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A microRNA expression signature for clinical response in locally advanced cervical cancer



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HIGHLIGHTS

- Nearly 50% of locally advanced cervical cancer patients have an unfavorable pathological response to conventional treatment.
- MicroRNAs are potential biomarkers in cervical cancer
- We identify microRNAs that can be used as molecular markers to predict pathological response in cervical cancer patients

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ABSTRACT

Objective. Nearly 50% of patients who are diagnosed with locally advanced cervical cancer have an unfavorable pathological response to conventional treatment. MicroRNAs (miRNAs) are potential biomarkers in cervical cancer; however, their role in identifying patients who do not respond to conventional treatment remains poorly investigated. Here, we identify a set of miRNAs that can be used as molecular markers to predict the pathological response in locally advanced cervical cancer patients receiving radiation and chemotherapy treatment.

Methods. Forty-one patients diagnosed with locally advanced cervical cancer were invited to participate in this study and enrolled after they signed an informed consent. Two patient cohorts were randomized for miRNA expression profiling, a discovery cohort (n = 10) and a validation cohort (n = 31); profiling was performed by means of a miScript miRNA PCR Array. After a median clinical follow-up of 45 months, statistical analysis was performed to identify miRNAs that could discriminate non-responders from complete pathological responders to conventional treatment.

Results. miRNA expression profiling identified 101 miRNAs that showed significant differences between non-responders and complete pathological responders (p < 0.05). Seven differentially expressed miRNAs were selected, and their expression patterns were confirmed in the validation phase; thus, miR-31-3p, -3676, -125a-5p, -100-5p, -125b-5p, and -200a-5p and miR-342 were significantly associated with clinical response. Expression of this miRNA signature above the median level was a significant predictor of non-response to standard treatment (p < 0.001).

Conclusions. These seven validated miRNA signatures could be used as molecular biomarkers of chemo- and radio-resistance in locally advanced cervical cancer patients.

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1. Introduction

Cervical cancer (CC) is an important health problem in developing countries, where it ranks as the second leading cause of cancer-related death [1]. Approximately 265,000 women died from CC in 2012; 87% of them were in less developed countries [2]. Despite early detection

programs, nearly 50% of these patients are diagnosed with locally advanced stages (LACC, stages IB2 to IVA according to FIGO) [3]. Standard treatment for LACC patients consists of radiotherapy in combination with cisplatin-based chemotherapy [4]. Unfortunately, nearly 50% of patients do not respond to standard treatment; these patients have a higher recurrence rate and worse survival in the first five years [5]. At present, no specific molecular markers can predict the clinical response of patients diagnosed with LACC.

MicroRNAs, or miRNAs, are small, non-coding RNAs that can post-transcriptionally inhibit their target genes due to the complementarity of bases to the 3' non-translatable region (3' UTR) of their target mRNAs. miRNAs are key regulators of gene expression because of their role in major biological processes, such as cell differentiation [6], cell growth [7], apoptosis [8], hematopoiesis [9], viral infections [10], and carcinogenesis [11–13].

Recently, several studies have investigated the role that miRNAs play in tumor radio- and chemo-resistance. For instance, in non-small cell lung cancer (NSCLC), nine miRNAs, including miR-29b-3p, miR-200a-3p, and miR-126-3p were significantly down-regulated, whereas miR-208a was up-regulated in the serum of NSCLC patients after radiation treatment ($p < 0.05$). Interestingly, miR-208a promotes cell proliferation and induces radio-resistance by targeting p21 with corresponding activation of the AKT/mTOR pathway [14]. In breast cancer, five miRNAs have been associated with overall survival in patients receiving systemic therapy [15]. With respect to CC, Ke et al. found that over-expression of miR-181a is linked to radio-resistance in locally advanced cervical tumor samples; moreover, they demonstrated that the pro-apoptotic protein kinase, PRKCD, is a validated target of miR-181a [16].

In the present study, we report a set of miRNAs that can be used as molecular markers to predict clinical outcomes in LACC patients receiving radiation and chemotherapy treatment. We hypothesized that primary tumors have a set of miRNAs capable of predicting the potential tumor response to conventional treatment; hence, the accurate identification of miRNAs that are involved in the innate resistance could be employed as a prognosis signature associated with clinical response. We identified a molecular signature consisting of seven validated miRNAs (miR-31-3p, miR-3676, miR-342, miR-125a-5p, miR-125b-5p, miR-100-5p and miR-200a-5p). Moreover, the bioinformatic analysis revealed new potential signaling pathways that could be involved in chemo-radiotherapy resistance phenotypes, such as the Wnt, Notch, and ErbB signaling pathways.

2. Materials and methods

2.1. Tumor samples

Upon diagnosis, 41 patients from the National Cancer Institute of Mexico (INCan) were invited to participate in this study and enrolled after they signed an informed consent form approved by the scientific and ethics committees of the INCan. As soon as the biopsy was obtained, the tumor samples were split into three pieces: one for pathologic confirmation of at least 80% of tumor cells, and the remaining two for RNA and DNA isolation.

