Tumor Evolution Inferred by Patterns of microRNA Expression through the Course of Disease, Therapy and Recurrence in Breast Cancer

1Dadiani Maya, 5*Bossel Ben-Moshe Noa, 2Paluch-Shimon Shani, 3Perry Gili, 3Balint Nora, 3Marin Irina, 3Pavlovski Anya, 1Morzayev Dana, 1Kahana-Edwin Smadar, 3Yosepovich Ady, 2Gal-Yam Einav Nili, 2Raanan Berger, 3,4Barshack Iris, 5Demony Eytan and 2,4Kaufman Bella.

1Sheba Medical Center, Cancer Research Center, Tel-Hashomer, Israel; 2Sheba Medical Center, Oncology Institute, Tel-Hashomer, Israel; 3Sheba Medical Center, Pathology Institute, Tel-Hashomer, Israel; 4Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel; 5Department of Physics of Complex Systems, Weizmann Institute of Science, Rehovot, Israel

* Equal contribution

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Corresponding author: Maya Dadiani; Chaim Sheba Medical Center, Cancer Research Center; Tel: 972-3-5307132 Fax: 972-3-5307522; email: Maya.dadiani@sheba.health.gov.il;

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Defining the evolutionary molecular path of breast cancer in each patient is challenging, as tumors are rarely diagnosed at their initial stages of development. Alternatively, to investigate how individual breast cancer disease evolves over time we can analyze the molecular changes in tumors at different stages of progression for the same patient. In this study we profiled miRNA expression of serial samples that represent the disease progression stages of breast cancer patients, from diagnosis through therapy until recurrence. Acquisition of such a dataset, accompanied by clinical data, has the potential for the development of predictive and prognostic signatures.

ABSTRACT

**Purpose**: Molecular evolution of tumors during progression, therapy and metastasis is a major clinical challenge and the main reason for resistance to therapy. We hypothesized that miRNAs that exhibit similar variation of expression through the course of disease in several patients have a significant function in the tumorigenic process.

**Experimental design**: Exploration of evolving disease by profiling 800 microRNAs (miRNAs) expression from serial samples of individual breast cancer patients at several time points: pre-treatment, post-treatment, lymph nodes and recurrence sites when available (58 unique samples from 19 patients). Using a dynamic approach for analysis, we identified expression modulation patterns and classified varying miRNAs into one of the 8 possible temporal expression patterns.

**Results**: The various patterns were found to be associated with different tumorigenic pathways. The dominant pattern identified a miRNA set that significantly differentiated between disease stages and its pattern in each patient was also associated with response to therapy. These miRNAs were related to tumor proliferation and to the cell-cycle pathway and their mRNA targets showed anti-correlated expression. Interestingly, the level of these miRNAs was lowest in matched recurrent samples from distant metastasis, indicating a gradual increase in proliferative potential through the course of disease. Finally, the average expression level of these miRNAs in the pre-treatment biopsy was significantly different comparing patients experiencing recurrence to recurrence free patients.

**Conclusion**: Serial tumor sampling combined with analysis of temporal expression patterns enabled to pinpoint significant signatures characterizing breast cancer progression, associated with response to therapy and with risk of recurrence.
INTRODUCTION

One of the main difficulties hindering advances in breast cancer treatment is the dynamic molecular evolution of tumors across the course of disease. Tumors may evolve in time during tumor progression and therapy, as well as in space, across different tumor clones and within metastases (1). The dynamics of tumor evolution is individual for each patient, shaped by intrinsic genetic factors along with extrinsic selective forces, such as cancer therapy. Most comprehensive studies, aimed at profiling the molecular players involved in breast cancer, were based on the primary tumor sample. However, the molecular evolution toward recurrence is a key to understanding the processes leading to metastases.

Ideally, to understand tumor evolution at the individual patient level, analysis of molecular changes in tumors at different progression stages for the same patient should be performed. This approach of serial assessments requires long-term maintenance of clinical databases and availability of biological samples along this timeline. Neo-adjuvant therapy (preoperative therapy), is an ideal setting for this purpose as we can compare samples at diagnosis (prior to therapy), and at surgery (following therapy). Neo-adjuvant systemic therapy is currently an accepted standard approach for women with locally advanced breast cancer and has been shown to reduce tumor size and allow for breast conserving surgery. Neo-adjuvant therapy can provide prognostic information and may also provide in vivo assessment of tumor sensitivity to therapy (2),(3). While some patients exhibit pathological complete response (pCR) with complete disappearance of the tumor, others respond only partially, or do not respond at all (4). Although pCR is an important prognostic factor, the risk of recurrence is not always in full concordance with it. The physician predicts the risk of recurrence based solely on statistics, taking into account the pathological response, with no considerations of molecular modulations. The treatment stage is considered as a “black box” for each individual patient and our knowledge and understanding of the molecular processes and changes that occur in cancer cells in response to treatment is very limited.

