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# A microRNA panel that regulates proinflammatory cytokines as diagnostic and prognosis biomarkers in colon cancer

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ABSTRACT

Colon cancer (CC) is the third most common neoplasm and the fourth cause of cancer-related death worldwide in both sexes. It has been established that inflammation plays a critical role in tumorigenesis and progression of CC. Immune, stromal and tumor cells supply the tumor microenvironment with pro-inflammatory cytokines such as interleukin 1β, TNFα, IL-6 and IL-11, to hyperactivate signaling pathways linked to cancerous processes. Recent findings suggest a putative role of microRNAs (miRNAs) in the progression and management of the inflammatory response in intestinal diseases. Moreover, miRNAs are able to regulate expression of molecular mediators that are linking inflammation and cancer. In this work a miRNA panel differentially expressed between healthy, inflammatory bowel disease (IBD) and CC tissue was established. Identified miRNAs regulate signaling pathways related to inflammation and cancer progression. An inflammation associated-miRNA panel composed of 11-miRNAs was found to be overexpressed in CC but not in inflamed or normal tissues (miR-21-5p, miR-304-5p, miR-577, miR-335-5p, miR-21-3p, miR-27b-5p, miR-335-3p, miR-215-5p, miR-30b-5p, miR-192-5p, miR-3065-5p). The association of top hit miRNAs, miR-3065-5p and miR-30b-5p expression with overall survival of CC patients was demonstrated using Kaplan-Meier tests. Finally, differential miRNA expression was validated using an inflammation-associated CC model induced by Azoxymethane/Dextran Sodium Sulfate (AOM/DSS) to compare miRNA expression in normal and inflamed tissue versus CC tissues. Based on these findings we propose the identified inflammatory miRNA panel as a potent diagnostic tool for CC determination.

## 1. Introduction

Colon cancer (CC) is reported as the fourth leading cause of cancer death in both sexes worldwide [1]. For patients diagnosed with this cancer at early stage the 5- year survival rate is 90.3% in contrast this rate drops dramatically to 12.5% for patients diagnosed in advanced stage [2]. According to the international guidelines diagnosis criteria, screening tests include colonoscopy and fecal occult blood test [3,4]. Nevertheless, these methods have limitations and require invasive procedures, a considerable variation in quality of practice and lower sensitivity of distal neoplasia. In addition to diagnosis, establishing

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reliable prognosis is also very important for clinical management of CC patients. Clinicopathological features as tumor location, size, nodal invasion and metastatic disease at diagnosis are utilized principally for prognosis assessment [5]. Also, molecular markers such as TP53, KRAS and BRAF mutations and microsatellite instability status have been studied in clinical trials as prognosis biomarkers [6]. Even though specific gene panels to diagnose CC are commercially available, their use is limited due to low predictive positive value, lack of clear treatment guidance and high costs [7]. Thus, establishing novel diagnostic and prognostic markers for early detection of colon cancer is currently of high clinical priority.

**Abbreviation's list**

| | |
|---|---|
| CC | Colon Cancer |
| TNFα | Tumor Necrosis Factor alpha |
| IL | Interleukin |
| miRNAs | micro ribonucleic acids |
| mRNAs | messenger ribonucleic acids |
| nt | nucleotide |
| TCGA | The Cancer Genomic Atlas |
| AOM | Azoxymethane |
| DSS | Dextran Sodium Sulfate |
| NP | Normal pouchitis |
| CP | Chronic pouchitis |
| CDLP | Crohn's-like disease of the pouch |
| COAD | Colon adenocarcinoma |
| qRT-PCR | quantitative Real-Time Polymerase Chain Reaction |
| IBD | Inflammatory Bowel Disease |
| FDR | False Discovery Ratio |

