared with identify the miR-320a expression changes in chemoresistance and chemosensitivity. We found that miR-320a expression was significantly decreased in nonresponders (i.e., chemoresistant samples). At the same time, the expression of TRPC5, NFATC3, and ETS-1 was significantly increased in nonresponders (Fig. 6 and Table 1), and the expression of miR-320a was significantly and negatively associated with the expression of TRPC5, NFATC3, and ETS-1 when analyzed by the Spearman correlation coefficient. Therefore, our data implicated miR-320a, TRPC5, NFATC3, and ETS-1 in clinical chemoresistance.

We later analyzed the predictive role of miR-320a in clinical samples by analyzing the distant relapse-free survival (DRFS) from the miRNA profiling data of Buffa et al. (2011) (NCBI accession number: GSE22216) involving 210 patients with 10-years of follow-up observation of clinical and demographic information. The samples were obtained at surgery followed by adjuvant chemotherapy and/or adjuvant hormone
therapy. The patients were divided into estrogen receptor (ER) negative (ER−) and ER positive (ER+) groups (patients aged >50 years were excluded as they were considered as a single group and treated with different regimens as indicated by Buffa et al.). The association of miR-320a with DRFS was analyzed by use of Kaplan-Meier. In the ER− groups (Fig. 7A), the miR-320a failed to predict the DRFS. By contrast, in the ER+ groups, miR-320a displayed clinical relevance for patient survival (Fig. 7B). We also tested the how well miR-320a predicted DRFS by measuring the AUC in the same data set of Buffa et al. A significant AUC for prediction of response to DRFS was found for miR-320a in the ER+ group (Fig. 7C).

Later, the predictive power of miR-320a was verified in the studies of Lyng et al. (2012) that included three miRNA profiling data sets for ER+ breast cancer patients (NCBI accession number: GSE37405); one of these data sets was abandoned because it lacked the detection of miR-320a. In the other two data sets (n = 40 and n = 51), the investigators indicated that the latter data set contained 52 individuals, but only 51 were found in the uploaded files, all patients received adjuvant tamoxifen as the only therapy, and the DFRS was recorded. In both groups, miR-320a was still able to predict the distant recurrence ratio (Fig. 7, D and E).

**Discussion**

MicroRNAs have attracted great interest from oncologists due to their versatile ability to regulate nearly every critical aspect of carcinogenesis: proliferation/apoptosis (He et al., 2007), motility (Tavazoie et al., 2008), angiogenesis (Dews et al., 2006), and the microenvironment (Soon and Kiaris, 2013) of cancer cells. Compared with targeting one molecule, interrupting one or more of the above signaling networks could be more effective in overcoming cancer (Hanahan and Weinberg, 2011). Because a single miRNA can target different genes that may intensively participate the whole process of developing a certain cancer property (Krek et al., 2005; Rajewsky, 2006), miRNA provides a promising therapeutic strategy in cancer treatment.

MicroRNA-320a has shown this potential to treat cancer chemoresistance. To date, only a few studies have implicated miR-320a in cancers (Tang et al., 2012; Wee et al., 2012), but the mechanism of action was unclear. Here, we not only showed that dysregulation of miR-320a was involved in the chemoresistance of cancer cells, but also demonstrated that it regulated the critical TRPC5-NFATC3 network during the developing of chemoresistance.

Previously, we showed that the effect of high TRPC5 expression on NFATC3 is to activate a calcium signaling pathway, which triggers the translocation of NFATC3 into the nucleus. The translocated NFATC3 binds to the promoter of the ABCB1 gene and induces ABCB1 expression. However, TRPC5 does not directly contribute to the high expression of NFATC3. Until this study, we were able to explain the reason for the high expression of TRPC5 and NFATC3—that is, they are both regulated by miR-320a. This is not the first miRNA that has been found to regulate several essential factors in one signaling network. The miR-200 family has been found to regulate the epithelial mesenchymal transition (EMT) of cancer cells by targeting modulators of the EMT such as ZEB1, ZEB2, and SIP1 (Peter, 2009); restoring the activity of miR-200 reverses the EMT and EMT-related chemoresistance (Li et al., 2009). Therefore, our studies and those of others suggest that focusing on one signaling pathway is a good way to explore and understand the synergic regulation of miRNA.