As longitudinal sampling can span several years over which preservation of archived tissues may vary, it is important to profile stable molecules, such as miRNAs, to obtain reliable information. miRNAs are a highly conserved group of short noncoding RNAs (~22bp) that play a major role in post-translational regulation of gene expression and are critical regulators of oncogenic pathways (5). miRNAs have been widely associated with breast cancer tumorigenesis (6), as well as with prognosis and response to treatment in breast cancer (7,8). A recent study by Dvinge et al. (9) describes the landscape of miRNAs expression in 1302 primary breast tumors accompanied by matched clinical information and genomic and transcriptomic data (10). This important dataset provides comprehensive information on the function of miRNA in breast cancer regulation. Nevertheless, analyzing miRNA modulations from serial samples prior to therapy, following therapy and at recurrence may highlight important molecular mechanisms that may be underestimated when analyzing only primary tumors.
In this study, we applied a longitudinal approach to identify expression signatures characterizing individual breast cancer progression. The analysis approach was to classify temporal modulation patterns observed through the course of disease. We hypothesized that expression modulations in individual patients throughout the course of their disease can directly identify miRNA sets that are differentially expressed at the various stages of the disease and are associated with disease progression or response to therapy and may have prognostic value.
MATERIALS AND METHODS

Tumor specimen collection. A prospectively maintained database that contains clinical data on ~600 cases who have undergone neo-adjuvant treatment at the Sheba Medical Center from 2003 was screened. Importantly, all patients received a uniform treatment: combination of doxorubicin, cyclophosphamide and taxane. The database was analyzed to select a cohort of patients having maximal available FFPE samples throughout the course of disease at the pathological archive (tumor pre-treatment (C), lymph-node pre-treatment (CL), tumor post-treatment (T), lymph node post-treatment (TL) and recurrence (R)) (Figure 1A). The main cohort consists of 10 patients with disease recurrence, of them, 8 /10 patients had samples from all three time points ( C, T and R) and 2 patients showing pCR (C and R only)(Figure 1B). A second independent cohort consists of 9 patients who remained disease-free (C and T samples), median follow-up 10 years (Figure S1). Lymph node samples for both cohorts were analyzed when available. For control normal breast, we utilized tumor adjacent normal epithelium for 3 patients as well as specimens from breast reduction (n=5). This study was approved by the Institutional Review Board (IRB).

Tissue processing and histology. All specimens were stored as Formalin-Fixed Paraffin-Embedded (FFPE) blocks at the Sheba Pathology department archive. Tissue slides were examined by expert breast pathologists to include a minimum of 70% cancer cells, otherwise were macro-dissected to eliminate contamination of stromal components. The extent of pathological response was evaluated by a pathologist, examining the residual tumor burden based on reduction in tumor cellularity, similar to Ogston et al. (Miller and Payne grade) (11). In addition, tumor and lymph node slides were evaluated to calculate the RCB class, as defined by Symmans, et al. (12)(Table S1).

Each tumor sample was sectioned and Total RNA was extracted using nucleic acid isolation kit (AllPrep® Qiagen).

Immunohistochemistry. Slides were immunostained for Ki67 on the Ventana Discovery autostainer (Ventana) using commercial Ki67 antibody (clone SP6, Thermo Scientific). Ki67 levels were assessed blinded to the clinical data using the automated Virtuoso image analysis algorithm (Ventana). Several representative regions of the tumor bed were selected for counting a total of at least 1000 tumor cells. The percentage of Ki67-positive cells to the total number of evaluated cells was calculated.

miRNA expression profiling. miRNA expression was assayed by NanoString nCounter® digital RNA transcript counting that assays 800 miRNAs. 58 unique samples were profiled for 19 patients. In order to estimate the technical and the biological noise, we profiled 3 technical and 3 biological repeats (consecutive sections from the same tumor sample).
Validation of the microarray results by qRT-PCR. The results were validated by qRT-PCR for 21 candidate miRNAs. We found a very good agreement between the nCounter® analysis and the qRT-PCR (Figure S2). cDNA generated from purified total RNA (100ng) by miScript Reverse Transcription Kit was quantified by qRT-PCR using miScript SYBR Green PCR Kit (Qiagen), using commercial primers (Qiagen). Reactions were performed in duplicates. RNU6 was used for normalizations. The relative expression levels were quantified using the 2^{-DDCt} method.

qRT-PCR of targeted genes. Primers for BUB1B and CDC25A were designed using Primer Express® and were commercially synthesized (Biosearch). All genes were normalized by three endogenous control genes: GAPDH, TBP and HPRT1. cDNA was synthesized from total RNA by qScript cDNA Synthesis Kit (Quanta Biosciences). To receive reliable results of mRNA from FFPE samples preserved across several years, we amplified the cDNA before the qPCR reactions using PerfeCTa PreAmp SuperMix (Quanta). (See Supp. Methods).

Data analysis. See Supp. Methods for full description; in brief, the data was normalized using a Lowess multi-array algorithm (13). Linear mixed effect model was used to identify miRNAs significantly different across the course of disease. These miRNAs were classified to the 8 varying expression patterns (Figure 1C) between C, T, R samples based on two thresholds: (1) Call increase/decrease for fold change ≥ 1.5 and (2) pattern observed in at least 3 patients (Figure 1D). Differential expression of the identified miRNAs in tumors vs. normal samples or high-grade vs. low-grade tumors was calculated on the METABRIC dataset (9). The CoSMic algorithm (14) with the METABRIC miRNA and mRNA expression data (10) (9) were used to identify targets for our 14 P1 candidate miRNAs. We applied the CoSMic algorithm with miRanda (15), TargetScan (16) or PITA (17) sequence-based prediction algorithms.