All patients in our study received the same standard treatment, consisting of 5 weekly cycles of 40 mg/m² of CDDP [cis-diamminedichloroplatinum (II)] and 67 days of radiotherapy for a total of 50–55 Gy in addition to 30 Gy of intracavitary brachytherapy. Patients had follow-up appointments approximately every 3 or 4 months for the first 2 years, every 6 months for the following 3 years, and annually thereafter. Patients who had a complete pathological response and “non-responders” (NRs) who had progressive disease were randomly selected from this patient cohort. After a clinical follow-up period of 45 months, we selected 41 patients who were categorized into two groups (21 patients who had a complete response (CRs) and 20 patients who had no response to clinical treatment or were NRs). In this

study, two patient cohorts were randomized for miRNA expression profiling, a discovery cohort ($n = 10$) and a validation cohort ($n = 31$).

2.2. Clinical definitions

Clinical responses were evaluated using the RECIST 1.1 criteria and computed axial tomography scans. The responses were classified as complete response (CR), defined as the disappearance of all signs of cancer in response to treatment, and no response (NR), defined as patients with partial, progressive, or stable disease [17].

2.3. HPV genotyping in the tumor samples

The MagNAPure instrument was used to extract DNA from the tumor samples according to the manufacturer's instructions. HPV genotyping was performed using two methods: linear array HPV genotyping and nested multiplex polymerase chain reaction (NM-PCR MY/GP primers) followed by direct sequencing of the PCR fragment according to previously reported processes [18].

2.4. RNA extraction

Total RNA was extracted from the tumor samples using Trizol reagent (Invitrogen, CA Cat. #15596-026) and subsequently purified with the miRNeasy Mini Kit (QIAGEN Cat. # 217004), according to the manufacturer's instructions. RNA quantification was performed using an Epoch spectrophotometer (BioTek Instruments Inc.).

2.5. miRNA expression profiles

miRNA expression profiles were measured using miScript miRNA PCR Array (SABiosciences Cat. # 331222). Briefly, 800 ng of RNA was used to synthesize cDNA; qPCR reactions were then performed on a LightCycler 480 (Roche, Mannheim). The raw data were analyzed using the Abs Quant/2nd Derivative Max method. Expression values were normalized with respect to six endogenous miRNAs: SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A and RNU6B/RNU6-2; differential expression was calculated using the Delta-Delta Ct method [19].

2.6. Statistical analysis

A *t*-test was used to identify significant differences in miRNA expression profiles between patients with a CR and those who were NRs. miRNAs with a $p < 0.05$ were considered to be significant. Unsupervised and supervised cluster hierarchical analyses were performed using Bioconductor and Biobase, bioDist, gplots and gene filter packages implemented in RStudio software (Ver. 0.98.501) [20].

2.7. Validation of the miRNA profile by RT-qPCR

Seven miRNAs were selected from the significant group to be validated by a second quantification method using TaqMan assays in the validation cohort ($n = 31$). Briefly, the reverse transcriptase reactions contained 10 ng of total RNA, 3 μ l of 5X RT primer, 1.5 μ l of 10X RT buffer, 0.25 mM of each dNTP, 50 U/ μ l of MultiScribe reverse transcriptase and 3.8 U/ μ l of RNase inhibitor. Reactions were performed in triplicate. Expression levels for both groups of patients (responders and non-responders) were determined using the Delta-Delta Ct method. We also used the *t*-test with Welch's correlation to identify significant differences between the complete response group and the group of patients without pathological response to conventional therapy.

2.8. Functional interpretation of miRNA profile

A bioinformatic approach based on miRNA-target databases was used to identify relevant mRNA targets and biological pathways. We used the list of miRNAs that were significant with $p < 0.05$ and had predicted mRNA-miRNA interactions. Finally, the resulting mRNA-miRNA interactions were visualized in KEGG-mirPath v.3 [21].

2.9. Disease-free survival

Disease-free survival (DFS) rates, as related to the expression of the validated seven-miRNA signature, were evaluated using the Kaplan-Meier method. The significance of the survival differences was determined by the log-rank test.

3. Results

3.1. Clinicopathologic characteristics

CC samples were collected from 41 patients with a clinical and pathological diagnosis of LACC (IB-IVA) at the INCan. All cervical samples were analyzed histologically to confirm a minimum of 80% of tumor cells; confirmed biopsies were then processed for nucleic acid isolation. Table 1 lists the major clinical characteristics of the patients. The median age at diagnosis was 48 years (range 32 to 63 years). The majority of patients were diagnosed as stages IIB (56.0%) and IIIB (26.8%); 85.3% had squamous cell carcinoma, and 14.7% had adenocarcinoma. The main HPV types were 16 (44.0%), 45 (24.4%) and 18 (19.5%). A relevant number of patients (17.1%) were infected with two or more HPV types. The median duration of clinical follow-up was 45 months. Twenty-one patients (51.2%) were classified as NRs, whereas 20 (48.8%) had a CR.

