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Ischemia caused by time to freezing induces systematic microRNA and mRNA responses in cancer tissue

Eldrid Borgan^{a,g,*}, Roy Navon^b, Hans Kristian Moen Vollan^{a,c,g}, Ellen Schlichting^c, Torill Sauer^d, Zohar Yakhini^{b,e}, Ole Christian Lingjærde^f, Therese Sørli^a, Anne-Lise Børresen-Dale^{a,g}

^aDepartment of Genetics, Institute of Cancer Research, Oslo University Hospital, Radiumhospitalet, Oslo, Norway

^bAgilent Laboratories, Tel Aviv, Israel

^cDepartment of Breast and Endocrine Surgery, Division of Surgery and Cancer, Oslo University Hospital, Oslo, Norway

^dDepartment of Pathology, Division of Surgery and Cancer, Oslo University Hospital, Oslo, Norway

^eDepartment of Computer Science, Technion–Israel Institute of Technology, Haifa, Israel

^fBiomedical Informatics, Department of Informatics, University of Oslo, Oslo, Norway

^gInstitute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway

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ABSTRACT

Time to freezing tumor tissue for RNA expression analysis will always vary to some extent. To evaluate the effect of ischemia time, tumor tissue from ten breast cancer patients was collected and aliquots of tissue were snap frozen at different time points after surgery (0, 0.5, 1, 3 and 6 h). Using miRNA and mRNA expression microarrays and statistical analysis, 56 miRNAs and 1788 mRNAs were found to be significantly altered with ischemia time up to six hours. Several of the 56 miRNAs have been reported to play a role in cancer, such as hsa-miR-663 and hsa-miR-125a-3p. Known stress response genes such as *GADD45B*, *JUND* and *FOSB* were among the mRNAs most significantly affected by time to freezing. A novel statistical method for identification of consistently correlated miRNA–mRNA pairs and miRNA-associated biological processes in time course data is presented. Application of this method revealed that several miRNAs, including hsa-miR-1228, hsa-miR-1225-5p and hsa-miR-574-5p, were associated through their correlation to mRNAs to biological processes such as “response to stimulus” and “stress response”. These miRNAs also showed enrichment of predicted targets among either their positively or negatively correlated mRNAs. The induced miRNAs may play both direct and indirect roles in biological responses. Caution should be taken when the miRNAs and mRNAs reported to be affected by ischemia time are included in a prognostic or predictive signature.

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Abbreviations: FDR, false discovery rate; GO, gene ontology; miRNA, microRNA.

* Corresponding author. Department of Genetics, Institute for Cancer Research, Oslo University Hospital, Radiumhospitalet, Montebello, 0310 Oslo, Norway. Tel.: +4722781381; fax: +4722781395.

E-mail addresses: eborgan@rr-research.no (E. Borgan), rnavon@gmail.com (R. Navon), Hans.Kristian.Moen.Vollan@rr-research.no (H.K.M. Vollan), elsc@uio.no (E. Schlichting), Torill.Sauer@ulleval.no (T. Sauer), zohar_yakhini@agilent.com (Z. Yakhini), ole@ifi.uio.no (O.C. Lingjærde), tsorlie@rr-research.no (T. Sørli), a.l.borresen-dale@medisin.uio.no (A.-L. Børresen-Dale).

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1. Introduction

Snap freezing of tumor tissue directly after surgery has been considered the gold standard for bio-banking of samples to be used in RNA expression analyses. However, snap freezing is not always feasible in clinical practice and time to freezing will always vary to some extent. Gene expression signatures have for the last decade been used to differentiate breast cancer into groups with observed differences in outcome (Perou et al., 2000; Sorlie et al., 2001; van de Vijver et al., 2002; Wang et al., 2005). More recently, several microRNAs (miRNAs) have been implicated in breast cancer, acting both as tumor suppressors (e.g. hsa-miR-125a/b, hsa-miR-34, hsa-let7 family) and as oncogenes (e.g. hsa-miR-21, hsa-miR-155, hsa-miR-373/520c) (O'Day and Lal, 2010). Measuring miRNA expression has been proposed as a valuable diagnostic tool for breast cancer, as miRNAs are more stable than mRNAs (Shi and Guo, 2009). Transcriptional variance related to the post-surgical procedure is essential to be aware of and affected transcripts should be avoided in prognostic and predictive signatures.