Pathway analysis was performed using the Pathifier (18) algorithm which infers a pathway deregulation score for each tumor sample and pathway, on the basis of mRNA expression data. We correlated the expression levels of our candidate miRNAs with the pathway deregulation scores of all KEGG pathways (19), calculated using Pathifier on the METABRIC dataset for samples with both mRNA and miRNA expression data (10). Gene Set Enrichment Analysis (GSEA) (20) was used to calculate the enrichment of an EMT-core list (21) in genes that are positively correlated with P5 miRNAs expression levels in the METABRIC dataset.
RESULTS

Identifying temporal miRNA expression patterns through the course of disease

We assembled a unique cohort of patients with all available matched samples through their course of disease: tumor sample before therapy, residual tumor post therapy, lymph nodes and recurrent tumors, to depict molecular modulations for each individual patient (total of 58 unique samples from 19 patients) (Figure 1A-B and S1). Clinical information is summarized in Table 1.

We hypothesized that expression modulations in individual patients through their course of disease and therapy can identify miRNAs that play a dynamic role in cancer progression. We therefore searched for miRNAs which exhibit modulation patterns that are shared between patients. There are 8 possible patterns that describe expression modulations between 3 time points across the course of disease: pre-treatment (C), post-treatment (T) and recurrence (R) (Figure 1C). To classify a particular miRNA to one of these patterns, we first filtered all miRNAs using linear mixed effect model to select only miRNAs that are significantly modulated over time. We then applied a threshold of 1.5 fold to define expression modulation between pairs of time points and considered only patterns that were observed in at least 3 patients (see Methods). For example, miR-125b exhibits pattern P1 in 4 out of 8 patients (having C, T, R samples), and therefore it was assigned to pattern P1 (Figure 1D).

The miRNAs sets classified for each pattern are presented in Figure 2. As can be seen from the patterns table and Figure 1C, the number of miRNAs classified to each pattern varied considerably. We focused on the main 4 patterns (P1, P2, P5 and P6). To investigate the potential involvement of these miRNAs in tumor progression, we examined their expression in an available dataset of miRNAs expression in breast cancer (METABRIC) (9). For example, the most abundant pattern, P1, is mainly composed of miRNAs that are down-regulated in breast tumors relative to normal samples according to the METABRIC dataset, while in pattern P2, which is the inverse complementary pattern to P1, most miRNAs (3 out of 4) are up-regulated in breast tumors (Figure 2). Furthermore, we performed a detailed and comprehensive search in the literature for evidence of the role of these miRNAs in breast cancer (Table S2). Finally, pathway analysis was used to map each miRNA to the most correlated biological pathway in breast cancer (18)(19) (see Methods). The entire information is summarized in Figure 2 and Table S3.

miRNAs in Pattern P1 are correlated with response to treatment

Notably, our method of pattern classification (shared by a minimum of 3 patients) was applied to a heterogeneous cohort of patients with distinct subtypes and diverse courses of disease. Therefore, it is reasonable that distinct groups of miRNAs can be assigned to the same pattern. Indeed, examining
miRNAs in pattern P1 across all patients revealed that pattern P1 is composed of two distinct groups (Figure 2). Most of the miRNAs assigned to P1 (upper P1 panel – 14 miRNAs) followed this pattern mainly in a subset of patients (patients 3, 4, 5 and 9), while few other miRNAs followed pattern P1 in other patients (lower P1 panel; patients 2, 7, 8 and 10). We noticed that the majority of miRNAs in the first P1 group are down-regulated in breast cancer according to the literature and the METABRIC dataset (Figure 2). We therefore redefined P1 miRNAs to include only this subset of 14 miRNAs as a more coherent group that exhibit the P1 pattern across the same group of patients and are down-regulated in breast tumors.

Next, we calculated the average expression levels of P1 miRNAs in each sample (defined as P1 score), and presented it for each patient individually (Figure 3A). Examining P1 scores of each patient across the course of disease (Figure 3B) revealed that P1 miRNAs show distinct patterns in different patients. We suspected that this can be associated with the extent of response to therapy. Therefore, we independently scored the level of pathological response to treatment by examining the reduction in tumor cellularity for each patient, by a blinded pathological examination (Miller and Payne –MP grade) (11) (Table S1). Surprisingly, there was full agreement between the expression pattern of P1 miRNAs and the pathological response to therapy (Figure 3B). Patients with moderate response to treatment (MP grade=3) showed an increase in P1 miRNAs post-treatment, as opposed to patients with minor (MP=2) or no response (MP=1). The log ratios of P1 between C and T were significantly different among the various response rates (ANOVA test, p=0.011), suggesting an association of P1 score with response to treatment.

