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Abstract

Conventional methods used for histologic classification and grading of endometrial cancer (EC) are not sufficient to predict lymph node metastases. MicroRNA signatures have recently been related to EC pathologic characteristics or prognosis. The aim of this study was to evaluate whether microRNA profiles of grade 1 – 2 endometrioid adenocarcinomas can be related to nodal status and used as a tool to adapt surgical staging in early-stage EC. MicroRNA expression were assessed in nine formalin-fixed paraffin-embedded (FFPE) EC primary tumors with positive lymph node and in 27 FFPE EC primary tumors with negative lymph node, matched for grade, stage and lymphovascular space involvement status. A microarray analysis showed that there was more than a two-fold significant difference in the expression of 12 microRNAs between the two groups. A qRT-PCR assay was used to confirm these results: the expression levels of five microRNAs (microRNA-34c-5p, -375, -184, 34c-3p, and 34b-5p) were significantly lower in the EC primary tumor with positive lymph node compared to those with negative lymph node. A minimal p-value approach revealed that women with a microRNA 375 fold change <0.30 were more likely to have positive lymph node (n=8; 53.3%) compared with those with a microRNA 375 fold change >0.30 (n=1; 4.8%), p=0.001. Furthermore, women with a microRNA 184 fold change <0.30 were more likely to have positive lymph node (n=6; 60.0%) compared with those with a microRNA 184 fold change >0.30 (n=3; 11.5%), p=0.006. This is the first study investigating the relative expression of mature microRNA genes in early-stage grade 1– 2 EC primary tumors according to the nodal status. This microRNA expression profile provides a potential basis for further study of the microRNA function in EC and could be used as a diagnostic tool for nodal status.

Introduction

Endometrial cancer (EC) is the most common gynecologic tumor in women in developed countries. The highest estimated incidences in 2012 are in the USA and Canada (19.1/100 000) and northern (12.9/100 000) and western Europe (15.6/100 000) (1)(2). Women are often diagnosed at an early stage (stage I of the International Federation of Gynaecology and Obstetrics (FIGO) classification), when the disease is still confined to the uterus, representing almost 75% of the cases. The most frequently occurring histologic subtype is endometrioid adenocarcinoma, which is a hormone-receptor-positive EC, with a good prognosis (3). Women can be classified with early-stage EC at low-, intermediate- or high-risk for recurrence by a combination of histologic criteria (i.e. depth of myometrial invasion and histologic type and grade) (4).

Standard treatments for early stage EC consist of primary hysterectomy and bilateral salpingo-oophorectomy. In this specific setting, many centers in the United States perform lymph node sampling, lymph node dissection or sentinel lymph node mapping in many or most EC, showing that indications for lymph node dissections are not well established. European guidelines have recently been modified and lymphadenectomy is no longer recommended in women with low- (FIGO stage IA, grade 1 and grade 2, endometrioid adenocarcinoma) or intermediate- (FIGO stage IA, grade 3 type 1 EC; FIGO stage IB, grade 1 and grade 2, endometrioid adenocarcinoma) risk EC (4). However, several authors have demonstrated that such an approach may lead to under-treatment as 10% of women of low-risk and 15% of intermediate-risk EC have nodal metastases (5,6), rates which can be higher in the case of lymphovascular space involvement (LVSI) on the primary tumor (7–9).

It is now well established that conventional methods used for histologic classification and grading of EC are not sufficient to predict lymph node metastases. This is probably due to considerable heterogeneity within endometrioid histologic subtypes (10). Hence, additional

tools including highly sensitive and specific molecular prognostic biomarkers are needed to select the women for whom complete surgical staging should be performed in order to better adapt adjuvant therapies.

MicroRNAs (miRNAs) are short length (~22 nucleotides), naturally existing RNAs functioning at the posttranscriptional level either by regulating mRNA degradation or by translating repression through binding of the 3'-untranslated regions (3'-UTRs) of mRNAs (11). The human genome encodes at least 1 500 miRNAs, and it is estimated that more than 30% of all mRNA expression is regulated by miRNAs (12). They have been linked to a variety of physiologic and pathologic processes, including carcinogenesis and can act as metastatic activators or suppressors (13)(14). Recently, miRNA signatures have been related to the pathologic characteristics and prognosis of EC (15). However, there are few data about nodal status-associated miRNA regulation in EC, especially in women at low and intermediate risk. miRNAs, with their remarkable stability, can be studied in formalin-fixed paraffin-embedded (FFPE) specimens (16) and are promising biomarkers for tumor staging.

The aim of this study was thus to evaluate whether miRNA profiles of grade 1 – 2 endometrioid adenocarcinomas can be related to nodal status and used as a tool to adapt surgical staging.

Materials and Methods

Experimental design

Approval for the present study was obtained from the local Medical Ethics Committee (CPP Ile-de-France V; e-4-15) and written informed consent was obtained from all women who provided the tissue samples used in this study. The experimental design for profiling the miRNA changes of nodal involvement in grade 1 – 2 early-stage FFPE primary EC tumor specimens is shown in Figure 1.

Thirty-six women with early-stage EC (i.e. women with primary tumor confined to the corpus uteri with or without nodal involvement) who underwent primary surgical treatment (including total hysterectomy, bilateral salpingo-oophorectomy and systematic nodal staging) between January 2003 and December 2012 in Tenon University Hospital - APHP were enrolled in the study. Nine of these women had lymph node (LN) metastases (positive LN) and met the following inclusion criteria: endometrioid adenocarcinoma, grade 1 or 2, FIGO stage I. The remaining 27 women, without LN metastases (negative LN), met the same inclusion criteria and were used as control subjects. One-to-three matching was performed according to the following three criteria: FIGO stage (IA, IB), histologic grade (grade 1, grade 2), and LVSI status (positive, negative). The exclusion criteria were as follows: previous malignancies, history of chemotherapy or radiotherapy, inflammatory disease, or Lynch syndrome (the search for a loss of expression of one of the Mismatch Repair proteins by immunohistochemistry and for tumor instability (microsatellite instability replication error repeats phenotype) were performed when EC occured before the age 50 years or when there was a suggestive family history).

The clinical and pathologic variables of the women were extracted from maintained EC databases and included age, parity, body mass index (BMI, calculated as weight in kilograms divided by the square of height in meters), comorbidities (diabetes, dyslipidemia), 2009 FIGO

stage, histologic type and grade, depth of myometrial invasion, LVSI status, and time between surgery and sample analysis.

The median age was 72 years [interquartile range (IQR): 65-78] in the negative LN group and 68 years [IQR: 64-76] in the positive group (p=0.84). The two groups were comparable for FIGO stage, histologic grade, and LVSI status. Furthermore, the comorbidities (diabetes and dyslipidemia), parity, BMI, tumor size, European Society of Medical Oncology (ESMO) risk group of recurrence and LVSI status did not differ according to the nodal status. The median time between surgery and sample RNA extraction was 32 months [IQR: 21-79] in the negative LN group and 30 months [IQR: 15-60] in the positive group (p=0.95). (Table 1)

Histologic Characteristics

Histologic grade 1 is defined by 5% or less of a solid nonsquamous, nonmorular growth pattern; histologic grade 2 by 6% to 50% of solid nonsquamous, nonmorular pattern; and histologic grade 3 by more than 50% of solid nonsquamous, nonmorular growth pattern (17). The presence of grade 3 nuclei involving more than 50% of the tumor increases the final tumor grade by 1. A tumor is considered LVSI positive when tumor emboli are found within a space clearly lined by endothelial cells on haematoxylin and eosin (H&E)-stained sections (18).

RNA extraction from FFPE tissues

FFPE tissues were obtained from the hysterectomy specimens. FFPE tissue blocks were sectioned on a standard microtome (Leica-microsystems RM 2145) to generate successive 10 µm sections which were evaluated by a pathologist. Regions of invasive carcinoma were confirmed and marked on each slide. For each sample, marked regions from two slides were

microdissected using a new, sterile blade and the dissected tissues were placed immediately into an RNase-free microcentrifuge tube.