Table 1
Clinical and pathological characteristics of all patients involved in this study.

Characteristic	Patients	
	N = 41	
<i>Age</i>		
Median	48	
Range	29–68	
<i>Histological type</i>		
Squamous cell carcinoma	35	85.3%
Adenocarcinoma	6	14.7%
<i>Tumor size</i>		
<4 cm	11	26.8%
≥4 cm	29	70.7%
Without data	1	2.5%
<i>Clinical stage (FIGO)</i>		
IB2	2	4.87%
IIA	0	0.0%
IIB	26	63.41%
IIIA	0	0.0%
IIIB	11	26.82%
IVA	2	4.87%
<i>VPH genotype (Frequency)</i>		
Type 16	12	29.26%
Type 18	7	17.07%
Type 45	10	24.29%
Others	4	9.75%
Patients with HPVcoinfection	7	17.07%
Not determined	1	2.43%
<i>Treatment outcome^a</i>		
Complete response	21	51.2%
No response	20	48.8%
Without date for desertion	0	0.0%

^a All patients received radiotherapy and cisplatin as co-adjuvant (50 Gy external radiation, 35 Gy intracavitary brachytherapy and 6 cycles of 40 mg/m² CDDP).

3.1.1. miRNA signature of LACC patients

The miRNA profile was analyzed on an initial discovery cohort (NR = 5; CR = 5), using miRbase v. 16, which contains 1066 mature miRNAs. The median expression of each miRNA for both groups of patients was calculated and the differences between them were determined using the *t*-test. In total, 101 miRNAs showed significant differences between NR versus CR patients ($p < 0.05$; Supplementary Table 1).

To identify a molecular profile of miRNAs that could discriminate between the NR and CR patient groups, we employed a supervised classification approach based on expression values for each miRNA *t*-test ($p < 0.05$) and a fold change ≥ 2 (Fig. 1). Interestingly, we found that four miRNAs (hsa-miR144-3p, hsa-miR3176, hsa-miR31-3p and miR3176) were over-expressed whereas 97 were down-regulated in NR versus CR patients (Supplementary Table 1). Several miRNA families were represented, including the let-7 family (hsa-let-7d, hsa-let-7d*, hsa-let-7g) located at 3p21.1 and hsa-let-7f-2* located at Xp11.22, the miR-10 family (hsa-miR10a and hsa-miR-10a* located at 17q21.32, hsa-miR-10b located at 2q31.1) and the miR-125 family (hsa-miR-125a-5p located at 19q13.41, hsa-miR-125b located at 11q24.1 and hsa-miR-125b-2* located at 21q21.1). These results suggest a coordinate transcriptional regulation of the aforementioned chromosomal regions.

3.2. Validation by qRT-PCR

To validate the above-mentioned miRNA signature in terms of clinical response, we used a subset of 7 miRNAs (miR-31-3p, -3676, -125a-5p, -100-5p, -125b-5p, and -200a-5p and miR-342), which were assessed in an independent group of 31 samples from LACC patients by TaqMan qRT-PCR assays (Fig. 2). The results were consistent with the single miRNA qRT-PCR experiment and global expression data.

3.3. miRNA targets and signaling pathways associated with clinical response

To understand the putative targets and signaling pathways associated with conventional treatment resistance in LACC patients, we used a novel bioinformatic strategy that involved the identification of miRNA-mRNA regulatory pathways [22]. Therefore, we employed the list of 101 significantly de-regulated miRNAs between both groups of patients. The algorithm selected those miRNA-mRNA pairs that had three matches in different databases; one of the databases was miRecords, which includes information from experimentally validated data. Therefore, these identified mRNAs were considered bona fide miRNA target genes [23]. The selected mRNA targets were associated with the signaling pathways in which they are involved. To obtain a complete visualization of the signaling pathways based on mRNAs and their respective miRNA regulators, the mRNA targets and their associated miRNAs were visualized using KEGG-mirPath v.3. With this approach, we identified 18 miRNAs and 149 unique mRNA targets. The miRNA-mRNA interactions were represented in 14 KEGG-pathways that are associated with LACC patients who had no response to conventional treatment (Supplementary Table 2).

Integrating the miRNA-mRNA target information, we found that the most significant biological pathways were previously involved in the maintenance of the tumor phenotype, including pathways in cancer ($p = 3.34 \text{ e-}19$), the ErbB signaling pathway ($p = 7.72 \text{ e-}14$), the Jak-STAT signaling pathway ($p = 2.793 \text{ e-}02$), Focal Adhesion ($p = 5.825361 \text{ e-}05$), the Wnt signaling pathway ($p = 2.06 \text{ e-}02$) and viral carcinogenesis ($p = 8.71 \text{ e-}11$) (Supplementary Table 2). Figs. 3 and 4 illustrate some of the most significant pathways that maintain radiochemo-therapy-resistant phenotypes in CC (the ErbB, JAK-STAT, Wnt and Focal Adhesion pathways); specific miRNA and mRNA interactions are highlighted in red.