Some studies have reported changes in gene expression levels in different types of tissue that was not immediately snap frozen after surgery (Almeida et al., 2004; Bray et al., 2010; Dash et al., 2002; De Cecco et al., 2009; Spruessel et al., 2004). A systematic compilation of transcripts and processes affected by ischemia time has to our knowledge not been published. Furthermore, no studies have so far reported the effects of ischemia time on non-coding RNA such as miRNAs. The clinical relevance of a systematic study of these effects is evident for all studies aiming to find good RNA based biomarkers. From a biological point of view, the induced stress response observed in excised tumor tissue presents an opportunity to study transcriptional responses to stimuli in human carcinomas that cannot easily be studied in vivo. Joint analysis of changes in miRNA and mRNA levels as a function of ischemia time may elucidate unknown biological roles of specific miRNAs.

In this study, independent time series from ten patients were used to identify miRNA and mRNA transcripts affected by ischemia time, that is, the length of time for which excised

breast cancer tissue was left at room temperature. In addition, using a novel statistical method for analyzing time course data, consistent miRNA–mRNA associations across tumors of different patients were identified and related to specific biological processes.

2. Materials and methods

2.1. Patient material and experimental design

Ten patients with primary operable breast cancer were included at time of surgery. These patients are a subset of an ongoing Norwegian study (Oslo-2), which has been approved by the regional ethical committee (approval number 1.2006.1607, amendment 1.2007.1125). Histopathological data and patient characteristics are given in Table 1. For five patients, a core needle biopsy was taken directly after tumor excision, placed in a cryo-tube and snap frozen in liquid nitrogen. For all ten patients four pieces of tumor tissue were macro-dissected by a pathologist to ensure representative tumor tissue for all time points. Cryo-tubes with each tumor samples were left at room temperature and frozen in liquid nitrogen at four different time points after surgery; 0.5, 1, 3 and 6 h. The experimental design and workflow of this study is illustrated in Supplemental Figure 1.

2.2. RNA extraction and microarray hybridization

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA concentration and quality was measured using NanoDrop (NanoDrop Technologies) and 2100 Bioanalyzer (Agilent Technologies), respectively. Total RNA was amplified and labeled using low-RNA input Linear Amplification RNA kit (Agilent Technologies) and cy3-CTP. Gene expression microarray experiments were performed with 400 ng of total RNA, using one-color Agilent Technologies 4 × 44 K Whole Human Genome Microarrays, according to the manufacturer's protocol One-Color Microarray-Based Gene Expression Analysis v5.5. The amount of cy3-labelled cRNA used for hybridization was 1.65 µg.

Table 1 – Histopathological characteristics for the study cohort. All patients were diagnosed with invasive ductal carcinoma (IDC).

Patient	Grade	Size (mm)	pT	pN	ER	PGR	HER2	Age at diagnosis
1	II	12	1c	0	pos(>90%)	pos(>56%)	pos	60
2	II	22 + 11	2	1a	pos(>75%)	pos(>75%)	neg	54
3	II	23	2	0	pos(>90%)	pos(>90%)	neg	49
4	II	13	1c	0	pos(>75%)	pos(>56%)	neg	48
5	III	8	1b	0	pos(>75%)	pos(>75%)	neg	61
6	I	8	1b	0	pos(>56%)	pos(>80%)	neg	52
7	II	23	2	0	pos(>90%)	neg	neg	49
8	II	52	3	2	pos(>80%)	pos(>80%)	neg	44
9	III	25	2	0	neg	weak pos(~25%)	neg	51
10	III	12	1c	1	neg	neg	neg	68

pT – primary tumor classification, pN – histological lymph node status, ER – estrogen receptor, PGR – Progesterone receptor, HER2 – HER2 status/amplification.

miRNA one-color microarray experiments were performed using Agilent Technologies “Human miRNA Microarray Kit (V2)” with 100 ng of total RNA according to manufacturer’s protocol miRNA Microarray System v1.5. Both types of microarrays were scanned using Agilent Technologies Microarray Scanner G2505B/G2505C. The data were extracted from the scanned images using Feature Extraction software (Agilent Technologies), version 10.1.1.1 and protocol miRNA-v1_10_Apr08 for miRNA and version 9.5.3 and protocol GE1-v5_95_Feb07 for mRNA.

2.3. Microarray preprocessing

The Feature Extraction (FE) files from the miRNA and mRNA microarray experiments were processed and normalized separately, using R (v2.10.1) and Bioconductor (Gentleman et al., 2004). The GeneView files from Feature Extraction were used for the miRNA microarray data, for which signal intensities for replicate probes had been averaged and log2 transformed. The miRNA data were shifted to represent only positive values. Each array was normalized separately by subtracting the 75th percentile from each probe on the array. Probes flagged as present in the GeneView files for less than 20 percent of the arrays were removed.