Total RNA was extracted using the miRNeasy FFPE Kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. Briefly, a deparaffinization solution was added to the FFPE samples. The tissues were then digested with protease and treated with DNase. After washing, the RNA, including the small miRNA fraction, was eluted with 20 µl distilled water. The concentrations and quality of the RNA recovered were measured using the Nanodrop 1000 A spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The median ratio of 260/280 was 1.87 (inter quartile range (IQR): 1.84-1.90) and the median concentration was 415.8 ng/µl (IQR: 327.1-568.2).

Microarray hybridization and data analysis

To offset the high cost of an individual miRNome, the 27 specimens of negative LN status were pooled by 3 in accordance with the matching criteria. In this way the influence of individual differences among the women were limited while maintaining opportunities for exploration of possible statistical variations. Microarray analysis was conducted on 18 distinct specimens: nine specimens with positive LN and nine specimens with negative LN. Microarray hybridization on miRNA 4.0 chips (Affymetrix) was conducted at the genomic platform of the Institut Cochin, Paris. After validation of the RNA quality with Bioanalyzer 2100, 1 µg of total RNA was biotin labelled following the FlashTag Biotin HSR RNA labelling kit (Affymetrix).

After overnight hybridization, the miRNA 4.0 chips were washed in the Fluidic Station FS450 (Affymetrix) following a specific protocol and scanned using the GCS3000 7G. The scanned images were then analyzed with Expression Console software (Affymetrix) to obtain raw data (CEL files) and metrics for Quality Control. No apparent outliers were detected. Specific

miRNA sanalysis was performed using Partek® Flow® software, version 3.0 Copyright ©; 2014 (Partek Inc., St. Louis, MO, USA). CEL files were imported and normalized using Robust Multi-array Averaging (RMA) (19).

Genes with a nominal p-value ≤ 0.05 were considered to be differentially expressed. Among these, genes showing a variation of 2 were retained for further analysis.

Validated target gene and enrichment analysis

To estimate the biologic effects of the differentially expressed miRNAs, lists of validated target genes were determined using currently available databases, including Tarbase® and Mirtarbase®. Gene Ontology (GO) enrichment analysis was performed on the lists using Genomatix GePS (release 2.4.0, Genomatix BH, Munich).

Validation of candidate miRNAs with reverse transcription (RT) and quantitative real time PCR (qRT-PCR)

One µg of total RNA was used in all RT reactions which were performed with the miScript®II RT Kit, using 5x miScript HiSpec Buffer method, according to the manufacturer's instructions (Qiagen, Courtaboeuf, France) with a Thermo Hybaid PXE 0.2 Thermal Cycler. cDNA samples were stored at −20°C for further use.

miRNA expression was analyzed by real-time PCR using miScript SYBR® Green PCR Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions with an initial activation step at 95°C for 15 minutes, followed by 45 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 70°C for 30 seconds. A final melting curve analysis was performed to verify that a single product was amplified. All steps were performed in duplicate using a LightCycler® 480 System (Roche). The results are expressed as Ct values and normalized on the calculated median Ct of each sample (ΔCt). miRNA primers were from Qiagen

(Courtaboeuf, France). Relative expression was calculated using the comparative Ct method (2-ΔΔCt). SNORD68 and RNU6 were both used as endogenous controls for data normalization.

For the results from the qRT–PCR on miRNA expression, data are expressed as means ± SEM. Means between two groups were compared using the Mann Whitney test. $p < 0.05$ was considered to be statistically significant. GraphPad Prism version 5 was used for analysis of tissue samples (GraphPad Software, La Jolla, CA, USA).

Principal component analysis (PCA)

PCA, a dimensionality reduction technique using a linear transformation applied on multidimensional data, was used to categorize miRNA (20).

PCA and biplot plotting were performed using custom code in R 3.1.3 software, available online.

Optimal miRNA fold-change cut-offs correlated with final nodal status

We calculate the ΔCt value by minus the Ct value of target microRNA by reference (endogenous control) microRNA. Normalized target microRNA expression level was calculated by $2^{-\Delta Ct}$. We further calculated the fold change of sample of interest to control sample by comparing their normalized target microRNA expression level.

For qualitative analysis, we calculated optimal cut-offs for each miRNA to correlate semi-quantitative expression and final nodal status. The optimal fold-change (FC) cut-off was determined by a minimal p-value approach. This involved dichotomizing the FC level into dummy variables with a cut-off every 0.01 units of its range of values. Fisher tests comparing the number of women with or without LN metastases for every dummy variable were then

calculated. The cut-off with the minimal p-value was chosen as the optimal cut-off for this variable.

Statistical analysis

Unless otherwise specified, data were managed with an Excel database and analysed using R 3.1.3 software, available online.

Results

Distinct miRNA signatures of type 1 EC primary tumors with positive LN

To establish a miRNA profile for primary endometrioid adenocarcinoma EC with positive LN, we performed a microarray analysis comparing endometrioid adenocarcinoma EC tumors with positive LN to those with negative LN. We focused our study on the 2,560 probes containing sequences for mature miRNAs. Among them, 142 probes had consistent normalized fluorescent intensities between the two groups (positive LN vs. negative LN) ($p < 0.05$) (Figure 2a). As illustrated by the volcano plot representation (Figure 2b), there was more than a two-fold significant difference in the normalized fluorescence intensity of 12 of these miRNAs between the positive LN vs. negative LN groups: seven miRNAs (miR 34c-5p, miR 375, miR 184, miR 34c-3p, miR 34b-5p, miR 148a-3p and miR 504-5p) had a decreased expression and five miRNAs (miR 129-5p, miR 5001-5p, miR 6068, miR 4467, miR 6850-5p) had an increased expression in samples from the positive LN group compared to samples from the negative group (Table 2).

GO term enrichment analysis was then performed using the list of all known validated targets for the miRNAs exhibiting at least a two-fold change, with significant value ($p<0.05$), in the EC primary tumors with positive LN. Genes related to cell transformation, genomic

instability, cellular metabolic process, neoplasm invasiveness, neoplasm cell transformation, growth arrest, malignant neoplasms, cancer progression, and microsatellite instability were specifically enriched (Figure 3**).**

Evaluation of miRNA expression by real-time qRT-PCR analysis

A qRT-PCR assay was used to confirm the expression of the miRNAs that were selected from the previous step. The expression levels of five miRNAs were significantly lower (miRNA-34c-5P, -375, -184, 34c-3p, and 34b-5p; p=0.006, 0.0008, 0.001 and 0.001, respectively) in the EC primary tumors with positive LN compared to those with negative LN. Two miRNAs (miR 148a-3p, miR 504-5p) and four miRNAs (miR 129-5p, miR 5001-5p, miR 6068, miR 4467) were down- and up-regulated, respectively, with no significant difference, in the EC primary tumors with positive LN compared to those with negative LN (Figure 4).

After eigenvalue decomposition of the 10 original dimensions (miR 34c-5p, miR 375, miR 184, miR 34c-3p, miR 34b-5p, miR 148a-3p, miR 504-5p, miR 5001-5p, miR 6068, and miR 4467), four principal components were found to be significant (with an eigenvalue >1). These components explained 75.72% of the variance in the data (Figure 5a). The two principal components explaining the greatest data variance are presented in Table 3. The first principal component, accounting for 32.02% of the variance, was dominated by tumor-suppressor miRNAs, especially of the miR 34 family, including miR 34b-5p, miR 34c-5p and miR 34c-3p. The second component, accounting for 24.31% of the variance, was dominated by miR 129-5p.

A biplot of the first and second components (those explaining the greatest variance) is shown in Figure 5b, demonstrating an opposition between miRNAs that were defined by microarray as being up-regulated miRNAs (miR 129-5p, miR 6068, miR 5001-5p, miR 4467)

and downregulated miRNAs (miR 34c-3p, miR 34c-5p, miR 34b-5p, miR 375, miR 504-5p, miR 184, and miR 148a-3p) in the positive LN group.