**miRNAs in Pattern P1 are differentially expressed across the various disease stages**

Remarkably, for most patients the expression levels of P1 miRNAs were lower in the recurrence tissue than in the primary tumor at diagnosis (Figure 3A-B). This led us to speculate that P1 miRNAs are indicative of disease progression. We compared P1 scores of all patients across the course of disease (Figure 3C). Interestingly, there was a significant difference in the absolute values of P1 scores across the entire cohort. We found a significant difference between P1 scores of primary tumors before and after treatment and between post-treatment tumors and recurrent tissues (Figure 3C, p=0.019 and 1.1x10^{-5}, respectively). Moreover, P1 scores at recurrence were significantly lower than at all other disease stages. All the recurrence samples had low P1 scores, independently of tumor subtype or metastatic niche. Thus, P1 miRNAs also differentiate between the stages of the disease, and are associated with disease progression.

To find out to what extent these miRNAs return to “normal” expression levels post-therapy, we quantified the expression levels of two miRNAs assigned to pattern P1 (miR-125b-5p and miR-100-5p) in normal breast tissue, either tumor adjacent normal epithelium or normal epithelium from healthy patients. Interestingly, although tumors exhibit higher post-treatment than pre-treatment expression levels, the expression level of adjacent normal tissues post-treatment was higher (Figure S3).
Importantly, expression levels of normal breast from healthy patients (breast reduction, n=5), although varied, were similar to the levels of normal breast tissue post treatment (no statistical difference between the expression of normal breast epithelium of healthy individuals versus normal epithelium post-treatment; Figure S3).

Next, we investigated P1 scores of involved axillary lymph nodes before and after treatment. We found that P1 scores were not significantly different between lymph nodes before and after treatment (p=0.47; Figure 3C), in contrast to the significant difference observed in the primary tumors before and after therapy. While there was no significant difference between primary tumors and lymph nodes before treatment (C vs. CL; p=0.88), the average expression levels were significantly lower in lymph nodes post-treatment compared to primary tumors post-treatment (T vs. TL; p=0.04) (Figure 3C). Examination of individual patient's profile of miRNAs from pattern P1 across their entire samples (Figure 3A) emphasizes these results, showing similar expression levels in the lymph nodes before and after treatment, suggesting reduced chemo-sensitivity in lymph nodes.

### miRNAs in Pattern P1 are associated with proliferation

As shown in Table S2, the down-regulation of P1 miRNAs is related to higher proliferation and invasion of breast cancer. Furthermore, by pathway analysis we found that P1 miRNAs are significantly negatively correlated to the cell cycle pathway and to other proliferative pathways such as the MAPK and JAK-STAT signaling pathways (1% FDR; see Methods and Figure 2). We further quantified miR-10b* (not included in the NanoString panel), which is a known inhibitor of the cell cycle in breast cancer cells (22) by qRT-PCR; its expression modulation exhibited a strong P1 pattern in patients 3 and 9 (Figure S4).

Next, we searched for putative target genes, which are connected to the cell cycle and to proliferative pathways, and are targeted by several miRNAs from the P1 group. We used CoSMic algorithm (14) that predicts context-specific target genes of a miRNA, utilizing sequence-based prediction scores as well as mRNA and miRNA expression data measured from the same samples (METABRIC). We identified several genes that are related to the cell cycle (8 genes), MAPK (10 genes) and JAK/STAT (6 Genes) pathways and are predicted targets of several miRNAs from P1 (Table S4). In addition, RAF1, which is an upstream activator of MAPK pathway is a predicted target of miR-125b-5p (Table S4).

We checked the expression levels of two candidates, BUB1B and CDC25A, known for their role in breast cancer progression (23,24). BUB1B is a kinase essential for the mitotic checkpoint and required for normal mitosis and a putative target of let-7c, miR-199a-3p, miR-199a-5p and miR-125b-5p. CDC25A is a phosphatase required for the progression from G1 to the S phase and is a putative target of miR-100-5p and miR-145-5p (Figure S5A). We quantified the mRNA levels of these genes by qPCR, specifically calibrated for quantification of mRNAs from archived samples. Expression levels of BUB1B and CDC25A were measured in several patients and were found to be inversely expressed to their
predicted regulatory miRNAs (Figure 4A-B). At recurrence, their levels were only partially inverted to the miRNAs levels (Figure S5B-C).

To further support the association of P1 miRNAs with proliferation we measured the level of the Ki67 proliferation marker in our cohort by immunostaining. Ki67 levels significantly varied between patients, mainly in pre-treatment samples and in recurrent samples (Figure 4C-D). Ki67 levels post-treatment were always lower compared to pre-treatment. We found a general agreement between P1 pattern in each patient and the modulations in Ki67 levels over time for individual patients (Figure 4E), and there was an overall moderate anti-correlation between P1 scores and Ki67 levels for all samples (cc=-0.26; Figure S6). However, P1 scores better differentiated between samples across disease progression, were associated with response to treatment and were less variable across all patients than Ki67 levels (Figure 3C). Importantly, in recurrence samples the average P1 scores were the lowest, whereas the average Ki67 levels were not the highest, and exhibited high variability.

Overall, the above observations suggest that P1 miRNAs are inhibitors of the cell cycle in breast cancer cells, and are highly informative regarding the proliferative state of the tumor.