The mRNA data were detrended for multiplicative effects and log2 transformed. Data from control probes, probes with good quality for less than 80 percent of the arrays (bad quality spots were defined as feature outliers from the FE file), and probes that were flagged as present on less than 20 percent of the arrays were omitted from the analysis. After setting spots flagged as outliers to NA, the average value of duplicate probes was used to represent each unique probe. The data were imputed using k-nearest neighbors (Troyanskaya et al., 2001) and quantile normalized. For transcripts represented by multiple different probes (based on GeneName, as provided by Agilent Technologies), the probe with the highest interquartile range (IQR) was chosen to represent each transcript. One mRNA array was excluded (Patient 8, time point 3 h) due to poor data quality; consequently the corresponding miRNA data were excluded. The final dataset included data for 507 unique miRNA transcripts and 24712 unique mRNA transcripts on 44 microarrays from biopsies representing independent time courses from ten patients.

2.4. Analyzing RNA integrity and hypoxia signature as a function of ischemia time

The RNA Integrity Number (RIN) value from the 2100 Bioanalyzer (Agilent Technologies) was used as the measurement for total RNA integrity. The effect of ischemia time on the RIN-value (from all 45 samples) was tested using the *reml* R package. Time (0, 0.5, 1, 3, and 6) was used as a continuous variable (fixed effect) while patient (1–10) was used as a blocking factor (random effect), in a linear mixed model. To assess whether the samples showed a more hypoxic phenotype with increasing ischemia time, the gene expression based Hypoxia classifier from Chi et al. (Chi et al., 2006) was applied. The original probes were matched using the R-package *BioMaRt* (Durinck et al., 2005), achieving a match of 70 percent. For each probe for each sample, the median expression value

across all samples was subtracted (median centering). A hypoxia score for each sample was calculated by taking the median expression of the hypoxia related genes (Chi et al., 2006). The calculated hypoxia score was used to assess whether ischemia time was associated with the variation in hypoxic gene signature, with the same linear mixed model as described above.

2.5. Analyzing the impact of delayed freezing on miRNA and mRNA levels

To explore the global expression patterns of miRNA and mRNA transcripts, hierarchical clustering and multidimensional scaling was performed on the two datasets separately. Also, transcripts with variation across samples that was not dominated by patient heterogeneity were selected. For each transcript a total correlation value was calculated by removing the transcript and calculating the sum of correlations between all pairs of biopsies, over all patients. The transcripts with the highest total correlations were regarded as the transcripts that contributed the least to the between-patient variation. Heatmaps of these miRNA and mRNA transcripts were made to visualize the mean variation with time of these transcripts.

To identify miRNA and mRNA transcripts that showed a systematic and significant variation with time, a supervised approach was taken. An established method for identifying such transcripts is implemented in the *Limma* package (R/Bioconductor) (Smyth, 2004). For both datasets, the log2 signal intensities of each probe were modeled as functions of time (0, 0.5, 1, 3, and 6) and patient (1–10). Time was used as a categorical variable, to capture all patterns of variation with time and not strictly linear trends, while patient was used as a blocking factor, as described by Smyth (Smyth, 2005). The miR and mRNA transcripts that were affected by ischemia time were identified by Empirical Bayes modified F-tests (Smyth, 2004), and the resulting *p*-values were adjusted using Benjamini Hochberg false discovery rate (Benjamini and Hochberg, 1995).

To test for enriched biological processes among the identified mRNA transcripts, a list was made of all gene names (as provided by Agilent Technologies) in the filtered microarray data, ranked according to effect of ischemia time on expression, with the most significant transcripts on top. This gene list was analyzed for Gene Ontology (GO) – term and transcription factor binding site (from the TRANSFAC database) enrichment using *GOrilla* (Eden et al., 2009). The *GOrilla* software searches for genesets that are enriched towards the top of the list compared to the rest of the list using the mHG statistics (Eden et al., 2007). The 8918 Biological Process (BP) terms and 615 transcription factor binding sites which were represented in the dataset were analyzed for enrichment.

2.6. Identifying consistently time-correlated miRNA–mRNA pairs

A procedure was tailored to identify miRNA–mRNA pairs for which expression was consistently time-correlated in several of the independent time courses (patients/tumors), illustrated in Figure 1 and described in details below. For each of the 10 patients, time correlations between every pair of miRNA and mRNA were calculated, restricting attention to 56 miRNAs

To assess the degree of consistency across patients of miRNA–mRNA correlation over time, methodology recently published by Navon et al (Navon et al., 2009) was utilized. The results were organized as 56 correlation matrices of dimension 1788×10 (each matrix representing a particular miRNA, rows representing mRNAs and columns representing patients). Consider one particular miRNA, and let C denote the 1788×10 matrix of correlations over time between the selected miRNA and each of the 1788 mRNAs, for each of the 10 patients (Figure 1, Step 1). For each column in C