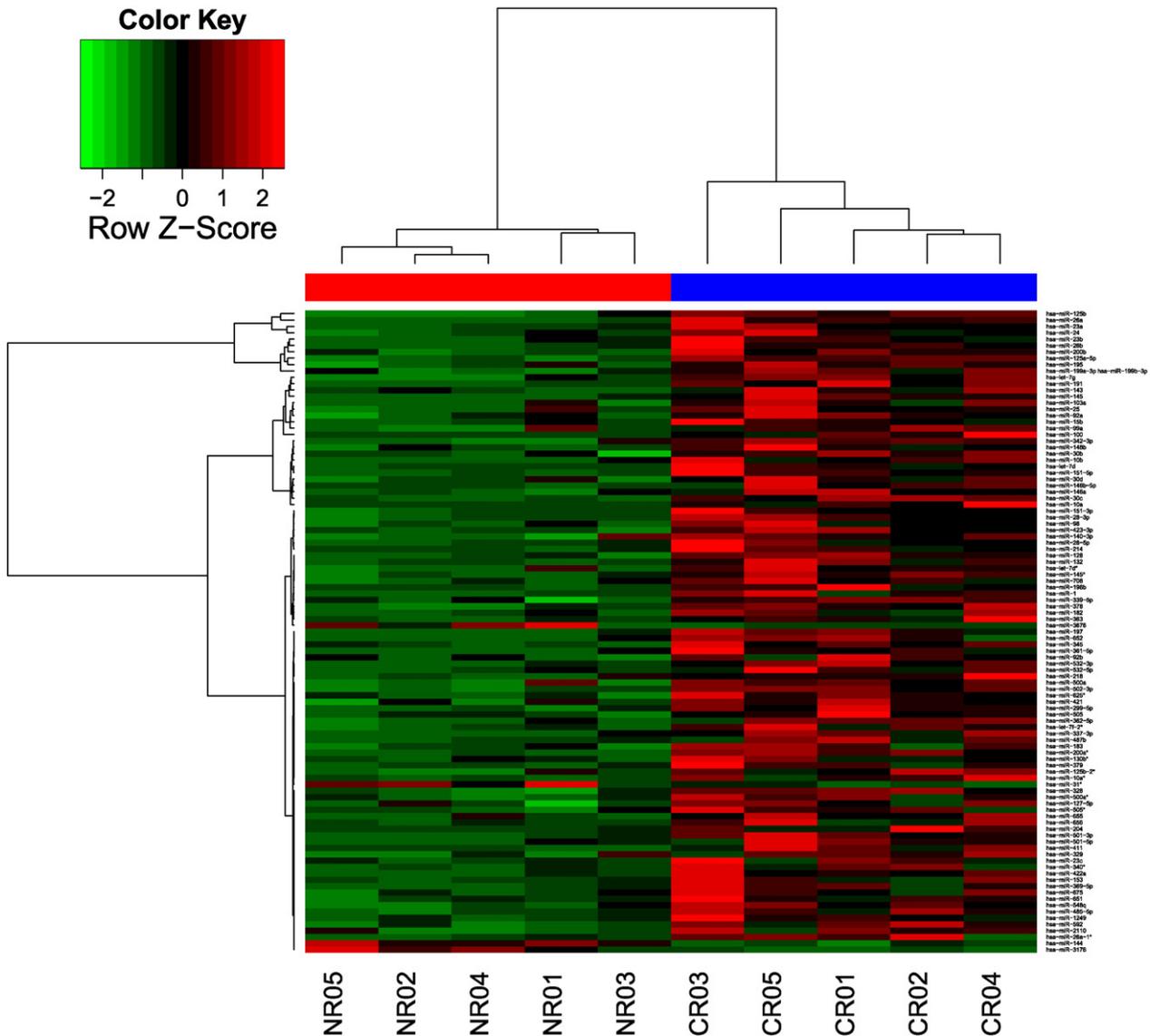


Fig. 1. Supervised hierarchical clustering of 10 cervical cancer samples using expression data from 101 miRNAs. We employed heatmap.2 function and Euclidean correlation; a $p < 0.05$ was considered to identify significantly differentially expressed miRNAs in patients with no response to conventional treatment. Columns display the clustering of tumor samples; rows indicate the clustering of miRNA names. Patient clinical outcome is indicated by colored squares: red for no response and blue for complete response to conventional treatment. All samples were properly assigned to the correct class based on the clinical outcome. The expression intensity of each miRNA in each sample varies from blue to red; for quantification of color codes, see color-bar on the left.

3.4. DFS

DFS was assessed relative to the seven miRNA signatures in LACC. Patients were dichotomized into two groups with low and high expression levels using the median value for each miRNA as the cutoff. Expression of a miRNA signature above the median level was a significant predictor of NR status to standard treatment ($p < 0.001$). The NR group had a mean DFS of 22 months; DFS has not yet been reached in the complete response group (Fig. 5).