Correlation between miRNA expression and nodal status in EC

Optimal cut-offs denoting the strongest correlation between quantitative expression of the miRNAs that were selected from the previous step and final LN status are summarized in Figure 6. The FC cut-offs defined were 0.30, 0.30, 0.0852, 0.09, 0.50, 1.45, and 1.75 for miR 375, miR 184, miR 34c-5p, miR 34b-5p, miR 148a-4p, miR 129-5p, and miR 4467, respectively. miR 375 and miR 184 had the most significant p-values: 0.001 and 0.006, respectively. We compared nodal status according to the cut-offs previously determined: women with EC and a miRNA 375 FC < 0.30 were more likely to have positive LN (n=8; 53.3%) compared with those with an miRNA 375 FC > 0.30 (n=1; 4.8%), p = 0.001; women with EC and an miRNA 184 FC < 0.30 were more likely to have positive LN (n=6; 60.0%) compared with those with a miRNA 184 FC > 0.30 (n=3; 11.5%), p = 0.006 (Table 4)**.**

Discussion

Our results show that in grade 1 – 2 early-stage EC, women with LN metastases have different miRNA profiles compared with those without metastatic LN. Moreover, we found that final LN status can be accurately predicted using miRNA expression level.

Nodal status in grade 1 – 2 early-stage EC is currently a major subject of debate. Although lymphadenectomy is no longer recommended in early-stage EC since the publication of a meta-analysis demonstrating no impact on survival, there are several discrepancies about how to manage women with early-stage/grade 1 – 2 EC in terms of surgical staging (21). The major limitation of preoperative imaging techniques (3), such as MRI and CT scan, is the poor detection rate of LN metastases (5). Moreover, a prospective

multicentre study showed that 12% of women with low- to intermediate-risk EC had LN metastases that would have been overlooked if LN staging had not been performed (6). This partially explains the great heterogeneity of recurrence rates in women with presumed early-stage tumors. Hence, the main challenge for physicians managing women with early-stage grade 1 – 2 EC is when to opt for lymphadenectomy and, in the absence of data on lymph node status, when to opt for adjuvant therapy.

To address this issue, we assessed miRNA level expression in primary tumors according to LN status. According to our chip analysis, seven miRNAs were downregulated and five upregulated more than two-fold between the positive LN vs. negative LN groups. Also, according to the enrichment analysis, the validated target genes for these differentially expressed miRNAs were exclusively involved in carcinogenic pathways, underlining the strength of our analysis. These results support data from the current literature showing that miRNAs can act as tumor suppressors or oncogenes in various cancers (13) including EC (22,23). Furthermore, recent evidence indicates that several miRNAs, including miR 34, regulate the metastatic process through the expression of epithelial to mesenchymal transition (EMT) – transcriptions factors (TF)s or EMT-activating signalling pathways (SNAIL1/SNAIL2, basic helix-loop-helix (bHLH), E47, E2-2, TWIST1/TWIST2, and ZEB (ZEB1/ZEB2) families) (24) that act as E-cadherin repressors (25) and, ultimately, enhance cell migration and invasiveness in various cancers including EC (26–28).

After validation by RT-qPCR of miRNA selected from the previous step, we found that miR 34b-5p, miR 34c-5p, miR 34c-3p, miR 375, and miR 184 emerged as being particularly relevant to determine positive or negative LN metastatic status in women with grade 1 – 2 early-stage EC. Furthermore, miR 34b-5p, miR 34c-5p, miR 34c-3p, and miR 184 dominated the first principal component of the PCA analysis. This is in accordance with current literature: the miR-34 family (miR-34a, b and c) is attracting a lot of attention since it

has been found to play a key role as a tumor suppressor in several cancers (29). Indeed, it is a direct target of the tumor suppressor gene p53 inducing apoptosis, cell cycle arrest and senescence when upregulated. It also negatively influences the viability of cancer stem cells and inhibits metastasis formation. The miR 34 family acts on apoptosis and cell cycle through the repression of many proteins involved in the regulation of these two biologic processes. In particular, the miR-34 family members bind to the 3'-UTRs of genes such as CDK4 and CDK6 (cell cycle proteins), Bcl-2 (an apoptosis regulator), SNAIL (epithelial mesenchymal transition) and CD44 (migration and metastasis), thus repressing their expression (29). Numerous studies have shown the dysregulation of miR 34 in various types of cancers, including hepatocellular, mesothelial and colon cancer, melanomas, leukemia, nasopharyngeal cancer (30), prostate cancer (31), neuroblastomas (32), glioblastoma (33) and breast cancer (34). Yet, little research has been conducted in EC, apart from a functional study by Li et al. demonstrating that miR-34c acts as a tumor suppressor in HEC-1-B cells, and that E2F3 protein may be a target of miR-34c (35).

In the same way, miR-184 functions as a tumor suppressor in various cancers (renal carcinoma (36), glioma (37,38), non-small-cell lung cancer (39), oral squamous cell carcinoma (40), breast cancer (41)) by repressing oncogenes such as TNFAIP2 (37), SND1 (38), CDC25A, c-MYC (39) and regulating the AKT/mTORC1 pathway (41). As suggested by various studies, miR 184 might be a useful diagnostic and therapeutic tool for malignant diseases (38,39). However, to the best of our knowledge, ours is the first study to provide data about miR 184 expression in EC.

Finally, a recent review by Yan et al. emphasized the emerging role of miR-375 in cancer and specified that miR-375 is frequently downregulated in multiple types of cancer, especially in hepatocarcinoma (42), esophagus cancer (43), osteosarcoma (44), lung cancer (45), and acts as a tumor suppressor by repressing many critical oncogenes such as PDK1,

JAK2, IGF1R (46), AEG-1 (42), and suppressing the PI3K/Akt pathway (44). Furthermore, miR-375 might be used as a diagnostic and prognostic biomarker in various cancers. In this setting, reduced miR-375 expression could be a predictor of poor outcome (HR: 12.8, 95% CI: 3 to 49) and distant metastasis (HR: 8.7, 95% CI: 2 to 31) in head and neck squamous cell carcinomas (47). Also, women with metastatic non-small-cell lung cancer (NSCLC) had lower plasma miR 375 expression than those with non-metastatic NSCLC ($p<0.05$) (45). However, miR-375 has only rarely been assessed in EC. One study found miR 375 to be associated with histologic type EC (downregulated in papillary-serous carcinoma compared with carcinosarcoma) (FC=-5 .1, p-value=3.8E-04) (48).

We focused our analysis on post-operative FFPE EC primary tumors since it is known that FFPE specimens can be used for real-time PCR-based quantitative miRNA expression studies (16). This meant we could draw on the entire specimen collection of the Pathology Department containing samples taken between 2003 and 2012, and select samples to form two groups fully respecting the matching criteria. However, to provide a diagnostic tool, this analysis should be validated on preoperative samples. Unfortunately, assessing miRNA expression in preoperative biopsy samples might be difficult due to the contamination of normal endometrial or myometrial tissue. A heterogenic sample, with distinct miRNA profiles (13), could lead to a wrong analysis. Interestingly, two recent studies have demonstrated that circulating miRNAs have a high accuracy in diagnosing endometrioid EC ((miR-15b, -27a, and -233) (49) and (miR-9/miR-1228 and miR-9/miR-92a) (50)). The miRNA described in our study may be preoperatively assessed in blood samples of women with grade 1 – 2 early-stage EC and might serve as a novel, non-invasive biomarker in the future, avoiding discrepancies in histologic type and grade between preoperative biopsies and final histology.

Some limitations of the study should be underlined. Because of the use of FFPE specimens with insufficient quality and RNA integrity, we could not assess the expression of

exact targets for the miRNAs validated in this study. Future research is needed to resolve this problem.

Conclusion

In conclusion, this is the first study investigating the relative expression of mature miRNA genes in early stage grade 1 - grade 2 EC primary tumors according to the nodal status. This miRNA expression profile may provide a basis for further study of the miRNA function in endometrioid adenocarcinoma, and be used as a diagnostic tool for nodal status.