The CC development is mostly associated with several environmental and non-inheritable factors, which include somatic mutations and chronic inflammation [8]. Proinflammatory cytokines such as IL-1β, tumor necrosis factor-α (TNF-α), IL6, IL-21 and IL17 are the principal effectors that link inflammation to CC development and progression [9]. These cytokines activate signaling pathways which lead to enhanced cells proliferation, invasion and migration in CC [10]. Emerging evidence increasingly demonstrates that microRNAs (miRNAs) can regulate inflammation signaling pathways during carcinogenesis and cancer progression [11]. The miRNAs are small non-coding RNAs composed by ~22 nt that bind to messenger RNAs (mRNAs) inducing its degradation and preventing its translation [12]. Interestingly, specific miRNAs have been proposed to be used as possible diagnostic and prognostic biomarkers for several types of cancer including breast and lung [13,14]. Similarly, for colon carcinomas, specific miRNAs such as miR-20a, miR-21, miR-106a, miR-181b and miR-203 were proposed as potential predictors of prognosis and therapeutic outcome [15]. However, currently miRNAs are not utilized in clinical practice for diagnosis and prognosis of CC.

In the present study, using TCGA database, we determined a group of miRNAs which were differentially expressed between CC and normal or inflamed colon tissue. Twelve of these miRNAs with known roles in the regulation of pro-inflammatory cytokines and key signaling pathways in cancer development and progression were examined. We found that miR-30b-5p and miR-3065-5p expression significantly correlated with a poor Overall Survival (OS) of CC patients from stage I to IV. Moreover, differential expression of miR21-5p, miR30b-5p, miR215-5p, mir3065-5p and miR155-5p was validated a murine model of colitis-associated CC, where differential expression of these miRNAs was noted in tumors as compared to normal or inflamed colon tissue. Based on these findings we propose this miRNA panel for CC diagnosis and specifically, miR-30b-5p and miR-3065-5p as prognostic biomarkers of CC.

## 2. Material and methods

### 2.1. Identification of miRNAs differentially expressed between normal, inflamed and colon cancer tissues

In order to identify miRNAs differentially expressed between normal and inflamed tissues we first obtained the hi-seq miRNA profiles of 6 normal pouchitis (NP), 40 chronic pouchitis (CP) and 139 Crohn's-like disease of the pouch (CDLP) from the Gene expression Omnibus accession GSE84779 [16]. Next, we assessed the differentially expressed miRNAs between Normal pouchitis vs chronic pouchitis and chronic pouchitis vs Crohn's-like disease using the R Bioconductor package DESeq2 [17]. We only considered those miRNAs that were differentially expressed in both chronic pouchitis and Crohn's like disease (IBD).

Next, we obtained the hi-seq miRNA profile from 8 normal and 409 tumoral tissues from stages I to IVB of the colon cancer project of the TCGA using the R TCGA biolinks package [18] and assessed the differential expression between normal and tumoral tissues using DESeq2. We only considered those miRNAs that were also differentially expressed in tumoral tissue and in both chronic pouchitis and Crohn's-like disease. Statistical significance was considered as False Discovery Ratio (FDR) $< 0.05$.

### 2.2. Identification of cytokines targeted by differentially expressed – miRNAs

The target prediction was assessed by the presence of miRNA-pro-inflammatory cytokine interaction in at least one predicted (DIANA-microT-CDS, ElMMo, MicroCosm, miRanda, miRDB, PicTar, PITA and TargetScan) and/or experimentally validated database (miRecords, miRTarBase, TarBase) using the Bioconductor package multiMir [19]. Correlation between miRNAs and cytokines was assessed by downloading the TCGA COAD mRNA profile using the R TCGA biolinks and the spearman correlation.

### 2.3. Pathway analysis

The pathway analysis was assessed using the WebGestalt platform [20] and the KEGG database with an over representation analysis; where we uploaded the miRNAs and all of their predicted targets.

### 2.4. Survival analysis

We downloaded the clinical data from the CC project from the TCGA using the Biocondutor TCGAbiolinks package (https://academic.oup.com/nar/article/44/8/e71/2465925) and divided the 409 available patients into two groups, high and low expression based on the media expression of each microRNAs. Kaplan-Meier tests were assessed using the R (v.4.0.1) survival package.