4. Discussion

Several studies have suggested the use of miRNAs as biomarkers in several types of diseases, particularly in cancer, in which the results have been encouraging [24–28]. To date, no miRNA subset that can identify which LACC patients will not respond to conventional therapy has yet been found. Here, we report a subset of 101 miRNAs that permit the accurate classification of LACC patients

regarding clinical response, from which seven miRNAs were validated in an independent tumor cohort. These seven validated miRNA signatures could be used as molecular biomarkers of chemo- and radio-resistance.

Our results are similar to those published in a seminal work by Hu et al. Those authors quantified 96 cancer-related miRNAs in 59 FFPE cervical tumor tissues by a real-time PCR-based miRNA approach and reported two miRNAs (miR-200a and miR-9) with the highest predictive value associated with CC survival [29]. In our study, miR-200a was one of the seven validated miRNAs that significantly distinguished patients who did not respond to standard treatment. In the qPCR experiments used to obtain the global expression profile of the miRNAs, miR-200a was under-expressed 5.82 fold in non-response patients ($p = 0.00028$), while miR-200a was four times under-expressed in the TaqMan probe validation experiments ($p = 0.0001$). Along the same research line, we identified the possible miR-200a molecular targets, which include NRAS, NR4A1, MAPK8, PDGFA, TCF4, DKK2, PSEN1, Fzd1, NOTCH2 and NOTCH4 and are associated with the MAPK, Wnt, Notch and ErbB signaling pathways. Some of these pathways are