Disclosure/conflict of interest

Geoffroy Canlorbe received support from Association pour la Recherche sur le Cancer (ARC). The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1 Flowchart describing the constitution of groups and the experimental design.
EC, endometrial cancer; FFPE: formalin-fixed paraffin-embedded; FIGO, International Federation of Gynaecology and Obstetrics; LVSI, lymphovascular space involvement; miRNA, microRNA; RT, reverse transcription; qPCR, quantitative real time polymerase chain reaction

Figure 2 (a) Heat map of microRNA deregulation in positive LN vs. negative LN FFPE grade 1-2 EC primary tumor specimen. 142 probes had consistent normalized fluorescent intensities between the two groups, negative LN vs. positive LN ($p<0.05$). **(b)** Volcano plot. Abscissa is the logarithmic value of the level of variation (LogRatio) and ordinate is the negative logarithm of the statistical value (-log (p)) of fluorescence intensities of the hybridized probes from positive or negative LN samples. There was more than a two-fold change in normalized fluorescence intensity of 12 microRNAs (blue triangle) between the positive LN vs. negative LN groups ($p < 0.05$): 7 have decreased intensity (on the left) and 5 have increased intensity (on the right).
EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LN, lymph node

Figure 3 Enrichment analysis. Gene ontology enrichment analysis was performed (Genomatix GePS (release 2.4.0, Genomatix BH, Munich)) using the list of all known validated targets for the miRNAs exhibiting at least a two-fold change, with significant value ($p<0.05$), in the EC primary tumors with positive lymph node.
EC, endometrial cancer

Figure 4 qRT-PCR assay. The expression levels of 5 microRNAs (miR 34c-5P, miR 375, miR 184, miR 34c-3p, and miR 34b-5p) were significantly lower in the grade 1-2 EC FFPE primary tumor samples with positive LN compared to those with negative LN. Mann-Whitney test, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; mean ± SEM

EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LN, lymph node

Figure 5 (a) After eigenvalue decomposition of 10 original dimensions (miR 34c-5p, miR 375, miR 184, miR 34c-3p, miR 34b-5p, miR 148a-3p, miR 504-5p, miR 5001-5p, miR 6068, and miR 4467), 4 principal components were found to be significant (with an eigenvalue >1). These components explained 75.72% of the variance components. **(b)** Biplot of the first and second components. There is an opposition between microRNAs that have been defined by microarray as being upregulated (miR 129-5p, miR 6068, miR 5001-5p, miR 4467) and downregulated (miR 34c-3p, miR 34c-5p, miR 34b-5p, miR 375, miR 504-5p, miR 184, and miR 148a-3p) in positive LN group.

PC, principal component; LN, lymph node

Figure 6 Optimal fold-change cut-offs denoting a correlation between microRNA expression and lymph node status in histologic grade 1-2 EC primary tumor specimens.

(a) miR 34c-5p; **(b)** miR 375; **(c)** miR 184; **(d)** miR 34c-3p; **(e)** miR 34b-5p; **(f)** miR 148a-3p; **(g)** miR 504-5p; **(h)** miR 129-5p; **(i)** miR 5001-5p; **(j)** miR 6068; **(k)** miR 4467

EC, endometrial cancer

Table 1 Epidemiologic and histologic characterizations between women with negative or positive LN.

EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LN, lymph node; IQR, interquartile range; BMI, body mass index; FIGO, International Federation of Gynaecology and Obstetrics; ESMO, European Society for Medical Oncology; LVSI, lymphovascular space involvement

		EC FFPE primary tumor Negative LN n=27	**EC FFPE primary tumor Positive LN n=9**	**P-value**
Age, median (IQR)		72 (65 - 78)	68 (64 - 76)	0.84
Diabetes n(%)		3 (11,1%)	0 (0%)	0.56
Dyslipidemia n(%)		3 (15.8%)	1 (20%)	1
Parity, median (IQR)		2 (1 - 3)	1 (0 - 3)	0.85
BMI, median (IQR)		24 (22 - 27)	25 (20 - 29)	0.92
FIGO Stage, n(%)				
	IA	13 (48.1%)	4 (44.4%)	1
	IB	14 (51.9%)	5 (55.6%)	
Histologic grade, n(%)				
	Grade 1	9 (33.3%)	3 (33.3%)	1
	Grade 2	18 (66.7%)	6 (66.7%)	
Tumor size (mm), median (IQR)		40 (25 - 40)	40 (25 - 42)	0.82
ESMO risk group, n(%)				
	Low risk	13 (48.1%)	4 (44.4%)	1
	Intermediate risk	14 (51.9%)	5 (55.6%)	
LVSI, n(%)				
	Yes	9 (33.3%)	3 (33.3%)	1
	No	18 (66.6%)	6 (66.6%)	
Time between surgery and sample analysis (months), median (IQR)		32 (21 - 79)	30 (15 - 60)	0.95

Table 2 List of the downregulated (fold-change<-2, p-value<0.05) and upregulated (fold-change>2, p-value<0.05) microRNA between positive LN *vs.* negative LN FFPE histologic grade 1-2 EC primary tumor specimen.

LN, lymph node; EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded

Downregulated microRNAs				**Upregulated microRNAs**		
Name	**Fold Change**	**P-Value**		**Name**	**Fold Change**	**P-Value**
miR 34c-5p	-5.92	0.009		**miR 129-5p**	2.90	0.033
miR 375	-5.39	0.020		**miR 5001-5p**	2.26	0.026
miR 184	-5.24	0.031		**miR 6068**	2.11	0.036
miR 34c-3p	-5.07	0.017		**miR 4467**	2.08	0.047
miR 34b-5p	-4.04	0.016		**miR 6850-5p**	2.06	0.042
miR 148a-3p	-2.48	0.031				
miR 504-5p	-2.06	0.025				

Table 3 Correlations between initial variables and principal components. The first principal component, accounting for 32.02% of the variance, was dominated by tumor-suppressor micro RNAs, especially of the miR 34 family, including miR 34b-5p, miR 34c-5p and miR 34c-3p. The second component, accounting for 24.31% of the variance, was dominated by miR 129-5p.

Principal component 1				**Principal component 2**		
Micro RNA	**correlation**	**p-value**		**Micro RNA**	**correlation**	**p-value**
miR 34b-5p	0.85	5.03e-11		miR 129-5p	0.71	1.30e-06
miR 34c-5p	0.85	8.83e-11		miR 34c-3p	0.65	1.56e-05
miR 34c-3p	0.64	2.93e-05		miR 6068	0.63	3.43e-05
miR 184	0.53	8.87e-04		miR 5001-5p	0.63	3.51e-05
miR 504-5p	0.50	1.80e-03		miR 4467	0.48	2.80e-03
miR 148a-3p	0.40	1.61e-02		miR 34c-5p	0.44	6.74e-03
miR 129-5p	-0.45	5.33e-03		miR 34b-5p	0.43	8.98e-03
miR 6068	-0.51	1.57e-03		miR 148a-3p	-0.45	6.06e-03
miR 5001-5p	-0.60	1.43e-04				

Table 4 Optimal fold-change cut-offs denoting the strongest correlation between micro RNA expression and LN status in histologic grade 1-2 EC primary tumor specimens.

EC, endometrial cancer; FFPE, formalin-fixed paraffin-embedded; LN, lymph node; FC, fold-change

		EC FFPE primary tumor Negative LN N=27	**EC FFPE primary tumor Positive LN N=9**	**p-value**
miR 375				
	FC > 0.30	20 (95.2%)	1 (4.8%)	0.001
	FC < 0.30	7 (46.7%)	8 (53.3%)	
miR 184				
	FC > 0.30	23 (88.5%)	3 (11.5%)	0.006
	FC < 0.30	4 (40.0%)	6 (60.0%)	
miR 34c-5p				
	FC > 0.0852	18 (90.0%)	2 (10.0%)	0.049
	FC < 0.0852	9 (56.3%)	7 (43.7%)	
miR 34b-5p				
	FC > 0.09	23 (85.2%)	4 (14.8%)	0.026
	FC < 0.09	4 (44.4%)	5 (55.6%)	
miR 148a-3p				
	FC > 0.50	22 (88.0%)	3 (12.0%)	0.012
	FC < 0.50	5 (45.5%)	6 (54.5%)	
miR 129-5p				
	FC > 1.45	3 (37.5%)	5 (62.5%)	0.013
	FC < 1.45	24 (85.7%)	4 (14.3%)	
miR 4467				
	FC > 1.75	3 (42.9%)	4 (57.1%)	0.0497
	FC < 1.75	24 (82.8%)	5 (17.2%)	