**P1 miRNAs differentiate between recurrence and recurrence-free patients**

The observations that the different stages of the disease are significantly different on their average P1 miRNAs expression levels, and that the average expression levels at recurrence are the lowest, indicates that this set of miRNAs may have a prognostic value. We calculated the average expression levels of P1 miRNAs for an independent cohort of patients that did not experience recurrence for at least 10 years. We found significantly lower expression levels of P1 miRNAs in pre-treatment tumors of patients with recurrence compared to the recurrence free patients (p=0.03; Figure 5A). Lower P1 miRNAs expression levels were observed also for the post-treatment tumors and lymph nodes of recurrent patients, but the differences were not significant. Importantly, there was no significant difference between RCB classes of both cohorts (Table S1). Moreover, Kaplan-Meier and COX proportional hazard model identified significant association between high P1 pre-treatment expression levels and better recurrence-free survival (p=0.016 and p=0.045, respectively; Figure 5B).

In addition, we tested the prognostic value of P1 miRNAs on the METABRIC dataset. Interestingly, P1 scores are significantly lower in patients who developed metastasis, relative to metastasis-free patients (p=0.001; Figure S7A); and higher levels of P1 scores are significantly associated with better disease-specific survival (p=5.9x10^{-4}; Figure S7B).

Since pattern P2 is the inverse complementary pattern to P1, and contains miRNAs that are up-regulated in breast tumors relative to normal samples, we calculated also the average expression levels of P2 miRNAs. As can be seen from Figure S8A, although the pre-treatment expression does not significantly differ between patients with and without recurrence, post-treatment expression levels in the tumor and lymph nodes are significantly different between patients with and without recurrent disease (p=0.001 and p=0.023, respectively). Moreover, Kaplan-Meier and COX analysis identified...
significant association between low expression levels of P2 post-treatment and better recurrence-free survival (p=0.0066 and p=0.0175, respectively; Figure S8B).

Importantly, since the recurrence-free group consisted of hormone-positive patients only, we further analyzed P1 and P2 using solely hormone positive patients from both cohorts. We found that P1 scores are still lower in patients with recurrence relative to recurrence-free patients (Figure S9A); although the result was not significant, probably due to the small sample size (7 vs. 9 patients). P2 was significant despite the small sample size, both in the post-treatment tumor samples and the post-treatment lymph node samples. Kaplan-Meier analyses performed on the hormone positive patients alone (16 patients) were significant both for P1 pre-treatment expression levels and for P2 post-treatment expression levels (Figure S9B).

Similarly, Kaplan-Meier analysis by P1 scores on the METABRIC dataset was also significant when taking into account only hormone positive samples (Figure S9C).

Thus, in our dataset P1 and P2 miRNAs expression levels pre- and post-treatment can significantly differentiate between patients experiencing recurrence and recurrence-free patients.

**miRNAs in patterns P5 and P6 are associated with the metastatic process**

miRNAs from patterns P5 and P6 exhibited a considerable modulation mainly at recurrence and not between pre- to post-treatment, which hints at their potential function during the invasive process. Importantly, these patterns were observed independently of the metastatic site (e.g. bone, lung etc.). Most P5 miRNAs, defined as low at recurrence, are associated in the literature with metastasis in their down-regulated state (Figure 2 and Table S2). Interestingly, some P5 miRNAs were up-regulated in primary breast cancer relative to normal breast but down-regulated in high grade tumors according to the METABRIC dataset (Figure 2). This opposite expression in primary and advanced tumors indicates a dual function at different stages of cancer. These miRNAs were found to be correlated with O-glycan biosynthesis; O-glycosylation has an important functional role in epithelial to mesenchymal (EMT) induction (25,26). In line with these results, we found that genes that are positively correlated with P5 miRNAs are enriched by genes that are up-regulated during EMT (21) (Figure S10).

As most available studies are performed on primary tumors, we found less evidence for the miRNAs of pattern P6 playing any potential role in cancer. Similarly, in the METABRIC dataset, most miRNAs from this pattern (which is characterized by miRNAs that are over-expressed specifically in the recurrent tissue), are not expressed at all.

Next, we checked the mean expression levels of P5 and P6 miRNAs across the course of disease. Interestingly, the mean expression levels of P5 miRNAs in the lymph nodes post treatment (TL) were decreased towards their expression in the recurrent tissue (Figure S11A). We observed the same trend also for P6 miRNAs, where the expression levels in the TL were increased towards their expression in the recurrent tissue (T vs. TL p=0.01; Figure S11B). This observation might hint that the lymph nodes post-treatment already acquired some invasive characteristics.
DISCUSSION

In this study we profiled miRNA expression of serial samples that represent the disease progression stages of breast cancer patients, from diagnosis through therapy till recurrence. The neo-adjuvant setting is an ideal platform for such a study, enabling the collection of matched pre-treatment biopsies and post-treatment residual tumors. Little is known about the evolution of solid tumors in individual patients, as assembly of such a longitudinal cohort that spans several years till recurrence requires comprehensive analysis of clinical databases and exploration of pathological archives. To our knowledge, no comprehensive temporal analysis that includes matched samples also from distant metastasis for several patients has been performed. The long timespan across the different disease progression stages can result in large variation in archived samples preserved at various years. In this case, miRNAs profiling is currently the most reliable method for such analysis, as miRNA molecules are very stable.