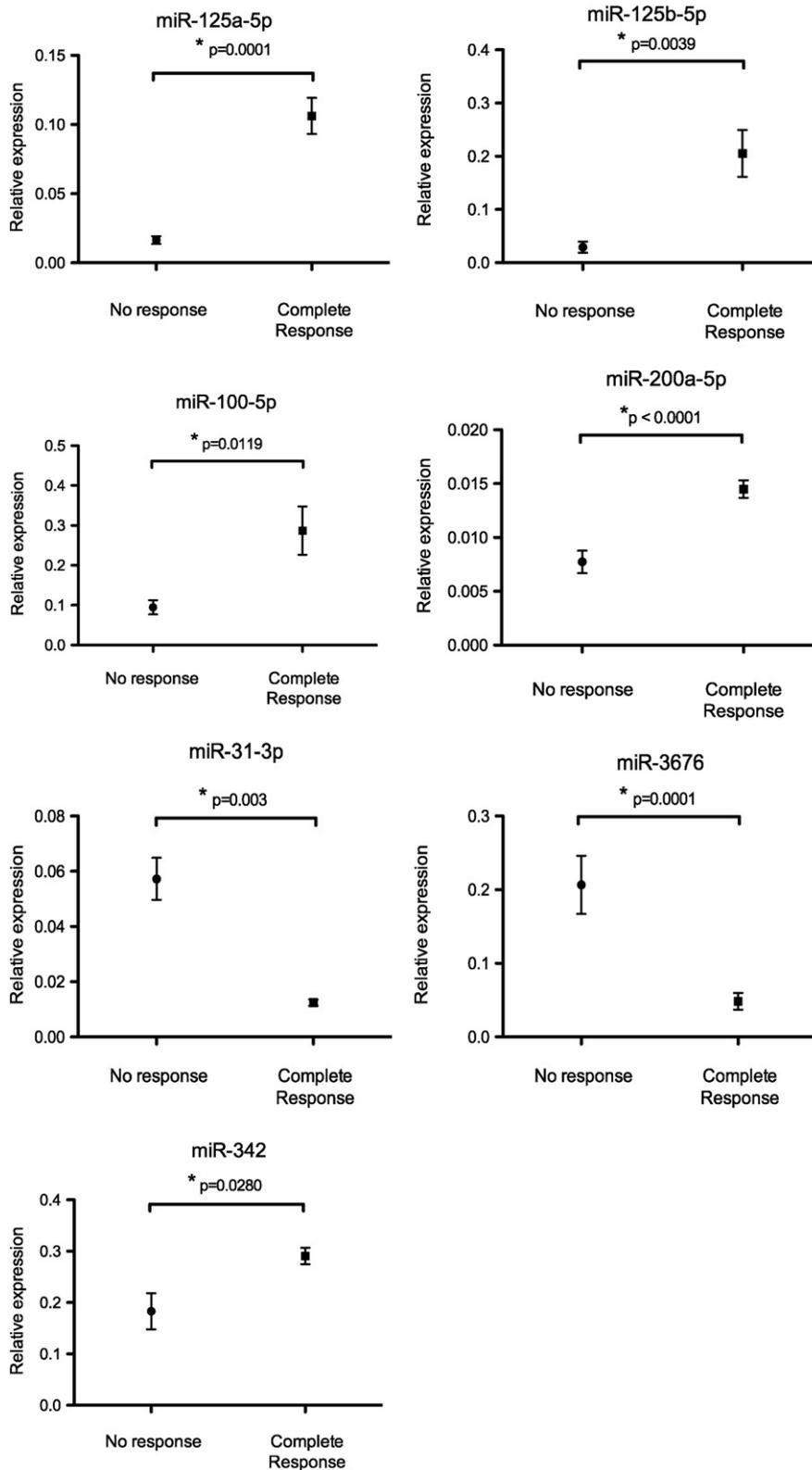


Fig. 2. Expression of seven differentially expressed miRNAs in 31 patients who constitute the validation cohort using TaqMan microRNA assays (NR = 15 and CR = 16). The relative expression values for hsa-miR-125a, miR-125b, miR-100 and miR-200a, miR-31-3p, miR3676 and miR-342 could distinguish between patients with no response and complete response to conventional treatment.

depicted in Figs. 3 and 4. Of note, the last two pathways have not been related to radio- or chemo-resistance in CC.

In addition, we found that miR-100 could be used as a potential molecular marker to identify resistance to conventional treatment in LACC patients, with significant p values ($p = 0.034$ in the global profile results

and $p = 0.011$ in the validation experiments). miR-100 was widely studied by Li et al. in a group of 125 cervical tissue samples, which included normal cervical epithelium, cervical intraepithelial neoplasias, CC tissues and five cancer cell lines (HaCat, SiHa, CasKi, HeLa and C33A). Li and colleagues reported that the expression of miR-100

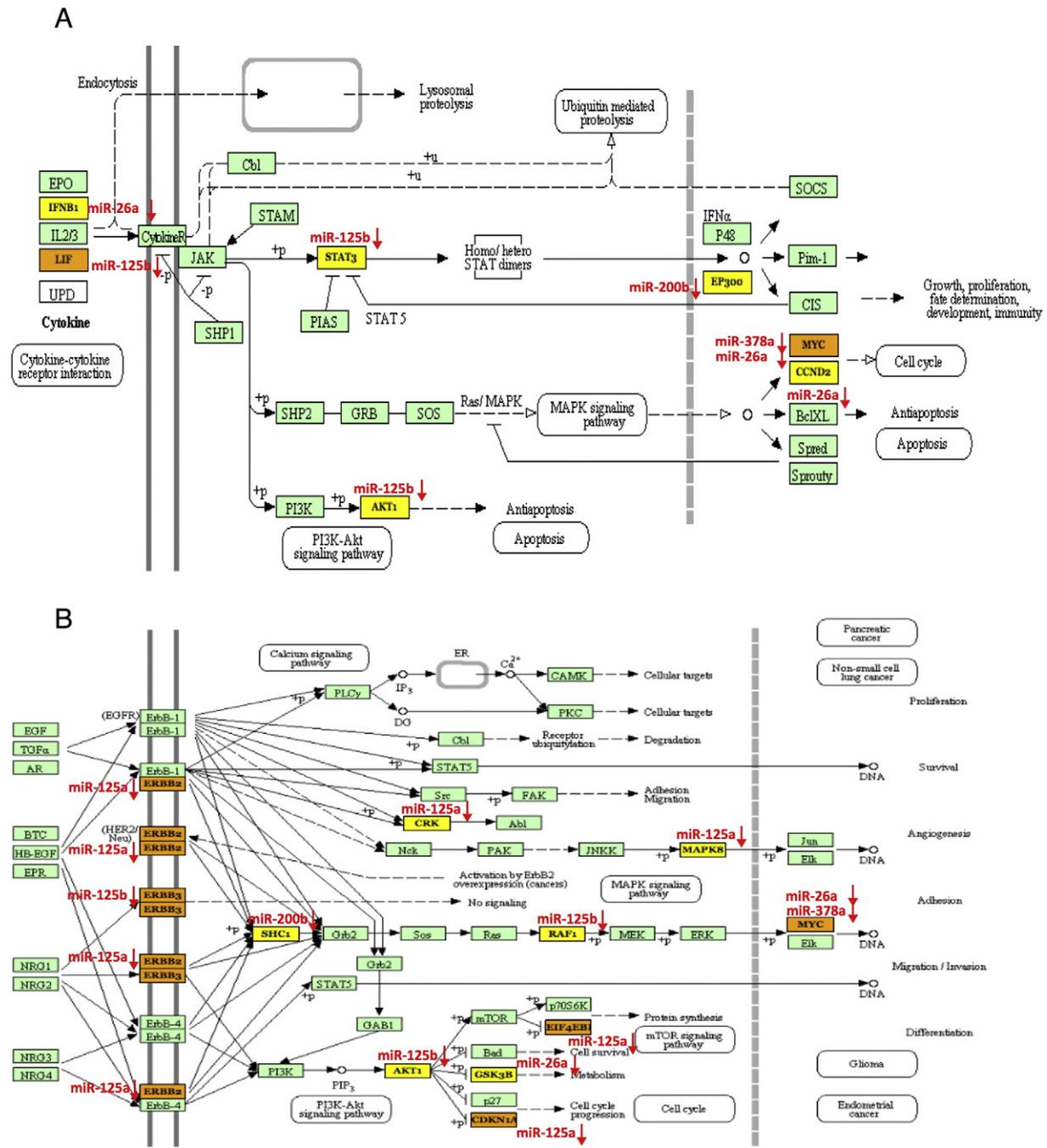


Fig. 3. miRNA:mRNA interactions in the STAT and ErbB molecular pathway associated with patients with no response to conventional treatment in LACC. Specific miRNAs are depicted in red and down-regulation is indicated with an arrow.