An mRNA with high rank across many patients is said to be consistently correlated to the miRNA in question. Using the reverse order of ranks consistently negatively correlated miRNA-mRNA pairs could be defined in a similar manner. Consider as before one particular miRNA and let $\mathbf{R}$ denote the 1788×10 matrix of ranks. To statistically assess whether at least m ($1 \leq m \leq 10$) patients display a high rank, a rank consistency score is defined. For every row (i.e. mRNA) k in the rank matrix $\mathbf{R}$ identify the m 'th smallest element $S(k;m) = R_{k,(m)}$ where $R_{k,(1)} \leq R_{k,(2)} \leq \dots \leq R_{k,(10)}$ denote the order statistics

for the elements in the k 'th row of R . Thus, $S(k;m)$ is the worst rank among the m highest-ranked correlations for the mRNA in question. A small value of $S(k;m)$ hence means that the correlation to this mRNA is highly ranked in at least m patients. To evaluate the statistical significance of any observed value of $S(k;m)$, the probability of obtaining the observed value or smaller (i.e. better) in random data drawn according to a null model was estimated, assuming that patients are independent and that ranks among mRNAs are uniformly distributed within each patient. It then follows (Navon et al., 2009) that the exact p -value when $S(k;m) = s$ is given by

$$p = \sum_{j=m}^{10} \binom{10}{j} s^j (1-s)^{10-j}$$

For various reasons, the degree of consistency between miRNA and mRNA expression across patients may differ between miRNA–mRNA pairs. A minimum rank consistency score which is the smallest p -value for $1 \leq m \leq 10$, was used to avoid restricting the value of m to a certain number. The corresponding p -value can be found in (Navon et al., 2009), and can be calculated in $O(N^2r^2)$ operations, where N is the number of mRNAs and r is the number of patients. This p -value (Figure 1, Step 3) was calculated using the software by Navon et al. which can be found at (<http://bioinfo.cs.technion.ac.il/people/zohar/RCoS/>). These p -values were corrected for multiple testing of 1788 mRNA transcripts, by Benjamini Hochberg false discovery rate (Benjamini and Hochberg, 1995), and were used to find consistently correlating miRNA–mRNA pairs (Figure 1, Step 4a).

2.7. Identifying miRNA associated biological processes

To identify biological processes that the 56 identified time-dependent miRNAs are involved in, the results from the consistent correlation analyses were utilized. For each miRNA, the 1788 mRNA transcripts that were altered with time were ranked according to the p -value from the consistent correlation analysis, resulting in 56 different rankings. To test for enriched GO-terms (biological processes) among the top correlated mRNAs, the ranked lists of mRNA transcripts were used as input to GOrilla (Eden et al., 2007, 2009). The significance threshold was set to p -value $< 10^{-7}$ (3289 biological processes tested). Hence, biological processes that were enriched among the mRNAs that co-varied with certain miRNAs could be identified (Figure 1, Step 4b). The same procedure was repeated for the negatively correlated miRNA–mRNA pairs.

2.8. miRNA target prediction analysis

To explore whether there was evidence that a miRNA consistently correlated to certain mRNAs could have direct effect on these mRNAs, mirSVR target prediction scores (Betel et al., 2010) and Gene Set Enrichment Analysis (Subramanian et al., 2005) were used. mirSVR scores are based on a machine learning method to rank miRNA target sites by a score based on sequence and contextual features from miRanda as well as functional data. Non-conserved target sites were included, and the mirSVR cut-off was set to scores < -0.1 . mirSVR scores for mRNAs with multiple binding sites were added together,

as the mirSVR scores were developed to linearly correlate with downregulation of log expression of mRNAs (Betel et al., 2010). For each miRNA present in the database and among the 56 miRNAs identified as affected by ischemia time, a gene set was created that included all predicted mRNA targets for that miRNA. Lists of the 1788 mRNAs were ranked by significance of consistent correlation to each of the 56 miRNAs. To assess whether there were more predicted targets towards the top of the list of mRNAs, GSEA was performed for each miRNA separately.

3. Results

3.1. The effect of ischemia time on RNA integrity

Although there was substantial variation in RNA integrity of the total RNA isolated from the 45 different tumor biopsies as measured using the RIN-value (Agilent Technologies Bioanalyzer 2100) (Supplemental Table 1), there was no significant evidence that this variation was related to time to freezing. Previous studies on breast cancer (De Cecco et al., 2009), normal breast (Ohashi et al., 2004), and on normal tonsil and colon (Micke et al., 2006) tissue show that RNA in excised tissue can remain intact even after many hours in room temperature. The evidence is not conclusive however, as other studies on colorectal (Bray et al., 2010; Opitz et al., 2010) and breast (Strand et al., 2007) cancer tissue have reported systematic degradation of RNA with ischemia time.