Our approach for analysis of the longitudinal dataset utilizes modulation patterns instead of comparing two time-points. Theoretically, each pattern can be associated with a particular scenario in tumor progression such as proliferation or invasion. Interestingly, some patterns were more abundant, hinting that some scenarios are more likely than others. Only few miRNAs were classified to patterns P3, P4, P7 and P8, thus these patterns are either not abundant (i.e. observed only for very few miRNAs) or are specific to too few patients.

P1, the most abundant pattern, exhibited an increase in expression post-therapy, followed by a decrease at recurrence. The current paradigm is that the highly proliferative cells from the bulk of tumor are eliminated at chemotherapy and the residual tumor is composed of dormant and/or chemo-resistant cells with low-proliferation rate. Since P1 miRNAs are associated with cell proliferation, this pattern can be considered as a “normalization pattern”, in which the overall expression level in the residual tumor approaches normal breast tissue levels. Indeed, the majority of P1 miRNAs are down-regulated in primary breast cancer vs. normal breast tissue. Importantly, we showed that expression levels in response to therapy are increasing towards expression levels of adjacent normal breast tissue but not reaching this level, hinting at the possibility that albeit the fraction of proliferating cells was reduced, they were not entirely eliminated. Moreover, up-regulation of P1 miRNAs following treatment was associated with better response to treatment. We assume that residual tumors of responders will be less proliferative, reflected by increased expression levels of P1 miRNAs, while patients not responding to treatment will not show an increase in P1 miRNAs expression. Moreover, we identified several target genes of P1 miRNAs that are associated with the cells cycle or MAPK pathways. Interestingly, some of P1 miRNAs were recently identified in a miRNA signature associated with hyperactive MAPK and poor outcome (27). Most of these shared miRNAs were correlated to...
MAPK pathway also in our pathway analysis. Several studies comparing pre-treatment to post-treatment samples were previously performed, identifying several genes and pathways that are differentially expressed between pre- and post-treatment samples (28–31). Magbanua et al., analyzed gene expression data of serial breast tumors samples during neo-adjuvant chemotherapy (31). In accordance with our results, they found that most differentially expressed genes are down-regulated following neo-adjuvant chemotherapy and are associated with the cell cycle and with response to treatment.

In the neo-adjuvant treatment, a complete eradication of the tumor (pCR) is a strong predictor of long-term disease-free survival; however, for partial response the prognostic power is very limited. Previous studies indicated that RCB-I class have also a prognostic value (12), however, there was no significant difference between the RCB class of both cohorts. Interestingly, although P1 miRNAs were identified by the pattern dynamics using only the recurrence cohort, there was a significant difference in the average expression levels of the pre-treatment samples between patients experiencing recurrence and recurrence-free patients. Importantly, any attempt to identify miRNAs that significantly differentiate recurrence and recurrence-free patients by standard t-tests were not successful; at 25% FDR none of the miRNAs differentiated recurrence from recurrence-free patients at pre- or post-treatment samples. Our data suggests that low pre-treatment expression levels of P1 miRNAs are indicative of higher probability of a patient to develop a recurrent event, and may have a prognostic value. Kaplan-Meier and COX analysis also supported this conclusion.

As we suggest that P1 miRNAs are a signature for tumor proliferation capacity, it is important to compare the results to Ki67, the gold-standard proliferation marker. The prognostic value of Ki67 score is controversial, due to its high variability and lack of reproducibility (32). Previous neo-adjuvant studies demonstrated that only post-therapy Ki67 is a significant independent prognostic factor (33) (34). Our findings, that the change in Ki67 correlates to P1 pattern, is in accordance with a recent study (35) showing that the change in Ki67 positive cells is an independent predictor of treatment outcomes rather than its absolute level. In contrast, another study found that Ki67 is a significant predictive and prognostic marker in pre-treatment biopsies over a wide range of cut points, but raises the point that the large variability of this marker may impair its usefulness as a prognostic and predictive marker (36). In our dataset post-treatment Ki67 levels were reduced in all patients, but were not correlated to the residual tumor burden as was shown for P1 levels. Ki67 levels were much more variable than P1 values, mainly in the recurrence samples, where P1 score was low in all samples, irrespective of the metastatic niche or subtype, in contrast to the variability observed in Ki67. This suggests that the integrated P1 score is a more informative proliferation marker than Ki67.

Exclusively to our study, the cohort contains an additional time point, at recurrence, enabling a broader analysis of molecular changes over the course of disease. Most studies involve the expression of primary tumors and only few studies compared expression levels between primary tumors and metastasis, mostly LN metastasis, showing a high concordance between primary and metastatic tissue
In contrast to our approach, most studies search for differential expression between the different groups rather than longitudinal analysis in each patient. Previous studies comparing proliferation rates of primary tumors and metastases found either no difference or up to two fold higher rates for metastases (39,40)(41). However, proliferation rates of matched primary and metastases were rarely estimated and to our knowledge compare only primary to matched lymph node metastases but not distant metastases. Pence et al. found no significant difference between matched primary breast tumor and lymph node metastases by Ki67 score (42). In agreement, we found that P1 scores of primary and lymph node metastases are not significantly different. However, our data indicates that recurrent metastatic samples are lower than the matched primary pre-treatment tumors, suggesting that tumor clones that survived and escaped chemotherapy have a similar or higher proliferation capacity.