maintains a gradual and significant reduction in expression levels in low to high-grade lesions including cancer and HPV-positive cellular lines [30]. Indeed, Polo-like kinase is a validated miR-100 target that is associated with radio-sensitization or radio-resistance, depending on the treatment schedule, in osteosarcoma and colorectal cancer [31].

Confirming these findings, Yang X et al. reported that miR-100 expression is down-regulated in radio-resistant colorectal cancer samples. Additionally, they demonstrated that miR-100 up-regulation sensitizes CCL-244 cells to X-ray irradiation [32]. Interestingly, miR-100 is down-regulated in cisplatin-resistant chondrosarcoma cells compared with parental cell lines. Meanwhile, the ectopic over-expression of miR-100 results in the sensitization of cisplatin-resistant cells [33]. This study and others confirm the role of miR-100 in the regulation of chem- and radio-resistance phenotypes in human cancer [34,35].

In our study, we identified several miRNAs that were previously associated with resistance to radiation and chemotherapy. For instance, miR-125a, which promotes paclitaxel sensitivity in CC by altering STAT3 expression, was down-regulated in non-responder LACC patients [36] (Fig. 2 and Supplementary Table 1). Up-regulation of miR-125b confers resistance of ovarian cancer cells to cisplatin through suppression of Bak1 expression [37]; in contrast, in our data, miR-125b was down-regulated in resistant tumors (Fig. 2, Supplementary Table 1). miR-30c inhibits breast tumor chemotherapy resistance by regulating TWF1 and IL-11 [38]; in the present study, miR-30c was also down-regulated in LACC patients with progressive disease (Supplementary Table 1). This finding suggests a common role of these miRNAs in the regulation of mechanisms leading to radio- and chemotherapy resistance in human tumors.