3.2. Changes in miRNA and mRNA levels related to delayed freezing

Biological variation between patients was the predominant variation in both the miRNA and mRNA data (Supplemental Figure 2). Trends of time-dependent variation were observed in the miRNA and mRNA data (Supplemental Figure 3), and this was tested statistically. Significantly altered transcript levels as a function of ischemia time up to 6 h after surgery were found for 56 miRNAs (Supplemental Table 2) and 1788 mRNAs (Supplemental Table 3) ($FDR < 0.05$). The top ten miRNAs and mRNAs are listed in Figure 2 A and C, respectively. All 56 significantly affected miRNAs displayed a trend of increased expression with time, plotted for hsa-miR-1224-5p, hsa-miR-1225-5p, hsa-miR-125a-3p and kshv-miR-K12-3 (Figure 2B). The most significantly affected mRNA transcripts also showed an increase in expression with time, plotted for DNA-damage-inducible 45 beta (GADD45B), LIM and senescent cell antigen-like domains 3 (LIMS3), jun D proto-oncogene (JUND) and FBJ murine osteosarcoma viral oncogene homolog B (FOSB) (Figure 2D). Among all 1788 transcripts, several transcripts also showed a mean decrease in expression with time or a more complex pattern of expression with time (Supplemental Table 3). Many of the 1788 significantly affected mRNA transcripts had unknown functions, such as ALU elements and pseudogenes. Among the gene transcripts with known function that were most significantly associated with ischemia time, 24 biological processes were enriched ($p < 10^{-5}$, $FDR < 0.0035$) (Figure 3). The most significant GO-terms were related to regulation of transcription. Several

Figure 2 – The most significantly differentially expressed miRNA and mRNA transcripts up to six hours in room temperature after surgery. (A) Top ten most significant miRNAs, (B) expression levels for each patient as a function of time for the four most significant miRNAs, (C) top ten most significant mRNAs, (D) expression levels for each patient as a function of time for the four most significant mRNAs (with known function).

transcription factor binding sites were also significantly enriched among the mRNAs most associated with ischemia time (Supplemental Table 4). Histone genes with the unknown motif KRCTCNNNMANAGC and genes with the CREB1 motif were the most significant hits. To assess whether the samples showed a more hypoxic phenotype with increasing ischemia time, the gene expression based Hypoxia classifier from Chi et al. was applied (Chi et al., 2006). Using the calculated hypoxia score, a slight increase in hypoxia with ischemia time was observed ($p < 0.0016$).

Focusing only on the first time points (0, 0.5 and 1 h), there was not significant evidence that any of the expressed miRNA transcripts were affected by ischemia time up to 1 h after surgery. Concerning mRNA expression, several transcripts were affected (FDR < 0.05, Supplemental Table 5); 119 transcripts showed differential expression between time point zero and 0.5 h (77/119 were lower expressed after 0.5 h) and 494 transcripts showed differential expression from time point 0.5 h–1 h (454/494 were lower expressed after 1 h). The majority of mRNA transcripts with lower expression between 1 h and 0.5 h were annotated as “Unknown” or as not being verified as protein-coding by Agilent Technologies. Among the mRNAs with higher expression levels at time 1 h compared

to time 0.5 h were a few of the most significant mRNAs from the 6 h analysis, such as JUN, ZFP36 and CD86.

3.3. Consistently correlated miRNA–mRNA pairs with time reveal miRNA-associated biological processes

Consistently correlated miRNA–mRNA pairs (FDR < 0.05) were identified, using the strategy illustrated in Figure 1. As an example, the correlation coefficients of the mRNAs that were consistently time-correlated to hsa-miRNA-125a-3p across patients are shown for each of the ten patients for (Figure 4). The significant miRNA–mRNA pairs between all 56 miRNAs and 1788 mRNAs affected by ischemia time are summarized in Figure 5. It should be noted that all 56 miRNAs that were found to be systematically altered with ischemia time showed a general increase in expression with time. This trend explains the pattern of consistent correlation (Figure 5), where an mRNA never is positively correlated to some miRNAs and negatively correlated to others.

Results from the consistent correlation analysis were combined with GO-term enrichment analysis to find miRNA associated biological processes. The biological processes significantly enriched ($p < 10^{-7}$) among the positively

Figure 3 – Enriched biological processes among the mRNA transcripts affected by ischemia time. A color-coded trimmed Directed Acyclic Graph (DAG) of all significantly enriched biological process GO-terms among the mRNA transcripts that are the most significantly affected by ischemia time, obtained using GOrilla (Eden et al., 2009).

correlated mRNAs of at least three miRNAs are summarized in Figure 6. The *p*-values for the miRNAs most significantly associated to the selected biological processes are included as a heatmap (Figure 6) and the DAGs for each of these miRNAs are shown in Supplemental Figure 4. The biological processes with the highest number of associated miRNAs were “response to stimulus” (32/56), “signal transduction” (17/56), and “cellular response to stimulus” (17/56) (Figure 6). There were no significant GO-terms among the negatively correlated mRNAs of the 56 miRNAs.