The modulation observed in the primary tumor following treatment was not observed in the lymph nodes compared to pre-treatment lymph node, suggesting that these cells are either resistant to therapy or are protected from the chemotherapy effect in the lymphatic site. Moreover, for P5 and P6 miRNAs, we observed that post-treatment lymph node levels approach the recurrence expression levels while the levels of tumor and lymph node pre-treatment were similar.

The METABRIC dataset, as most other available datasets of breast cancer, includes only primary breast cancer samples and not metastatic samples. Therefore, the information on miRNAs that are expressed only at invasive stages, such as in patterns P5 and P6 are limited. Similarly, the identification of pathways and genes that are correlated to metastatic events is limited. Notably, most miRNAs from pattern P5 were up-regulated in initial stages of breast cancer but down regulated in high grade tumors. This dual behavior is reminiscent of TGFB, known to exert a dual function in cancer. In normal cells and early carcinoma it acts as a tumor suppressor, while in aggressive and invasive tumors it promotes cancer progression, migration and invasion (43,44). TGFB in its pro-metastatic arm regulates epithelial to mesenchymal transformation (EMT) that involves the transition from adherent epithelial cells to motile mesenchymal cells, a process necessary for the primary tumor in order to migrate and invade. The reverse process is MET - mesenchymal–epithelial transition, shown to be involved in the metastatic process, by promoting epithelial properties, thereby, facilitating their settlement in distant organs. Indeed, most P5 miRNAs are correlated with O-glycan biosynthesis. O-glycosylation has an important functional role in EMT induction; in addition, our GSEA analysis revealed that most P5 miRNAs are enriched by genes up-regulated during EMT.

In summary, identifying molecular players that modulate in the various stages of the disease can pinpoint pathways that are alternately activated, depending on the disease context. Enlargement of our cohort and validation of our findings in additional patients will strengthen our conclusions; moreover, performing the analysis on each molecular subtype will identify processes which are unique to each subtype. However, as discussed before, it is very hard to assemble such unique cohorts of patients with matched pre-treatment, post-treatment and recurrent samples. Remarkably, notwithstanding the small size of our cohort and it heterogeneity, we identified sets of miRNAs which
behave similarly across several patients and have implications for tumor progression, response to treatment and recurrent disease.

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**FIGURE LEGENDS**

**Figure 1:** Identifying temporal miRNA expression patterns through the course of disease. (A) The longitudinal collection of samples: from each individual patient, core biopsy (C) and lymph nodes (CL) are collected at diagnosis before the neo-adjuvant treatment; tumor sample (T) and lymph node (TL) are collected at surgery post-treatment; for patients with recurrent disease (local or distant metastasis), recurrent tissue (R) is also collected. (B) The types of samples that were collected for each patient; when present, the box is colored as in (A). (C) 8 possible varying expression patterns (P1-P8) across the three selected time points: C, T and R. The number of miRNAs that were classified to each pattern is indicated. (D) A flow chart for assigning a miRNA to a pattern (actual data for miR-125b-5p is shown as an example): First, Linear mixed effect model is used to select all miRNAs that are differentially modulated over time. Then, a corresponding pattern is assigned for each patient using a threshold of 1.5 fold change. Next, if the miRNA expression follows the same pattern in 3 patients or more, the miRNA is assigned to this pattern (see Supp. Methods for miRNAs that are assigned to more than one pattern).

**Figure 2:** List of miRNAs that were classified to each pattern. Results obtained from the METABRIC dataset: red / green arrows indicate whether the miRNA is up /down regulated (FDR=1%) in tumor versus normal tissue (column 3) and in grade 3 relative to grade 1 tumors (column 4). miRNAs that are not expressed in the METABRIC dataset are indicated by gray entries. Evidence from the literature: whether the miRNA is up (red arrow) or down (green arrow) regulated in breast tumors versus normal samples (column 5), and whether it’s up or down regulation is associated with metastasis (column 6) or resistance to therapy (column 7). Columns 8-17 indicate whether the miRNA’s expression levels in the METABRIC dataset are significantly correlated (1% FDR) with the Pathway Deregulation Scores of the corresponding KEGG pathways (see text for details). Green / red entries represent negative / positive correlation. Columns 18-25 indicate for each miRNA whether it is expressed, in each patient, according to its assigned pattern (black entries if consistent with fold-change threshold of 1.5, gray for 1.4).

**Figure 3:** miRNAs in Pattern P1 are correlated with response to treatment and disease progression. (A) Expression matrices of the subset of 14 miRNAs from P1 (see text), presented for each patient across all its available samples. Colors indicate expression levels after centering and normalizing each miRNA (row) across all patients, with red denoting relatively high expression and blue relatively low expression (see colorbar at bottom right). The mean expression level of the miRNAs (“P1 score”) is presented below each matrix. In all matrices the miRNAs are ordered as shown for patient 2. (B) Log-ratios of P1 scores, relative to the core biopsy (C), are plotted for each patient. Pathological response to treatment was scored by Miller and Payne grade (MP) and is denoted by colors (see legend). Log
ratios of P1 between C and T are significantly different among the various MP grades (ANOVA test, p=0.011). (C) Box-plot of P1 scores in core biopsies pre-treatment (C), tumor samples post-treatment (T), recurrent tissues (R), lymph node before treatment (CL) and lymph node after treatment (TL). Each box contains patients for which the corresponding information is available. * indicates p<0.05 and ** indicates p<0.01.