Inclusion criteria

- Primary surgical treatment for EC with total hysterectomy and systematic nodal staging
- Endometrioid adenocarcinoma EC
- Grade 1 - Grade 2 EC
- FIGO stage IA - IB

Exclusion criteria

- Previous malignancies
- History of chemotherapy
- History of radiotherapy
- Lynch syndrome
- Inflammatory disease

FFPE EC primary tumor with positive lymph node n=9

Matching criteria1:3

- FIGO stage
- Histologic grade
- LVSI status

FFPE EC primary tumor with negative lymph node n=27

MicroRNA screening phase

Material: hybridization on miRNA 4.0 chips (Affymetrix)

Samples: 9 (FFPE EC primary tumor with positive lymph node) vs. 9 (27 FFPE EC primary tumor with negative lymph node, pooled by 3)

Statistical analysis: microarray analysis (p-value ≤ 0.05 and variation > 2); enrichment analysis

Validation by RT-qPCR

Analysis of 10 candidate microRNAs

Material: RT-qPCR

Samples: 9 (FFPE EC primary tumor with positive lymph node) vs. 27 (FFPE EC primary tumor with negative lymph node)

Statistical analysis: principal component analysis; minimal p-value approach

Figure 1

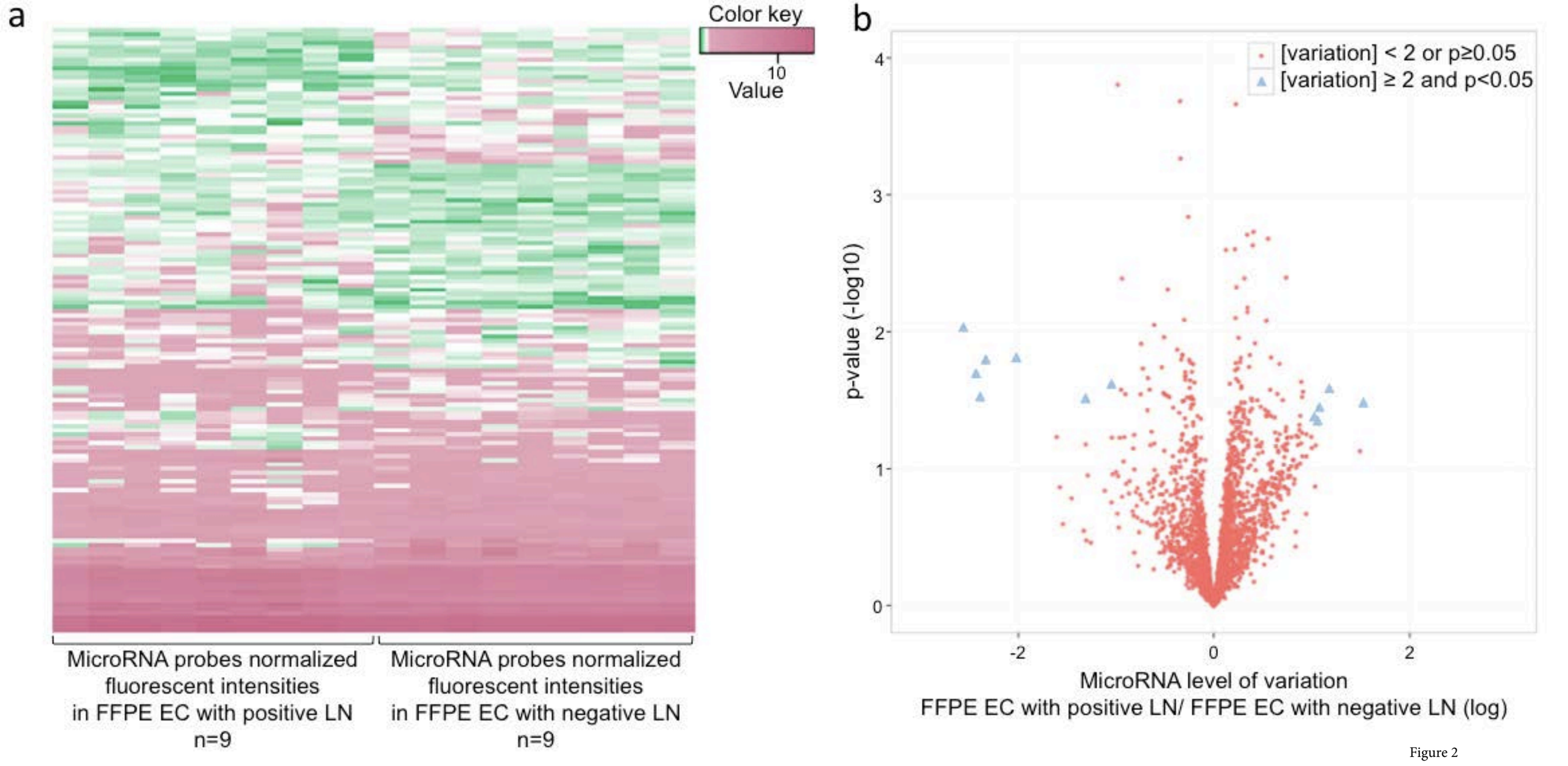

a
Color key
10
Value
MicroRNA probes normalized fluorescent intensities in FFPE EC with positive LN n=9
MicroRNA probes normalized fluorescent intensities in FFPE EC with negative LN n=9
b
[variation] < 2 or p≥0.05
[variation] ≥ 2 and p<0.05
p-value (-log10)
4
3
2
1
0
-2
0
2
MicroRNA level of variation
FFPE EC with positive LN/ FFPE EC with negative LN (log)

