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Orchestrating the Tumor Microenvironment in Colorectal Cancer

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Abstract Colorectal cancer (CRC) is one of the most common cancers worldwide. Despite the improvement of treatment options that bettered advanced-stage patient's overall survival, the clinical outcome is still poor. The tumor microenvironment is thought to be an essential participant in the metastasis process. Liver metastases are the most frequent metastases that occur in CRC patients. It is believed that colorectal cancer has the liver as a preferential site for metastasis, and this is possible due to the multiple interactions that happen in the tumor microenvironment and due to the tumor stem cells input. As a means to investigate this biological process, microRNAs (miRNAs) have established a name for themselves as putative biomarkers in cancer and especially colorectal cancer. In this pilot study, we explored miRNAs expression that by influencing the tumor stem cells can help prevail liver metastasis in advanced-stage CRC patients.

Keywords: *colorectal cancer, liver metastases, tumor microenvironment, tumor stem cells, miRNA*

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1. Introduction

Colorectal cancer (CRC) is considered one of the most frequent cancers worldwide for both men and women [1]. In recent years, significant progress has been made in understanding the molecular mechanism behind colorectal cancer invasion and metastasis. Therefore, a new door has opened for improving diagnosis, prognostic and therapeutic measures [2]. Liver metastases are the most prevalent type of distant metastasis for colorectal carcinoma and the central menace of clinical outcome [3]. The disease is heterogeneous, and despite all the efforts made, new molecular biomarkers are still needed.

Cancer microenvironment has established its role in cancer progression. The tumor microenvironment exerts an impact on cancer cells and plays a crucial role in tumor growth, invasion, angiogenesis, resistance to treatment and metastasis [4].

MiRNAs are small non-coding RNAs of approximately 21-25 nucleotides in length that target mRNA to promote mRNA degradation or inhibit protein translation [5]. It is known that miRNA aberration can play essential roles in cancer progression and development [6]. With recent advances in understanding tumor suppressor and oncogenic miRNAs and figuring out their roles in different cancers, there is an excellent chance of using these small molecules for diagnostic and prognostic features [7]. Preclinical models and clinical trials have shown great potential in the therapeutic instance for miRNAs [8].

Tumor stem cells are a category of tumor cells that poses the propriety of self-renewal and differentiation in different solid tumors and also hematological malignancies [9]. These rare immortal cells within a tumor can divide and form new cell types that constitute the tumor and contribute establishing tumors [10].

This pilot study aims to evaluate the potential of several miRNA expression that modulate tumor stem cells to predict liver metastasis in advanced-stage patients. This is the first step proposing a protocol for miRNA analysis that can bring new information about the invasive phenotype of CRC.

2. Materials and Methods

To bring new information about the invasive potential and aggressiveness of the CRC phenotype, we established a study design protocol. The sequence miRNAs expression - tumor stem cells modulation- liver metastasis in CRC was followed. We investigated a set of 84 miRNAs associated with tumor stem cell modulation in two groups of patients with different CRC evolution. The difference between the two groups was that we selected patients who evolved to liver metastases and patients that did not get liver metastases in the evolution of their disease.

The patient's selection was done according to the pathological stage, age, the presence or absence of liver metastases and the imagistic evaluation measure. All patients were evaluated by CT scan. We included in this pilot study a number of 11 patients with different clinical outcomes. The age of the patients ranged from 41 to 84 years old. The distribution for sexes was well balanced. The study included 6 men (FFPE blocks: 7899/5/10; 6151/4/12; 6761/4/11; 6377/4/10; 6097/4/10; 9278/3/12) and 5 women (FFPE blocks: 2099/7/11; 7889/3/11; 8056/5/11; 5099/4/11; 3710/8/12). The staging included in this study ranged between stage IIA and IIIB at the moment of diagnosis according to the AJCC 7th edition staging system.

As mentioned before, 6 patients presented liver metastases in their evolution (FFPE blocks: 2099/7/11; 7889/3/11; 7899/5/10; 8056/5/11; 6151/4/12; 6761/4/11) and 5 patients didn't get liver metastases in their evolution (FFPE blocks: 5099/4/11; 6377/4/10; 6097/4/10; 3710/8/12; 9278/3/12.).