**Figure 4: miRNAs in Pattern P1 are associated with proliferation and cell cycle.** (A) Expression levels of P1 miRNAs that target BUB1B – let-7c, miR-125b-5p, miR-199a-3p and miR-199a-5p. Presented are the log-ratio expression levels (NanoString) of tumor post-treatment relative to core biopsy pre-treatment in patients 9 (black), 5 (gray), and 2 (white); and for BUB1B in the same samples and patients as measured by qRT-PCR. (B) Same as (A) for miR-100-5p and miR-145-5p which target CDC25A. (C) Representative images of Ki67 immunostaining in patients 2, 3 and 9 in C, T, R samples. (D) Box-plot for %Ki67 positive cells in C, T, R samples. (E) Comparison between %Ki67 positive cells (blue; left y-axis) and P1 scores (green; right y-axis) in individual patients (indicated in the titles) across C, T, R samples.

**Figure 5: P1 miRNAs differentiate between recurrence and recurrence-free patients.** (A) Comparison between P1 scores in recurrent (REC; patients 1-10) and recurrence free (REC FREE; patients 11-19), for three different types of tissues: C, T and TL (see text). * indicates p<0.05. (B) Kaplan-Meier analysis for the association between the pre-treatment expression levels of P1 scores and recurrence-free survival; done on both cohorts together (19 patients). HR=Hazard Ratio; CI=Confidence Interval, as calculated by COX proportional hazard model.
REFERENCES


Table 1: Clinical and pathological characteristics of the cohorts

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* Median follow up time, 11 (10-12) years

Abbreviations: AC-T - doxorubicin (Adriamycin), cyclophosphamide and paclitaxel (Taxol); AC-TH - as AC-T with trastuzumab (Herceptin); CR - pathological complete response; PR - pathological partial response; SD - stable disease; RFS - recurrence free survival.
Figure 1

A. Diagnosis
- Neo-adjuvant treatment
- Tumor surgery
- Post-treatment
- Recurrence

Core biopsy (C)
Tumor sample (T)
Lymph node (CL)
Lymph node (TL)
Local/Distant metastasis (R)

B. Table

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C. miRNA Analysis

- P1: 20 miRNAs
  - C: 10, T: 10, R: 0
- P3: 1 miRNA
  - C: 11, T: 0, R: 0
- P5: 7 miRNAs
  - C: 12, T: 11, R: 9
- P7: 1 miRNA
  - C: 11, T: 0, R: 0
- P2: 4 miRNAs
  - C: 10, T: 9, R: 1
- P4: 2 miRNAs
  - C: 12, T: 10, R: 0
- P6: 7 miRNAs
  - C: 11, T: 11, R: 10
- P8: 0 miRNAs
  - C: 0, T: 0, R: 0

D. Identifying miRNAs significantly different across time (1% FDR)

1. Check miRNA expression
2. Assign to a pattern in each patient
3. If expressed in the same pattern in 3 or more patients, assign to this pattern

- miR-125b-5p
  - P1
  - P5
  - P1
  - P1
  - P8
  - P1
  - P8
  - P1
  - P1
  - P5
  - P8

Fold change: 1.5
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Figure 4

(A) P1 miRNAs and their target gene expression levels in different patient samples. The log-ratio post-to pre-treatment expression is shown for miR-125b-5p, miR-199a-3p, and miR-199a-5p. The target gene expression is also shown for BUB1B.

(B) P1 miRNAs and their target gene expression levels in different patient samples. The log-ratio post-to pre-treatment expression is shown for miR-100-5p, miR-145-5p, and CDC25A.

(C) Representative images of tissue samples from pre-treatment (C), post-treatment (T), and recurrence (R) stages for patients 2, 3, and 9.

(D) Bar graph showing the %Ki67 expression levels in C, T, and R stages for Patient 2. The median, interquartile range, and range are indicated.

(E) Graph showing the P1 score (%Ki67) and recurrence (R) for Patients 2, 3, 7, 8, 9, and 10.
Figure 5

A

P1 miRNAs

REC FREE  REC  REC FREE  REC  REC FREE  REC
Pre-treatment  Post-treatment  Post-treatment
core biopsy (C)  Tumor (T)  Lymph node (TL)

B

Recurrent-Free

High P1 scores (n=9)

Kaplan-Meier $P=0.016$

HR=0.3 (95% CI: 0.09-0.97); $P=0.045$

Low P1 scores (n=10)

Time (months)
Clinical Cancer Research

Tumor Evolution Inferred by Patterns of microRNA Expression through the Course of Disease, Therapy and Recurrence in Breast Cancer

Maya Dadiani, Noa Bossel Ben Moshe, Shani Shimon-Paluch, et al.

Clin Cancer Res  Published OnlineFirst March 8, 2016.

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