### 2.5. Azoxymethane/Dextran Sodium Sulfate colon cancer model

C57BL/6J mice (Jackson Laboratories) aged 8–14 weeks were used in all experimental procedures, with approval by the Institutional Animal Care and Use Committee (IS00012286). Humane handling of animals was in accordance with the institutional guidelines. All mice were maintained under specific pathogen-free conditions at the Northwestern University, Feinberg School of Medicine animal facilities. For all experiments, animals were age and sex matched, and both male and female mice were equally utilized.

To induce colon carcinogenesis, C57BL/6J mice (Jackson Laboratory) ages 8–10 weeks were given a single injection of Azoxymethane (AOM, Sigma Alrich); 12.5 mg/kg body weight, i.p., followed by 3 cycles of 3.0% Dextran Sodium Sulfate (DSS, Thermo Scientific). Each DSS

cycle consisted of 5 days DSS administration in drinking water followed by 16 days recovery with normal drinking water. First DSS cycle was started on day 7 following AOM injection. Throughout all DSS cycles, colitis disease index (body weight, stool consistency and blood in stool) were monitored. Starting with cycle 3, tumor development was monitored by high resolution endoscopic imaging (bi-weekly). At the end of cycle 3, colons were excised and non-tumor and tumor tissue was surgically dissected and processed in Trizol for RNA isolation as recommended by manufacturer.

### 2.6. Dextran sulfate sodium colitis model

Mice were allowed free access to food and drinking water containing 3% (wt/vol) DSS for 7 days. Daily clinical assessment was performed as previously described [21].

### 2.7. MiRNA expression

Tissue miRNA expression analyses by qRT-PCR were performed using stem-loop primers as previously described [21]. The following primers were used in the above analyses: Forward miR-21-5p: 5'-TAG CTT ATC AGA CTG ATG TTG A-3', Forward miR-30b-5p: 5'-TGT AAA CAT CCT ACA CTC AGC T-3', Forward miR-215-5p: 5'-ATG ACC TAT GAT TTG ACA GAC CAA-3', Forward miR-3065-5p: 5'-TCA ACA AAA TCA CTG ATG CTG GA-3', Forward miR-155-5p: 5'-ACA CTC CAG CTG GGT TAA TGC TAA ACG TGA T-3'. For miRNAs a universal reverse: 5'-CCA GTG CAG GGT CCG AGG T-3' was used.

## 3. Results

### 3.1. Clinical characteristics of patients with CRC from TCGA cohort

All patients with CC were selected from the TCGA database (n = 409). Of them, 52.07% were male and 47.92 % were female patients. The median age of patients was 68 years. Histological type of all tumors was colon adenocarcinoma, the TNM tumors stages included 69 patients diagnosed with stage I (16.9%), 162 with stage II (39.4%), 117 with stage III (28.6%) and 61 with stage IV (14.9%).

### 3.2. Differential expressed miRNAs during the progression from IBD to CC are able to regulate pro-inflammatory cytokines

We performed differential analyses comparing the miRNAs expression levels between normal versus inflamed tissues (IBD or Crohn's) and normal versus tumoral tissues. We identified miRNAs with different expression levels between the three groups of tissues. Eighteen miRNAs (let-7i, miR-146b, miR-148a, miR-182, miR-199b, miR-21, miR-223, miR-27b, miR-3065, miR-30b, miR-335, miR-338, miR-340, miR-424, miR-5571, miR-577, miR-629, miR-653) were differentially expressed between colon tumors compared to normal and inflamed tissues (Fig. 1).