Figure 2

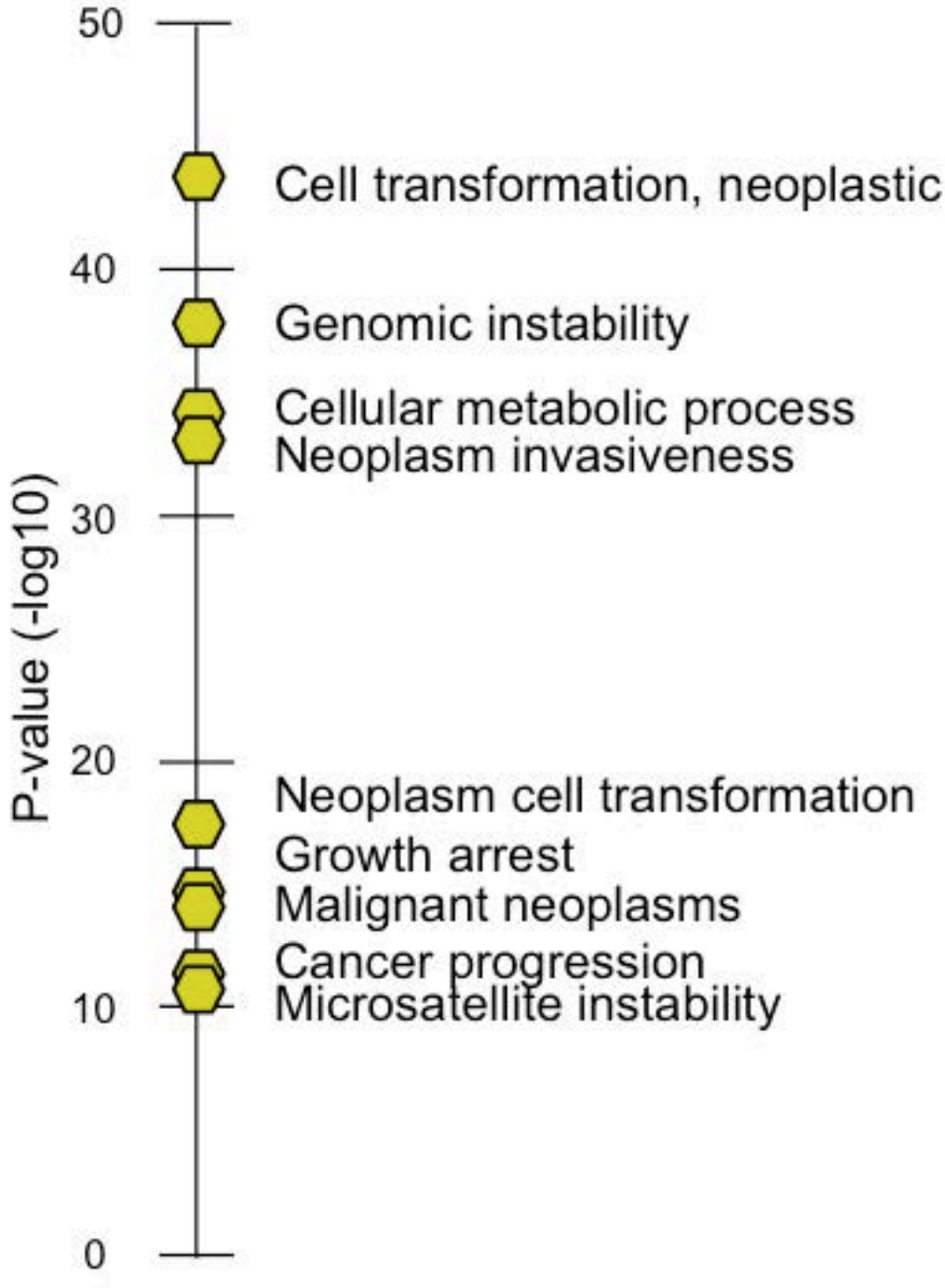
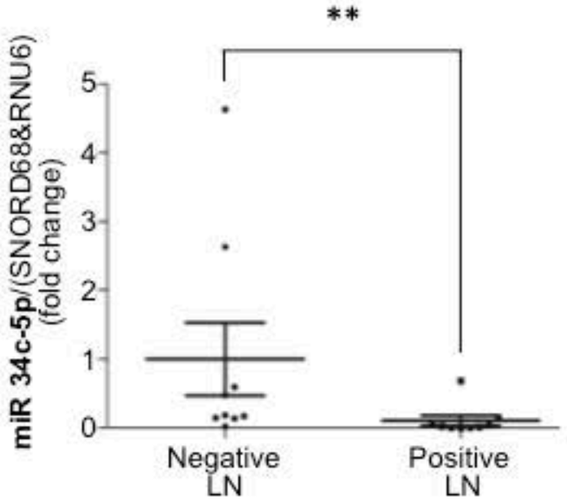
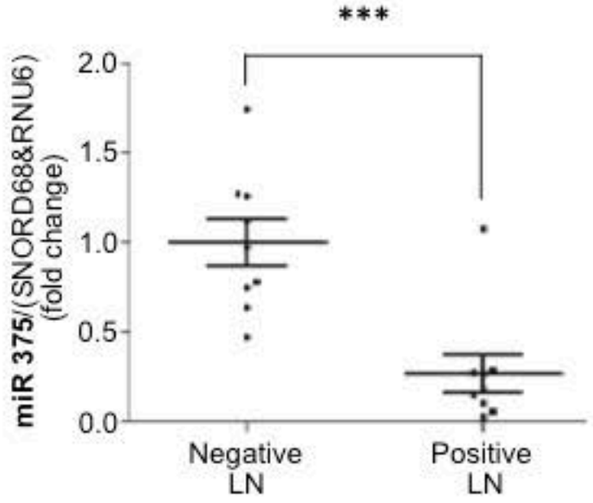
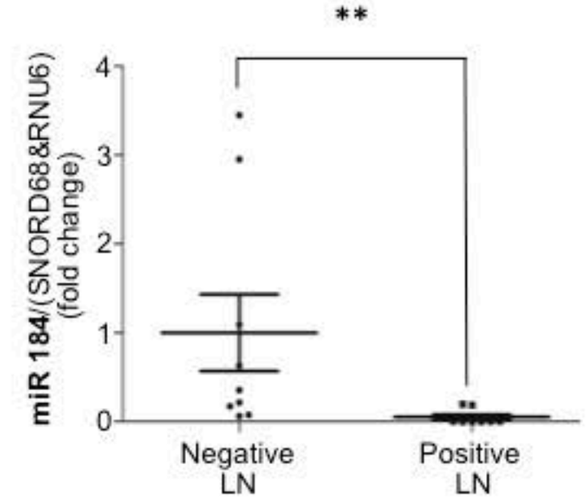
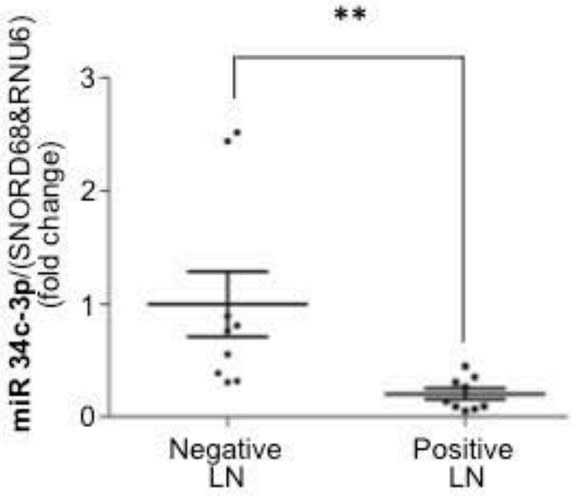

50
40
30
20
10
0
P-value (-log10)
Cell transformation, neoplastic
Genomic instability
Cellular metabolic process
Neoplasm invasiveness
Neoplasm cell transformation
Growth arrest
Malignant neoplasms
Cancer progression
Microsatellite instability