In breast cancers, miR-320a is generally down-regulated, and it is reverse-regulated by activation of ETS-1 (Tang et al., 2012; Wee et al., 2012); our study further explained how methylation is involved in miR-320a down-regulation and ETS-1 up-regulation, especially in chemoresistant breast cancer cells. Previously, Wee et al. (2012) analyzed the methylation status of miR-320a in nine breast cancer cell lines, and argued that its down-regulation in these cells was independent of methylation. However, we not only found, for the first time, the effect of miR-320a in chemoresistant cancer cells but also that miR-320a can be regulated by methylation. The difference between our results and those of others may be due to the differences in the

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**Fig. 5.** Expression of miR-320a in other chemoresistant cancer cells. (A) (a) miR-320a was down-regulated, while (b) TRPC5 and (c) NFATC3 were up-regulated in paclitaxel-resistant MCF-7/PTX cells and fluorouracil-resistant HCT-8/5-FU cells as measured by RT-PCR. (B) miR-320a–mediated chemoresistance in MCF-7/PTX and HCT-8/5-FU cells was analyzed by MTT. **P < 0.01 versus chemo-sensitive MCF-7/WT and MCF-7/WT cells. *P < 0.05 versus untransfected with miR-320a mimic.**
regulatory systems of chemoresistant and normal cancer cells, suggesting that methylation of the miR-320a coding sequence is an effective signature to distinguish chemoresistant from chemosensitive cancer cells. In addition, we noted that Wee et al. (2012) only characterized the promoter of miR-320a by luciferase reporter assay; by combining 5'-RACE with luciferase assays, we were able to locate the promoter and 5'-UTR more precisely. These regions covered more than 2000 bp upstream of miR-320a and included three main CpGis, one of which was newly identified by us and shown to be hypermethylated in chemoresistant cancer cells.

After DAC globally hypomethylated the MCF-7/ADM cells, the chemoresistant characters of the cells, including down-regulation of miR-320a as well as up-regulation of TRPC5 and

**Fig. 6.** Dysregulation of miR-320 (original magnification 63×, oil immersion), TRPC5 (100×), NFATC3 (63×, oil immersion), and ETS-1 (63×, oil immersion) was analyzed in postchemotherapeutic samples (n = 8) from patients receiving anthracycline-taxane–based neoadjuvant chemotherapy. The sections were stained with DAPI (blue) to identify the nucleus. The miR-320a was then detected by fluorescence in situ hybridization with red fluorescence; TRPC5, NFATC3, and ETS-1 were detected by immunohistochemical staining with N,N-dimethyl-4-aminoazobenzene (DAB) and green fluorescence, respectively.

### TABLE 1
Statistical summary of the expression of miR-320a, TRPC5, NFATC3, and ETS-1 in clinical samples

<table>
<thead>
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<th>Expression score</th>
<th>miR-320a</th>
<th>TRPC5</th>
<th>NFATC3</th>
<th>ETS-1</th>
</tr>
</thead>
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<tr>
<td>Responder (n = 4)</td>
<td>4.00 ± 0.70</td>
<td>4.25 ± 0.62</td>
<td>4.75 ± 0.71</td>
<td>3.16 ± 0.44</td>
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<td>Nonresponder (n = 4)</td>
<td>1.98 ± 0.28</td>
<td>8.22 ± 0.90</td>
<td>7.23 ± 0.70</td>
<td>5.48 ± 0.49</td>
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<td>P value</td>
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<td>Correlation versus miR-320a</td>
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<td>−0.77</td>
<td>−0.78</td>
<td>−0.72</td>
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NFATC3, were reversed, confirming that methylation is involved in miR-320a regulation as well as confirming the relationship between miR-320a and TRPC5/NFATC3. It should be noted that because there is no effective way to specifically demethylate the promoter of miR-320a, we cannot exclude the influence of other methylation-regulated factors that could regulate miR-320a at the same time. Therefore, based on the current results, we still cannot draw an explicit conclusion that promoter methylation is the cause of miR-320a down-regulation, and can only conclude that the methylation is involved in regulating miR-320a.