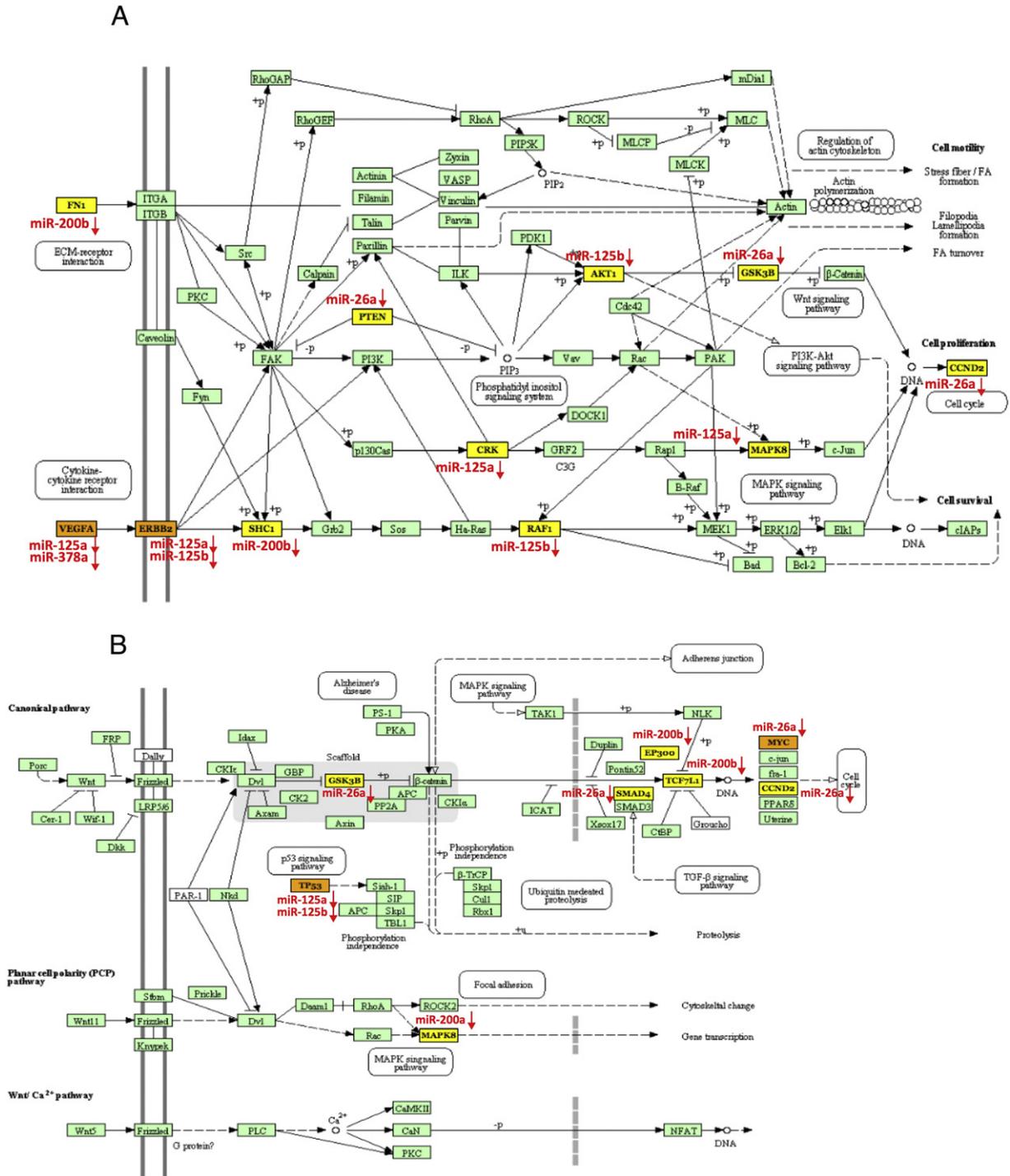


Fig. 4. miRNA:mRNA interactions in the Focal Adhesion and Wnt molecular pathways associated with patients with no response to conventional treatment in LACC. Specific miRNAs are depicted in red and down-regulation is indicated with an arrow.

In this report, we identified a group of miRNAs that belong to the let-7 family (hsa-let-7d, hsa-let-7d*, hsa-let-7f-2, and hsa-let-7g). Previous studies have shown that the expression of let-7g is modulated by ionizing radiation in human endothelial cells [39]. In our findings, let-7g had a 3-fold overexpression in patients with complete response. Similarly, Arora and colleagues reported that let-7g enhances sensitivity to ionizing radiation by the suppression of NFκB1 in lung cancer [40]. An extensive review of published studies had reported deregulation of eight members of the let-7 family (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g and let-7i) in response to ionizing radiation on distinct cancer-derived cell lines [41].

More significantly, the miRNAs reported here, including miR-3176, miR-3676, miR-502, miR-128, miR-145, miR-651, miR-299, miR-548, and miR-345, are for the first time associated with the development of resistance in LACC patients.

The list of miRNAs identified in this study has been associated with the development of resistance to radio- and chemotherapy through the regulation of key genes that participate in the MAPK and Wnt signaling pathways, apoptosis, and the cell cycle. However, we identified three signaling pathways that were not previously associated with resistance: Jak-STAT, Notch and ErbB. Thus, the association of the aforementioned molecular pathways with chemo- and radio-resistant

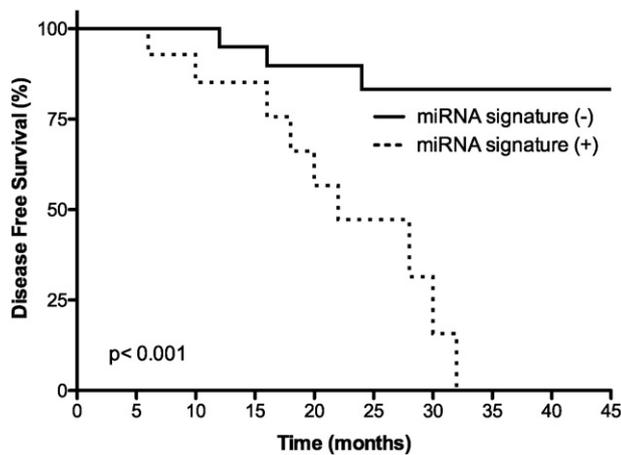


Fig. 5. Kaplan-Meier DFS analysis based on 7 validated miRNA signatures. The behavior of the seven-miRNA profile for patients with no response to conventional treatment is shown by a dotted line, and that for patients with a complete response is shown by a continuous line; both groups are clearly separated within the first months ($n = 41$ patients). Clinical response was assessed according to RECIST 1.1. The log rank Mantel-cox test identified significant differences ($p = 0.001$) between the groups of patients (95% CI, 0.014–0.052).

phenotypes provides insights into new pharmacological targets that could be used in the near future to develop better therapeutic strategies for CC patients.

Our results propose a subset of miRNAs related to radio- and chemo-resistance in LACC patients. Deeper and more exhaustive validation analyses with functional assays to evaluate the specific roles of the identified miRNAs in the regulation of the tumor cells' radio- and chemotherapy responses are needed.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ygyno.2016.07.093>.

Authors' contributions

APT processed the microarrays, acquired and analyzed the data, and interpreted the data. APT, CPP and NJH planned and drew all figures and discussed the manuscript. JFR, OPZ and DCL monitored the patients who participated in this project. APT, JFCC and CLC performed the bioinformatic analysis. CPP planned, organized, and conceived the original idea.

Competing interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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