Next, we analyzed potential targets of the differentially expressed miRNAs and determined the biological role of these miRNAs resulting in 4564 unique genes (supplementary file 1). Finally, we examined the signaling pathways in which these miRNAs could regulate. The top pathways upregulated by these miRNAs were the inflammatory IL-17 pathway and WNT pathway, both strongly correlated to tumorigenesis, evolution and progression of CC [22,23]. In contrast the most downregulated pathways were cGMP-PKG and Cholinergic synapse (Fig. 2A), the downregulation of this pathways have been correlated to tumor growth, tumor location, and prognosis of CC [24]. Thus, these findings indicate that miRNA expression profile in colon cancer can regulate key signaling pathways involved in CC development and progression.

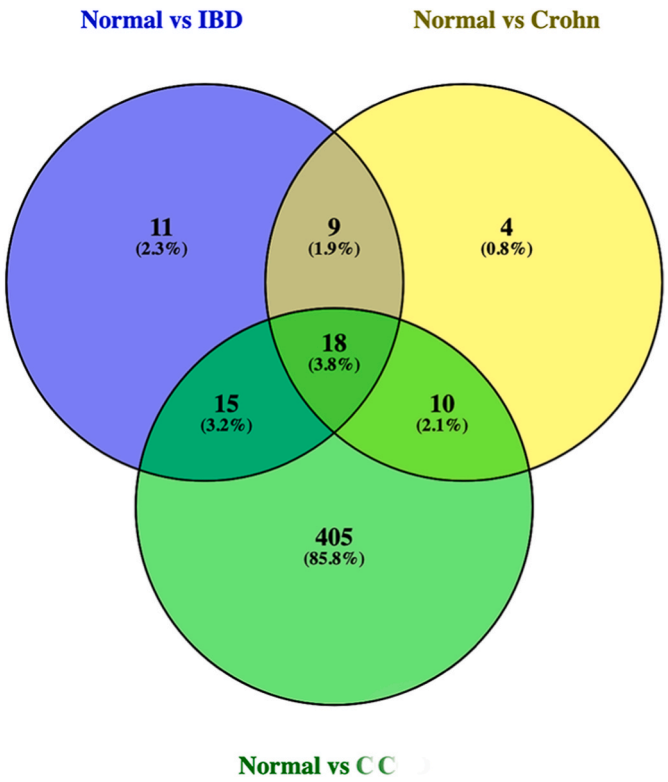


**Fig. 1. Comparison of miRNA expression analyses in IBD, Crohn's disease and COAD.** The Venn diagram represents the miRNAs with differential expression for each group of tissues. At the center are showed the number of miRNA differentially expressed between the three groups.

### 3.3. Inflammation associated miRNAs as diagnosis biomarkers in colon cancer tissues

One of the main pathways regulated by these miRNAs was IL-17 suggesting that these miRNAs can regulate/impact inflammatory responses in the tumors. To further explore this, we focus on miRNAs with an established role in regulating proinflammatory cytokine expression. Our results showed that 12 out of the 18 miRNAs can regulate cytokines such as; TNFα, IL-1β, IL21, IL22, IL17A and IL7 (Fig. 2B). Next, we wanted to establish whether the identified miRNAs positively or negatively corelated with their cytokine target. To achieve this, we correlated the expression of each miRNA with their target using the TCGA COAD Dataset. As shown in the Supplementary Table 2; the expression levels of miR-3065-5p, miR-432-5p, miR-577, miR-30b-5p and miR-335-3p showed a negative (inverse) correlation with the expression of their targets. On the other hand, the expression levels of miR-21-3p, miR-21-5p, miR-27b-5p, miR-215-5p and miR-192-5p were positively correlated (expression levels high or low of both) with their target cytokines. While miR-340-5p and miR-335-5p showed positive or negative association depending on the evaluated target. These data highlight the potential role of miRNAs in regulating proinflammatory cytokine expression.