The RNA extracted from formalin-fixed paraffin-embedded (FFPE) blocks from the two groups of patients was used for an exploratory analysis by PCR array, investigating a set of 84 miRNAs associated with tumor stem cell modulation. We used biological material that was extracted from the FFPE block of every patient before any administred treatment. After macrodissection, the miRNeasy FFPE extraction kit (Qiagen) was used according to the manufacturer specifications. By applying statistical tests, significant miRNAs associated with liver metastases would be identified.

Our protocol used the PCR array method for the gene expression assessment. For every 100 ng RNA, cDNA synthesis was performed using the RT² First Strand Kit. Afterward, the cDNA was preamplified and used for analysis using quantitative PCR analysis. The statistical methods used for measuring the gene expression levels were Man Whitney or Student's t-test. This study and protocol derived from this was carried out according to the University of Medicine and Pharmacy 'Iuliu Hatieganu" Ethics Committee.

3. Results

The expression level of the miRNA set involved in modulating tumor stem cell phenotype was investigated using PCR array analysis. The evaluation of the fluorescent signal that quantifies the level of gene expression was performed using the miScript SYBR Green PCR kit. The PCR reaction was performed with the LightCycler480 (Roche) and the amplification process according to their protocol. For each miRNA, the specific fluorescent signals were recorded as amplification curves.

Because we had to do the preamplification step, we only considered the miRNAs as being expressed when they were detected at a CT (number of cycles when the fluorescent signal was assessed) value of < 30. The expression levels >30 CT weren't considered as important for the statistical analysis.

In our pilot study, the percentage of miRNAs expressed in the metastatic group was comparable with the percentage in the group without metastases (78.8% vs. 79.8 %).

The gene expression assessment was realized simultaneously for all 84 miRNAs of interest using PCR array. The normalizer used was miRNA-16-5p.

The results showed that three miRNAs (miR-185-5p, mir-125b-5p, miR-150-5p) were significantly expressed from the two groups. The levels of expression of these miRNAs were diminished in the metastatic group compared with the non-metastatic one. These miRNAs were considered for their role associated with liver metastases.

Further validation on an extended series of samples (31 samples) that included the samples for the PCR array analysis showed that only miR-185-5p and miR-125b-5p were statistically significant.

4. Discussion

Recent studies have revealed that miRNAs can be key players in all cancer processes, and they can play a role in colorectal cancer prognostic profiles [11]. Furthermore, miRNAs are linked with diagnostic, prognostic, and therapeutic instances in CRC by their features as non-invasive reliable biomarkers [12]. Exploring the vast tumor microenvironment can bring new data for CRC clinical outcome improvement [13]. Our pilot study and protocol development found that two miRNAs (miR-185-5p;miR-125b-5p) with their altered expression could differentiate patients with liver metastases and frame them in a poor prognostic group. Of course, these results need to be further assessed in more significant trials on larger cohorts of patients to gain clinical applicability.

Several studies have demonstrated the role of miR-125b-5p in colorectal cancer. A study by Chen et al. [14] showed that miR-125 expression is underexpressed in colorectal cancer and could be a prognostic biomarker. Another study showed that miR-125 targets and inhibited VEGF. This could be used as a therapeutic target [15]. Considering miR-185-5p as a potential prognostic biomarker, Dong Xu et al. showed that miR-185 is a tumor suppressor in CRC cells and can inhibit the proliferation of cancerous cells [16]. A study by Zhang et al. showed that miR-185 targets STM1 and can be assessed as a potential biomarker for CRC [17].

Other relevant miRNAs are associated with clinical outcome for colorectal cancer. For example, miR-21 is associated with poor prognostic in CRC patients and can predict chemoresistance for these patients [18,19].

The study encompasses our protocol to analyze the link between miRNAs that modulate tumor stem cells and help predict liver metastases in advanced-stage patients.

There are a series of limitations to our pilot study. The low number of patients enrolled could only suggest the prognostic significance of these miRNAs without a very relevant statistical significance. There is a need for further studies with larger cohorts of patients .This study could serve as a first step in conducting other well structured more extensive studies.

The perspectives of miRNAs research are dependent on improving expression technologies of these small molecule's sensitivity and specificity. Concluding, miRNAs can

provide future opportunities for individualized prognostic and therapeutic strategies.

5. Conclusions

CRC is a heterogeneous disease. Exploring the tumor microenvironment can lead to a revolution in individualized treatment strategies and help develop new drugs. Our results revealed that in advanced stage CRC, miR-125b-5p and miR-185-5p could provide new information about the patient's potential of developing liver metastases.

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