Significant enrichment of predicted targets among the most correlated mRNAs was observed for six miRNAs among their positively correlated mRNAs, and four miRNAs among their negatively correlated mRNAs (FDR < 0.10) (Table 2). hsa-miR-1228 was significantly associated with six of the twelve identified biological processes and showed enrichment of predicted targets among its positively correlated mRNAs. Several miRNAs such as hsa-miR-663, hsa-miR-939, and hsa-miR-1228 were consistently positively correlated with and predicted to target FOSB. Other transcripts coding for

Figure 4 – mRNA transcripts consistently correlated to hsa-miR-125a-3p. Heatmaps visualizing the correlation between hsa-miR-125a-3p and its most consistently (A) negatively or (B) positively correlated mRNA transcripts. Each column represents a patient, and the rows are sorted according to consistent correlation.

Figure 5 – Consistently time-correlated miRNA-mRNA pairs in the ten patients. Significant ($FDR < 0.05$) miRNA-mRNA pairs among the miRNAs and mRNAs associated with ischemia time are indicated in a heatmap. (miRNAs and mRNAs that were involved

members of the AP-1 complex, such as *JUND*, *JUN*, *MAFF* and *ATF3* were also predicted targets of one or more of their positively correlated miRNAs. hsa-miR-1225-5P, hsa-miR-574-5p, and hsa-miR-557 were all associated to “stress response” and there was enrichment of predicted targets among their negatively correlated mRNAs. THAP domain containing 6 (*THAP6*) was one of the mRNAs consistently negatively correlated to all these miRNAs. For hsa-miR-574-5P, there was also enrichment of predicted targets among its positively correlated mRNAs, indicating that this miRNA may interact directly with both positively and negatively correlating mRNA transcripts.

Several viral miRNAs were among the 56 miRNAs that showed altered expression with ischemia time, such as the herpes family virus miRNAs kshv-miR-K12-3 (Kaposi sarcoma-associated herpesvirus miR-K12-3), hcmv-miR-US4 (Human cytomegalovirus miR-US4), hsv1-miR-H1 (Herpes Simplex Virus 1 miR-H1), and ebv-miR-BART13 (Epstein Barr virus miR-BART13). These miRNAs displayed similar expression patterns as the human miRNAs and were consistently correlated with many human mRNA transcripts over time (Figure 5). hcmv-miR-US4 was also the miRNA that was most strongly associated with the biological process “response to wounding” (Figure 6). It should be noted that hiv1-miR-H1 (Human immunodeficiency virus 1 miR-H1 stem-loop) was among the 56 miRNAs, but that the existence of viral miRNAs in HIV has been questioned (Lin and Cullen, 2007; Pfeffer et al., 2005).

4. Discussion

In this study, 56 miRNA and 1788 mRNA transcripts that were affected by ischemia time have been identified and miRNA-associated biological processes such as “response to stimulus”, “response to wounding” and “stress response” have been suggested based on covariation between miRNA–mRNA pairs. Two of the identified miRNAs, hsa-miR-125a (Li et al., 2009; Scott et al., 2007) and hsa-miR-663 (Lehmann et al., 2008) have been suggested to act as tumor suppressors in breast cancer. The mature 3' transcript of hsa-miR-125a, hsa-miR-125a-3p, which was found in this study has also been shown to be downregulated in non-small cell lung cancer (Jiang et al., 2010). hsa-miR-125a-3p and hsa-miR-663 were consistently positively correlated to many mRNAs and there was evidence that hsa-miR-663 had a direct effect on stress response through several of its correlated mRNAs, such as FOSB. Several other miRNAs affected by time in our study have been reported to show aberrant expression in cell lines or tumors of other cancers such as hsa-miR-518c*, hsa-miR-198, hsa-

in less than five pairs ($\text{FDR} < 0.01$) were excluded). Rows and columns are ordered by hierarchical clustering with Euclidean distance and complete linkage. Note that none of the miRNAs show a decreasing expression with time. Viral miRNAs are indicated by red color. Color-coding of heatmap corresponds to red: positive correlation, $\text{FDR} < 0.01$; orange: positive correlation, $\text{FDR} < 0.05$; green: negative correlation, $\text{FDR} < 0.05$; blue: negative correlation, $\text{FDR} < 0.01$.