Next, we examined the expression levels of these 12 miRNAs, comparing colon inflamed tissue versus CC tissue. The results showed that 11 miRNAs were overexpressed (miR-21-5p, miR-304-5p, miR-577, miR-335-5p, miR-21-3p, miR-27b-5p, miR-335-3p, miR-215-5p, miR-30b-5p, miR-192-5p, miR-3065-5p) and only miR-432-5p was downregulated in CC tissue (Fig. 2C). Interestingly, the expression levels of these 11 miRNAs were higher in all stages of colon cancer analyzed (I-IV) versus healthy tissue (Fig. 2D). Moreover, miR-335-5p and miR-3065-5p showed higher expression levels (twelve and six times more respectively) compared with healthy tissues in which are not expressed. These results suggest the role of these miRNAs as potential biomarkers to

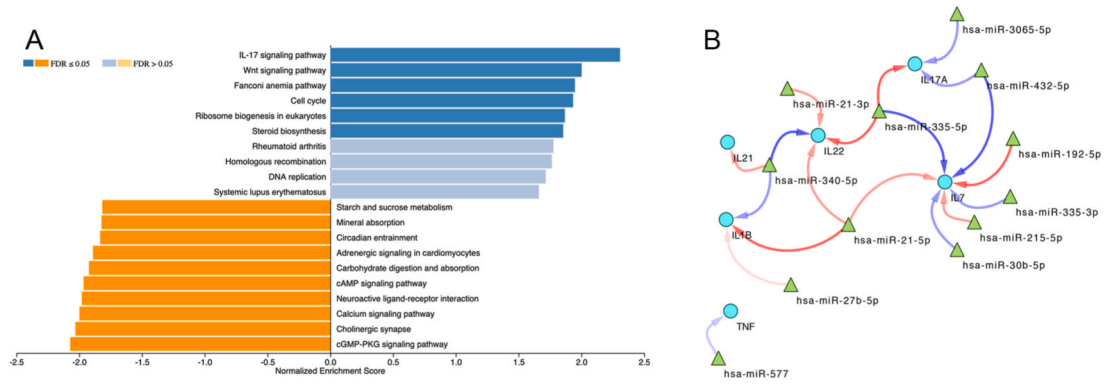


**Fig. 2. miRNAs associated to inflammation regulates cancer progression related pathways. A)** The bar plot shows the normalized enrichment score of the main pathways regulated by the differentially expressed miRNAs. Orange represents negatively enriched pathways and blue positively enriched pathways. **B)** Network analyses shows the regulation of pro-inflammatory cytokines by the miRNAs. miRNAs are represented as green triangles and cytokines as blue circles. The color of the arrows represents the correlation miRNA-cytokine. Blue is for negative and red is for positive correlation.

distinguish cancer tissues from normal and inflamed colon tissues.

### *3.4. The overall survival (OS) of CC patients is associated with miR-30b-5p and miR-3065-5p overexpression*

We analyzed if the 11 overexpressed miRNAs could be associated with the OS of CC patients. The results showed that only the higher expression of miR-3065-5p ($p = 0.0016$) (Fig. 3A) and miR -30b-5p ($p = 0.0037$) (Fig. 3B) was associated with a poor OS of the patients. These results support the idea that the overexpression of these miRNAs could function as biomarkers predictive of OS in CC patients.

### *3.5. miRNA signature as biomarker for development of colitis-associated colon cancer*

To validate the differentially expressed of miRNA panel in colitis-associated colon cancer, the expression levels of miR-3065-5p, miR-30b-5p (associated to poor OS) and miR-21-5p and miR-215-5p (associated to inflammation) were measured in murine model of acute colon epithelial injury/colitis (DSS) and a murine model of colon cancer (AOM/DSS). Developing colon tumors were monitored by high resolution endoscopic imaging (Fig. 4A). Once tumors were formed mice were sacrificed and colon/tumor tissue samples were recovered and the relative expression of selected miRNAs was determined. As we have seen in human tissue, expression of the analyzed miRNAs changed in colon inflammation and in the development of colon cancer (Fig. 4B). Inflammatory miR-155-5p was used as a positive control for inflammation