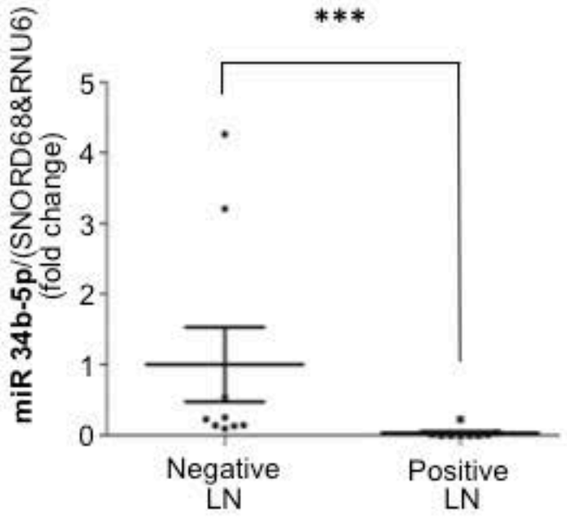
Figure 3

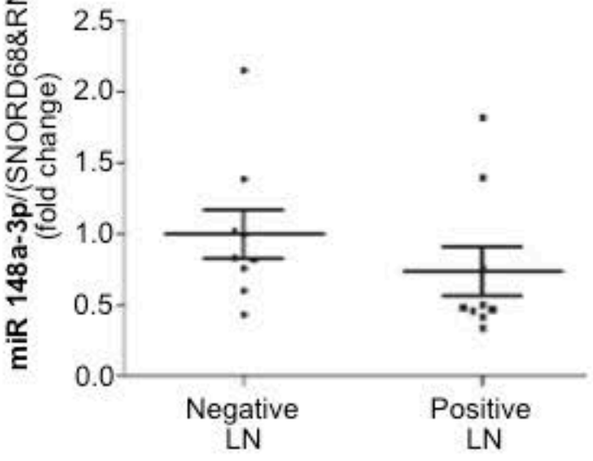

**
miR 34c-5p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN

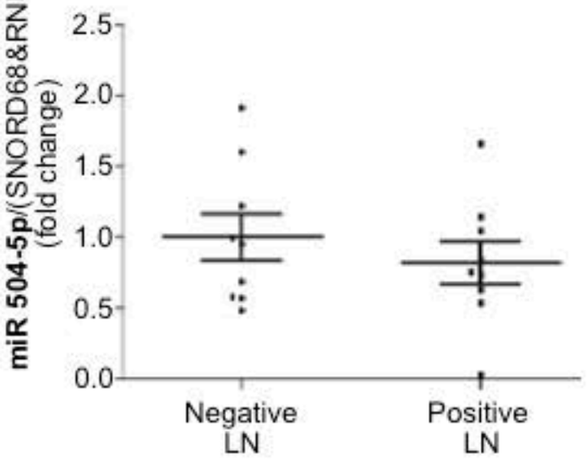

***
miR 375/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN

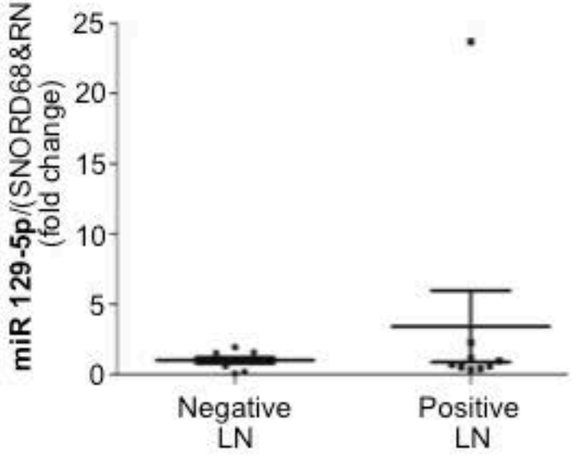

**
miR 184/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN

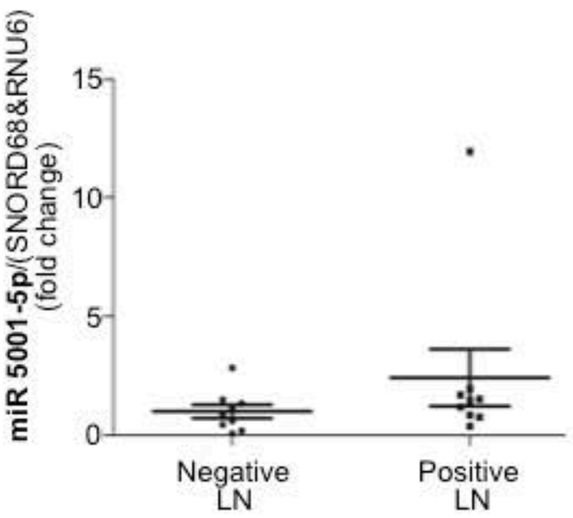

**
miR 34c-3p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN

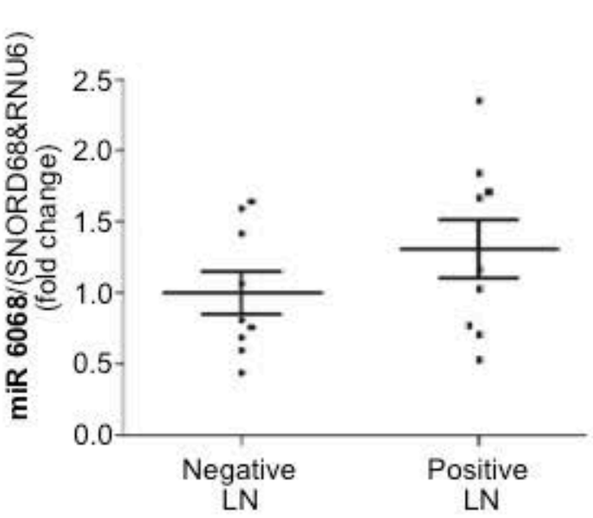

***
miR 34b-5p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN


miR 148a-3p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN


miR 504-5p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN


miR 129-5p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN


miR 5001-5p/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN


miR 6068/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN

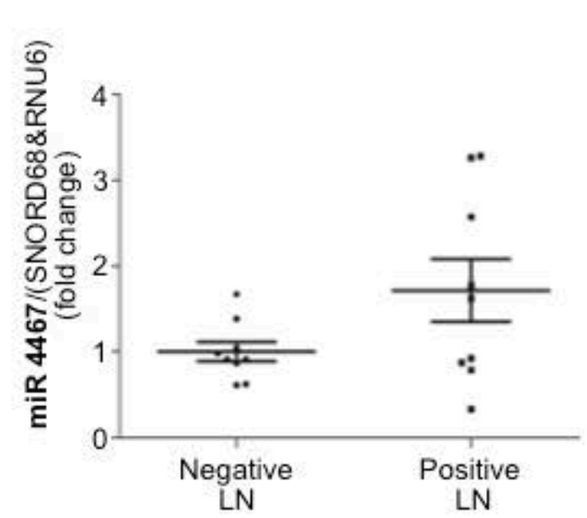
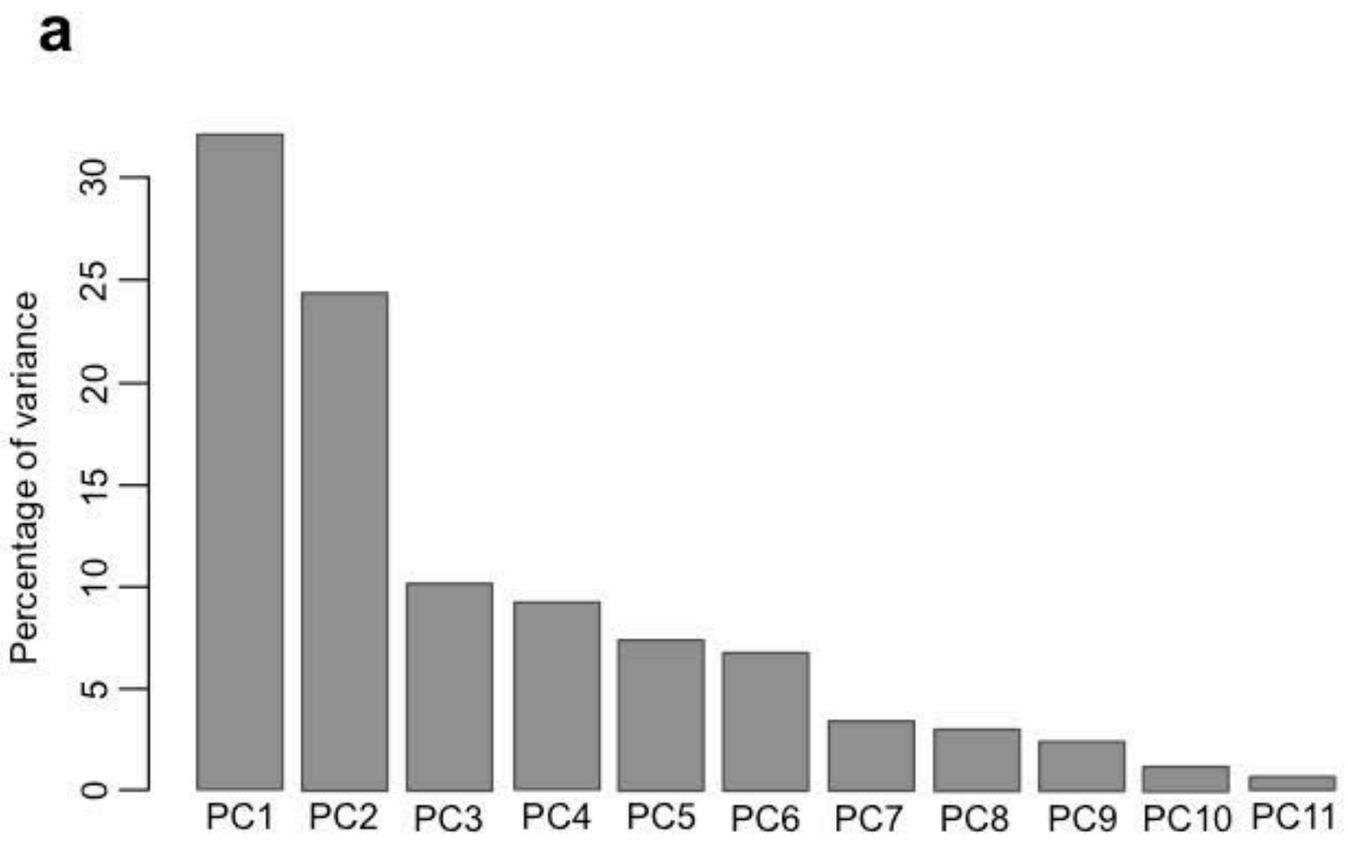

miR 4467/(SNORD68&RNU6) (fold change)
Negative LN
Positive LN

Figure 4



	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11
Eigenvalue	3.52	2.67	1.12	1.01	0.80	0.74	0.37	0.34	0.27	0.14	0.02
Percentage of variance	32.02	24.31	10.20	9.19	7.29	6.68	3.36	3.06	2.44	1.25	0.20
Cumulative percentage of variance	32.02	56.33	66.53	75.72	83.02	89.70	93.06	96.12	98.55	99.80	100.00