The changes of TRPC5 and NFATC3 tended to be greater than miR-320a, suggesting that other factors controlled by methylation also regulate TRPC5 and NFATC3, and this needs further study. It should be noted that DAC treatment did not dramatically (but significantly) restore the expression of miR-320a because a small amount was still detectable in MCF-7/ADM cells. However, neither of these factors completely inhibited miR-320a because a small amount was still detectable in MCF-7/ADM cells (Fig. 2A). Therefore, both methylation and ETS-1 only partially and independently regulate miR-320a expression, and deletion of methylation partially diminishes the inhibition of miR-320a.

These data also demonstrate how the opposite status of DNA methylation contributed to the development of chemoresistance in the same population of cells. The complicated mechanism of regulation by methylation is triggered in tumor cells when they confront a disadvantageous environment and is determined by the pressure of selection. Genes that inhibit tumor progression are suppressed by methylation, while those that promote proliferation are highly activated. Finally, the most favorable changes are selected and MCF-7/ADM cells develop from MCF-7/WT cells, making them no longer sensitive to ADM.
Methylation-based therapies with DAC have already been approved by the U.S. Food and Drug Administration to treat myelodysplastic syndromes and chronic myelomonocytic leukemia (Kaminskas et al., 2005; Steensma, 2009). However, DAC is a nonspecific agent, so global hypomethylation by DAC may trigger tumor progression (Gius et al., 2004). Furthermore, appropriate techniques to detect DNA methylation-based biomarkers clinically are lacking. On the other hand, specific interfering miRNAs are relatively easily realized by miRNA mimics or inhibitors, and fluorescence in situ hybridization can effectively detect miRNA markers on small tumor sections. Here, we also demonstrated an easy and effective way of using miRNA tools to detect and inhibit chemoresistance, suggesting good potential for the development of miRNA-based therapies.

When the miR-320a-mediated chemoresistance was examined in PTX-resistant MCF-7 cells and 5-FU-resistant HCT-8 cells, we found that although miR-320a was down-regulated in both cell lines, it only significantly modulated chemoresistance in breast cancer cells, suggesting that a role of miR-320a in chemoresistance may be found more frequently in breast cancers. The hypothesis was then supported by the clinical studies, in which chemoresistant breast cancers were accompanied by low miR-320a expression.

Neoadjuvant chemotherapy samples are good sources of chemoresistant/sensitive cancer cells; tumors that do not respond to chemotherapy (nonresponders) are defined as chemoresistant and vice versa. In this study, we confirmed the high expression of TRPC5 and NFATC3 and low expression of miR-320a in nonresponders to two-agent neoadjuvant chemotherapy, suggesting that miR-320a is a common factor hijacked by cancer cells to induce chemoresistance regardless of the type and number of chemotherapeutic drugs.

Chemoresistant cancer cells have greater opportunities for relapse because they may gain features of stem/mesenchymal cells that easily metastasize (Polyak and Weinberg, 2009). Indeed, by mediating chemoresistance, miR-320a also showed its clinical relevance and predictive power on DRFS in ER+ breast cancer patients, but not in ER− patients who by nature have a poor prognosis (Putti et al., 2005); higher miR-320a expression in ER− patients seems not to reverse their malignancy. However, ER+ breast cancer patients have lower risk of recurrence and a better 10-year survival ratio, but 30–50% develop resistance to adjuvant tamoxifen or other chemotherapies through mechanisms that remain largely unclear (Early Breast Cancer Trialists Collaborative Group studies). Based on our study, we suggest that low expression of miR-320a in ER+ patients contributes to their drug resistance, and acts as an unfavorable breast cancer prognostic marker for DRFS or recurrence-free ratio.

In Fig. 8, we provide a schematic of the regulatory network of miR-320a. Hypermethylation and hypomethylation of the promoters of miR-320a and ETS1 respectively suppress miR-320a expression in chemoresistant cancer cells, thus increasing the expression of TRPC5 and NFATC3, which are targeted by miR-320a and essential for P-gp-induced chemoresistance. Our study provides potential therapeutic targets for the suppression of chemoresistance in breast cancers.

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Authorship Contributions

Participated in research design: He, Ma, Jin.
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