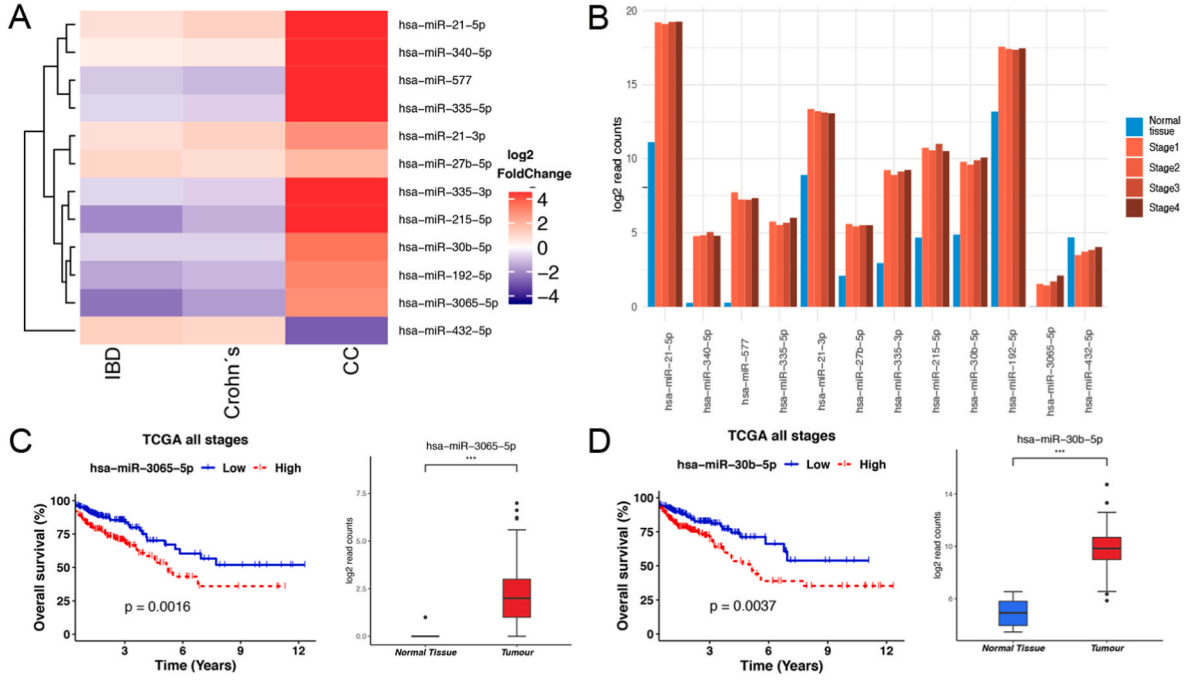


**Fig. 3. A miRNA panel is differentially expressed in IBD, Crohn's disease and CC and is related to overall survival of CC patients. A)** Heatmap of miRNA panel expression in inflammatory diseases and CC. Blue color represents low expression and red color represents high expression. **B)** Expression levels of 12 miRNAs in the different stages of COAD (red bars) compared with the expression in normal tissues (blue bars). **C)** Differences in OS of the COAD patients with high/low miRNA expression. Red lines represent patients with a higher expression and blue with lower expression of miRNAs. **D)** The boxplots represent the normalized log2 of the read counts of each miRNA, where *** represent $p<0.001$.