Figure 6 – miRNA associated biological processes. Biological processes GO-terms that were significantly ($p < 10^{-7}$) enriched among mRNAs that were consistently positively correlated to at least three miRNAs are ordered according to median p -value of the significant miRNAs. The number of miRNAs with significant enrichment for each biological process, boxplots of p -values for all miRNAs significantly associated with each biological process, and a heatmap of the ten miRNAs with the lowest p -value for any of the biological processes are shown. Viral miRNAs are indicated by red color.

miR-498 in retinoblastoma (Zhao et al., 2009), hsa-miR-320, hsa-miR-498 in colon cancer (Scheperle et al., 2008), hsa-miR-134 in small cell lung cancer (Guo et al., 2010), hsa-miR-483-5p in adrenocortical cancer (Soon et al., 2009), and hsa-miR-765 in adrenal pheochromocytomas (Tombol et al., 2010). Interestingly, several viral miRNAs from viruses of the herpes family were also found to be affected by ischemia time. However, further studies would be needed to confirm the presence and elucidate the roles of these viruses and viral miRNAs in breast carcinomas. Nevertheless, instead of omitting these miRNAs from the analysis as is often done, they were included even though the reason for their presence is not fully understood.

The most significantly affected mRNAs showed an increase in expression with time. The most striking mRNA response is the transcripts coding for the transcription factors of the Jun, Fos, Maf, and ATF subfamilies, which form AP-1 dimers. This is in agreement with previous studies that have shown that expression levels of AP-1 transcription factors were altered with ischemia time in prostate (Dash et al., 2002) and colon cancer tissue (Spruessel et al., 2004). The overrepresentation of transcription factors among the altered mRNAs is reflected by enriched biological processes among these mRNAs, such as “regulation of gene expression”. Additionally, several of the AP-1 transcripts have transcription factor binding sites, such as the CREB1 motif, that were enriched among the mRNAs most affected by ischemia time. It is also worth noting that many of the affected mRNA transcripts were not annotated with a known function, and that some of these represent

non-coding RNA (ncRNA) that could play a regulatory role in stress response. Examples are ALU elements, that have been shown to be expressed in response to cellular insults (Liu et al., 1995), and pseudogenes, which have been suggested to play a role in regulation either by destabilizing their protein-coding mRNAs (Hirotsume et al., 2003) or by acting as miRNA sponges (Poliseno et al., 2010). There were particularly many ribosomal protein pseudogenes that showed lower expression after 1 h.

Several of the miRNAs associated with specific biological processes showed enrichment of predicted targets among either their positively or negatively correlated mRNAs. This suggests that miRNAs associated to biological processes may have either direct or indirect effects. Identified miRNA–mRNA pairs such hsa-miR-574-5P – CIRBP represent the classical notion that miRNAs are inversely correlated to their mRNA targets. Interestingly, hsa-miR-574-5P has been suggested as serum biomarker for early detection of non-small cell lung cancer (Foss et al., 2011). hsa-miR-574-5P was the only miRNA with enrichment of predicted targets among both its positively and negatively correlated mRNAs, and was associated to biological processes such as “immune system process”. Among the positively correlating miRNA–mRNA pairs with predicted target binding, pairs that may be involved in the observed stress response are e.g. hsa-miR-663 – FOSB, hsa-miR-939 – JUND, and hsa-miR-1228 – CDKN1A. A positive regulatory role has been reported for certain miRNAs during cell cycle arrest (Vasudevan et al., 2007), which supports that some miRNAs could be directly

Table 2 – Predicted target enrichment among the mRNAs consistently correlated to each miRNA. mirSVR scores < -0.1 (not required to be conserved) were used for target prediction (Betel et al., 2010). GSEA was used to analyze if there was enrichment of predicted targets among the most correlated mRNAs (Subramanian et al., 2005).