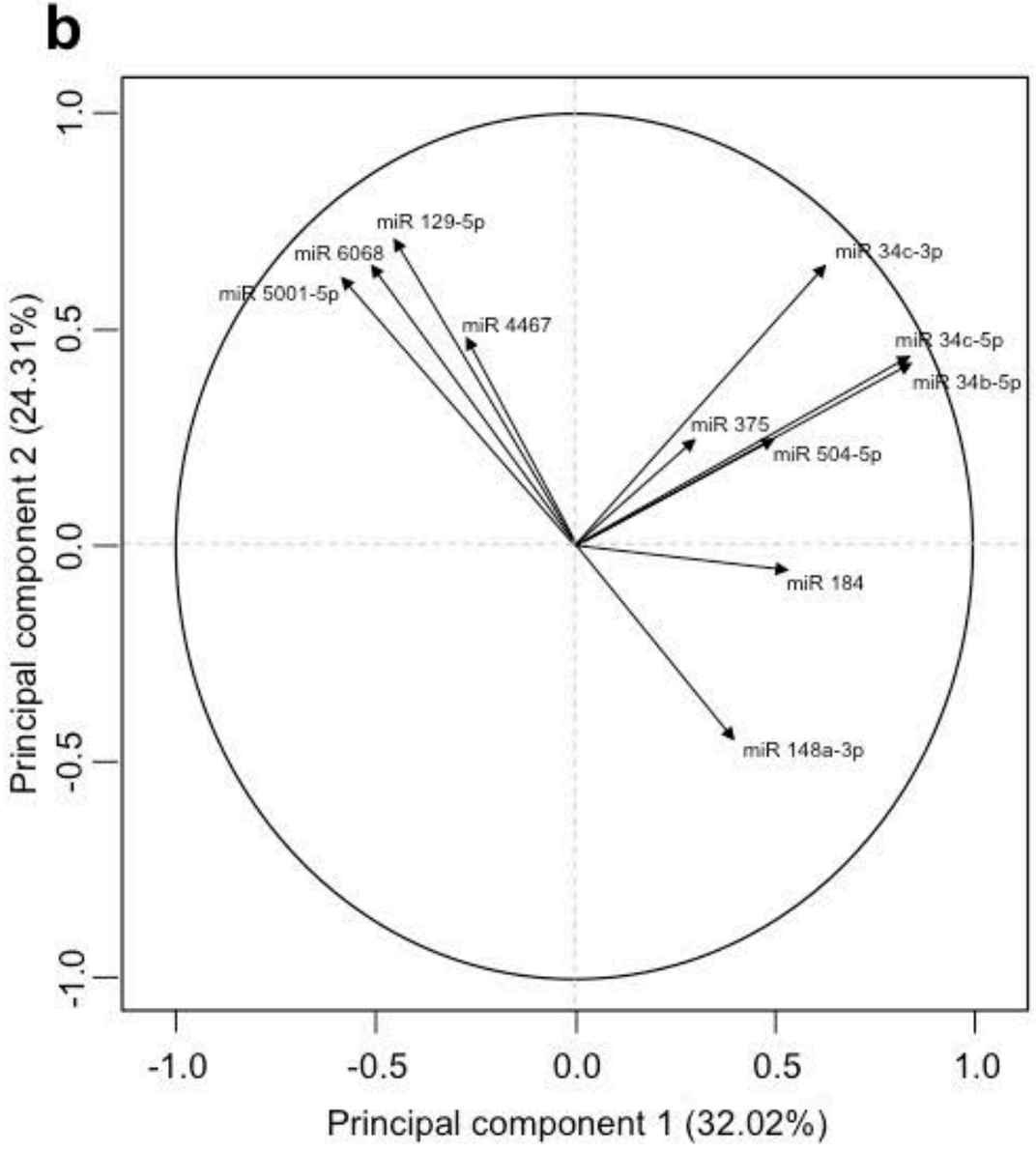


Figure 5

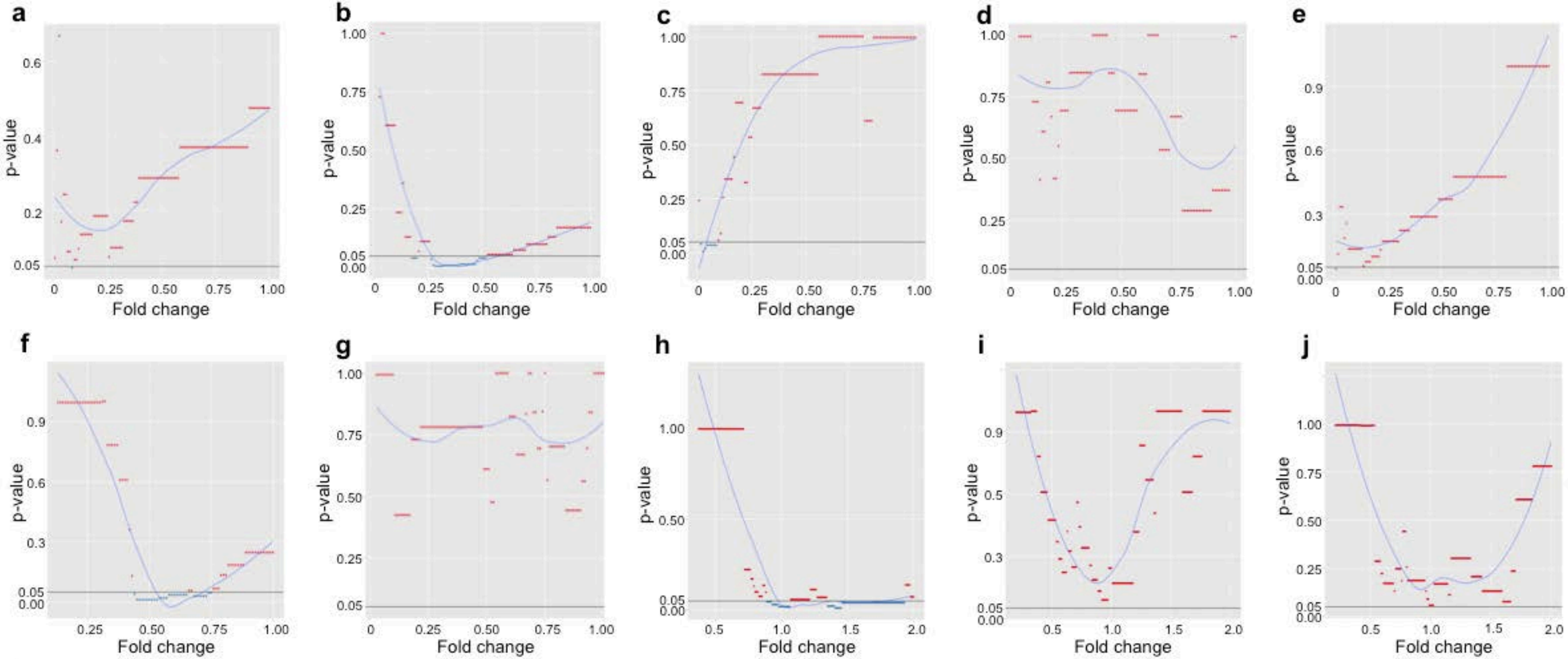

a
b
c
d
e
f
g
h
i
j
k
p-value
Fold change
Significativity
Non significant (p≥0.05)
Significant (p<0.05)

Figure 6