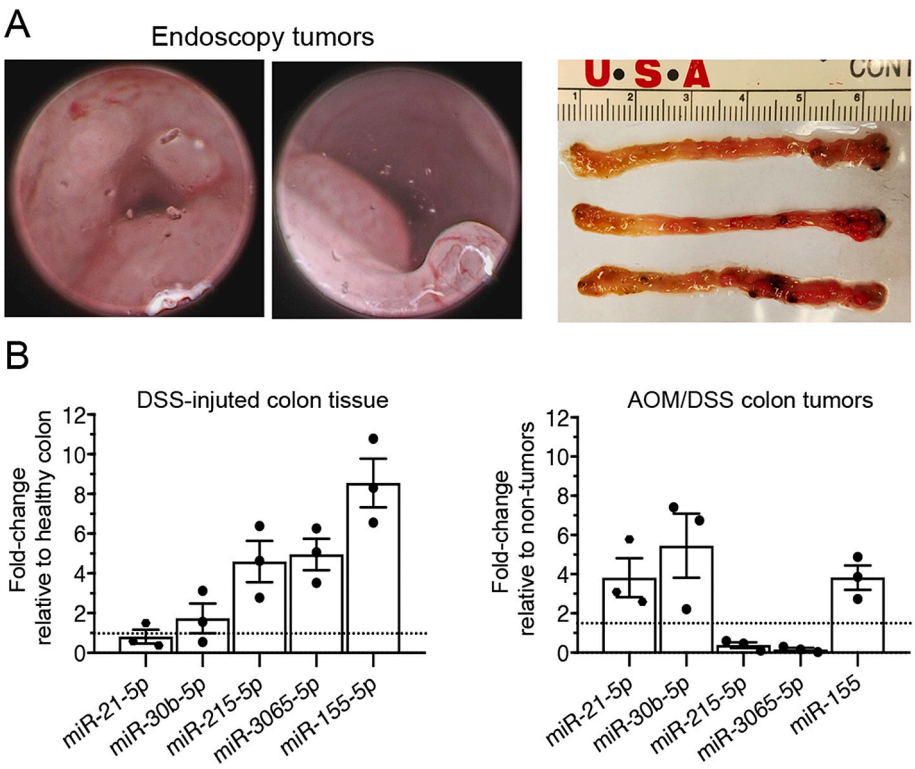


**Fig. 4. Differentially-expressed miRNA panel in murine models of acute colitis and colitis-associated CC. A)** Representative endoscopy image (left panel) and of a dissected colon (right panel), depict developing colon tumors following AOM/DSS treatment. **B)** Expression analyses of differentially expressed - miRNAs in DSS-injured colon tissues and AOM/DSS colon tumors. All data was normalized to pair non-inflamed/non-tumor colon tissue. N = 3 mice per condition.

in these samples as it is known to be elevated both in DSS and CC [25]. Compared to expression in healthy tissue, miR-21-5p and miR-30b-5p were not significantly altered with colon injury (by DSS) however both were significantly elevated in CC (4- and 5-fold respectively). In contrast, miR-215-5p and miR-3065-5p were induced in DSS treated tissues but downregulated in CC. These results suggest an important role of evaluated-miRNAs in inflammation/injury and cellular transformation, indicating distinct expression patterns in acute inflammation/injury and cancer. These findings further support the idea that changes in the expression of inflammation-associated miRNAs can distinguish/predict normal versus inflamed and cancerous tissue.