Systematic Name	SIZE	ES	NES	NOM p-val	FDR	Top correlated genes among predicted targets
Positively correlated						
hsa-miR-663	40	0.53	1.71	0	0	FOSB, GRASP, PIK3R5, NR4A1, MAFF, FLT1, ADRBK1, RHOB, ARHGEF1, PYY2
hsa-miR-760	77	0.44	1.54	0	0	ITPKC, NOL12, PER1, MAP3K11, FOSL2, MAFF, YPEL5, HIST1H2AL, ZBTB7B, ATF3
hsa-miR-940	148	0.42	1.54	0	0	VPS18, PLCB2, CD86, ZNF197, JUN, ZNF713, RAP1GAP, NUMB, PRH2, JUND
hsa-miR-939	65	0.44	1.49	0	0	JUND, PHF1, FOSB, ADM, LNX2, SPRY3, CFLAR, ZCCHC6, RAP2B, CENPT
hsa-miR-1228	75	0.44	1.52	0.001	0.029	CDKN1A, FOSB, ZNF197, RHOB, NFKBIZ, IFRD1, BAHCC1, NR4A1, ARHGEF1, ADAMTS1
hsa-miR-574-5P	52	0.44	1.48	0.002	0.056	VPS18, EP400NL, ARID5B, LOC389043, WDR33, CFLAR, NFKBIZ, PPM1B, TCP11L2, TRIB1
Negatively correlated						
hsa-miR-574-5P	52	0.44	1.47	0.001	0.027	THAP6, MIS12, CIRBP, QTRTD1, C15orf17, ZFP14, ZNF396, CEP192, LRBA, LYSMD3
hsa-miR-1225-5P	39	0.48	1.56	0.002	0.052	THAP6, WDR36, PDCD6IP, ZNF526, KIAA1370, METT5D1, FBXL2, TM2D3, ZHX3, GRIA4
hsa-miR-638	22	0.55	1.66	0.002	0.052	CIRBP, TMED2, ALPK1, PMS1, USP47, CNOT4, PRKAB2, RBM17, SDCCAG8, SNAP23
hsa-miR-557	98	0.40	1.42	0.0030	0.072	TTC30B, THAP6, DNAJA3, CNOT4, ELF1, LRBA, DFFB, FBXL2, C1orf109, C9orf45
SIZE: number of predicted targets among all the 1788 genes, ES: enrichment score, NES = normalized enrichment score, NOM p-val: nominal p-value.						

targeting their positively correlated mRNAs. Another explanation could be that these miRNAs act as fine tuners that decrease the abundance of the mRNA targets slightly over time, which would be captured as positive correlation over these time courses.

The biological relevance of the observed miRNA–mRNA covariation and identified miRNA-associated biological processes is of course limited by studying responses in excised tumor tissue. However, the fact that the most significant changes in both miRNA and mRNA levels were increased expression with time, and that several of the identified mRNA transcripts are known early response and stress related genes, suggests that the tumor cells may still regulate gene expression despite their loss of blood supply and attachment to the surrounding tissue. Also, a number of the 56 miRNAs found to be affected by ischemia time in this study have previously been reported to be involved in response to epidermal growth factor (EGF) stimulation after serum starvation in mammary epithelial cell lines (Avraham et al., 2010), namely hsa-miR-498, hsa-miR-198, hsa-miR-320, hsa-miR-564, hsa-miR-638, hsa-miR-601, hsa-miR-671, hsa-miR-134, hsa-miR-188, hsa-miR-765, hsa-miR-663 and hsa-miR-572. These miRNAs showed an increase in expression in this study, which is in agreement with Avraham et al., where these miRNAs showed an immediate decrease in abundance after EGF stimulation. This response was followed by an increase in levels of immediate early genes such as *JUN*, *FOSB* and *EGR1*, contradictory to this study where a steady increase in levels of the same mRNAs was observed with ischemia time. Further studies are needed to understand the

interaction between these miRNAs and mRNAs in response to different stimuli.

For clinical biobanks, it is reassuring that no miRNA and few mRNA transcripts with known function were found to be significantly affected during the first hour. A limitation of this sub-analysis is that the core needle biopsy at time point zero was only performed for five of the patients. However, it should be noted that typical snap freezing in clinical cohorts does not actually occur at mathematical time zero, due to surgical or pathological evaluation to choose which part of the specimen to collect and freeze. In practical terms, the 30 min time point may be more relevant for many published studies. To capture the “true” biological baseline of mRNA expression, a core needle biopsy would have to be taken before surgery. This was confirmed in a similar study on prostate cancer biopsies pre and post surgery (Lin et al., 2006). Among mRNAs found to increase in expression levels post surgery by Lin et al., there was an overlap of 30% (19/61, p -value $< 1.22 \times 10^{-10}$, hypergeometric test) with the present study. This overlap, including the various AP-1 transcription factors, indicates that the stress response induced by the surgical procedure continues and increases in magnitude if snap freezing is delayed. From a clinical point of view, the relevance of this study is therefore evident in that caution must be taken to include the miRNA and mRNA transcripts presented here in any prognostic or predictive signature. This is especially important when different cohorts are compared for which the sampling routines differ, and a systematic bias in these miRNA and mRNA transcripts due to prolonged time to freezing may be confused as biologically relevant differences.

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Appendix. Supplemental material

Supplementary data related to this article can be found online at [doi:10.1016/j.molonc.2011.08.004](https://doi.org/10.1016/j.molonc.2011.08.004).

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