## 4. Discussion

Molecular mediators such as microRNAs (miRNAs) have an important role during colon cancer progression [26]. In an effort to expand the knowledge of miRNAs that regulate molecular events in inflammation and that could serve as diagnostic and prognostic indicators in colorectal cancer, in this study we focused on determining differentially expressed miRNA in CC tissues versus healthy colon and inflamed colon tissue samples. The identified miRNAs have an important role in the regulation of signaling pathways that lead to cancer development and progression as proinflammatory IL-17, Fanconi anemia pathway [27], homologous recombination DNA repair [28], cGMP-PKG, Cholinergic synapse and calcium signaling pathways [24]. In this regard, inflammation has been established as a main risk factor for developing CC [26]. Moreover, cytokines have a crucial role in CC development and progression. Pro-inflammatory cytokines such as IL-1, TNF, IL-6 and IL-23/IL17 play an important roles in colitis-associated CC and in sporadic colon cancer, where they trigger similar molecular mechanisms to promote tissue injury or tumorigenesis [29]. In this context, our results showed association between the cytokines and miRNAs panel. The miRNA-cytokine positive association suggests that miRNAs could regulate and promote expression of inflammatory cytokines. Although the upregulation pattern of miRNAs-target is not common, the "relief of repression" mechanism described by Vasudeban [30] could explain the positive associations that we found. Moreover, a reciprocal regulatory role between miRNAs and cytokines is demonstrated [31]. We observed a positive association between miR-21-5p and expression levels of proinflammatory cytokines including IL-1β and IL-22. Recently, it has been shown that high levels of miR-21-5p in macrophage-derived exosomes induced cancer call migration and invasion trough BRG1 downregulation. This was dependent on Wnt/β-catenin activation and promoted colon cancer growth and spreading [32]. In contrast, negative association observed between miRNA-cytokine could be explained by a direct targeting of miRNA to the cytokine mRNA or by repression of components of signaling pathways that regulate cytokine expression. In addition, we observed different expression patterns of miRNAs when comparing normal, inflamed tissues and CC tissue. We found 11 miRNAs that showed higher expression in colon tumors versus inflamed tissue, and remains overexpressed in colon tumors from clinical stage I to IV suggesting a possible role as master regulators miRNAs. The consistent expression of miRNAs in all stages of cancer could be considered as a master regulator factor for the maintenance of the oncogenic phenotype [33]. Indeed in colorectal cancer several miRNAs have been proposed as master regulators of cancer progression [34].

Other studies have previously proposed miRNA panels for colon cancer diagnosis [35,36]. However, these studies considered miRNAs expression levels in normal versus colon cancer tissue only, thus, an important contribution of our work is the identification of miRNA panel that could allow for distinguishing between inflamed and normal tissue from cancer tissue even in early-stage tumors. Additionally, the association of higher miR-3065-5p and miR-30b-5p expression with poor overall survival of CC patients was established. Although miR-3065-5p showed anti-tumor effects in melanoma cells [37], how these miRNAs impact colon cancer development is not clear. Moreover, miR-30b-5p function can vary depending on cancer type [38]. In colon cancer low levels of miR-30b-5p were reported as a poor prognostic markers for

5-year disease free survival, suggesting an oncogenic role [39]. The overexpression of miR-30b-5p in colon cancer tissue that we observed supports this idea. Further studies focused on the regulatory functions of these miRNAs in CC are needed. Additionally, low levels expression of miR-432-5p was reported to associate with colon cancer progression trough migration and invasion inhibition [40,41], suggesting its putative role as prognosis marker.

Finally, studies using murine CC model, miR-21-5p and miR-30b-5p showed higher expression in tumors compared to inflamed or normal colon tissue, supporting a potential regulation of chronic inflammation and carcinogenesis by these miRNAs. Also, expression levels of miR-215-5p and miR-3065-5p were higher in inflamed compared to CC tissues, contrasting with the changes observed in human IBD and colon cancer tissues. This data in line with findings by Josse *et al*, who previously demonstrated inverse expression levels of the same miRNAs between AOM/DSS induced murine colon cancer and human colon cancer [42]. Notwithstanding the differences observed, these miRNAs can serve as biomarkers in mouse or human colon cancer and could be helpful in early detection of this disease. Moreover, the expression of two of these miRNAs was associated with overall survival, so they could be proposed also as potential biomarkers of overall survival in CC.

## Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

## Authors' contributions

**Conception and design:** A.M.G., B.C.L., C.P.P. and E.A.P.Y.

**Development of methodology:** A. M. G., B. C. L., T. M. B., M. M. R., R. S. and E.A.P.Y.

**Analysis and interpretation of data:** A. M. G., B. C. L., R. S, A. D. C. P., G. C. R., E.O. M.S., C.P.P. and EAPY.

**Writing, Review and/or revision of the manuscript:** A. M. G., B. C. L., R. S., M. M. R., A. D. C. P., G. C. R., D.C. DL., E.O. M.S., C. P.P. and E. A. P. Y.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101252.

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