1 Tissue micro-RNAs associated with colorectal cancer 2 prognosis: a systematic review 3

4 Micro-RNAs associated with colorectal cancer prognosis 5

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29

30 **Abstract**

31 Colorectal cancer (CRC) is a multifactorial disease commonly diagnosed
 32 worldwide, with high mortality rates. Several studies demonstrate important
 33 associations between differential expression of micro-RNAs (miRs) and the prognosis
 34 of CRC. However, only a few systematic reviews emphasize the most relevant miRs
 35 able to contribute to the establishment of new prognostic biomarkers in CRC
 36 patients. The present study aimed to identify differentially expressed tissue miRs
 37 associated with prognostic factors in CRC patients, through a systematic review of
 38 the Literature. Using the PubMed database, Cochrane Library and Web of Science,
 39 studies published in English evaluating miRs differentially expressed in tumor tissue
 40 and significantly associated with the prognostic aspects of CRC were selected. All
 41 the included studies used RT-PCR (Taqman or SYBR Green) for miR expression
 42 analysis and the period of publication was from 2009 to 2018. A total of 115 articles

accomplished the inclusion criteria and were included in the review. The studies investigated the expression of 102 different miRs associated with prognostic aspects in colorectal cancer patients. The most frequent oncogenic miRs investigated were miR-21, miR-181a, miR-182, miR-183, miR-210 and miR-224 and the hyperexpression of these miRs was associated with distant metastasis, lymph node metastasis and worse survival in patients with CRC. The most frequent tumor suppressor miRs were miR-126, miR-199b and miR-22 and the hypoexpression of these miRs was associated with distant metastasis, worse prognosis and a higher risk of disease relapse (worse disease-free survival). Specific tissue miRs are shown to be promising prognostic biomarkers in patients with CRC, given their strong association with the prognostic aspects of these tumors, however, new studies are necessary to establish the sensibility and specificity of the miRs in order to use them in clinical practice.

56

Key-words: colorectal cancer, micro-RNA, prognosis, systematic review, oncology

58

59 Introduction

Colorectal cancer (CRC) is one of the most prevalent neoplasms in the world, being the second type of cancer more frequent in women and the third type more frequent in men. In Brazil, for the 2018-2019 biennium, it is estimated the occurrence of 36.030 new colorectal cancer cases with 17.380 cases in men and 18.980 cases in women¹.

65 The survival of patients with CRC is directly associated with the pathological
66 stage (pTNM) of the disease which is determined after microscope analysis of the
67 sample obtained by biopsy or surgical resection of the tumor ^{2,3}. The pTNM stage is a
68 system of stage classification proposed by the American Joint Committee on Cancer
69 which determines the degree of tumor development according with the T, N and M
70 categories. The T category informs about tumor growth, depth and stage of adjacent
71 tissues invasion. The N category informs about lymph nodes metastasis and the M
72 category describes the quantity of affected anatomical sites by distant metastasis ³.

73 On one hand, it is estimated that the survival of patients diagnosed at the first
74 stages of the disease is up to 90%, but on the other, 30 to 45% of the patients with
75 late diagnosis will present disease recurrence ^{4,5}. Factors such as low degree of
76 differentiation and high levels of serum carcinoembryonic antigen (CEA) are also
77 described as important for the increase of CRC recurrence after tumor resection ^{2,6}.
78 However, results from recent studies have demonstrated that the analysis of micro-
79 RNA expression in CRC can be used as a more sensible prognostic biomarker due
80 to its potential of predicting disease recurrence, survival and therapeutic response ⁷⁻
81 ¹¹.

82 MicroRNAs (miRs) are small non-coding RNA molecules of approximately 21
83 to 24 nucleotides in length that modulate gene expression by binding with the 3'
84 untranslated region (3'UTR) of messenger RNAs resulting in its translational
85 repression or cleavage ¹². In the literature, more than 2000 miRs have been already
86 described which demonstrate an essential role in the regulation of eukaryotic cells
87 and in the pathogenesis of several diseases, including cancer ^{13,14}.

Biogenesis of miRs begins with the activity of RNA polymerase enzyme II which transcribes the primary miRs (pri-miRs) recognized afterwards by DGCR8 protein associated with DROSHA enzyme creating a complex which allows the cleavage of the pri-miRs originating the precursor miRs (pre-miRs). The pre-miRs are carried to the cell cytoplasm by Exportin-5 and cleaved by DICER, generating double stranded miRs which will be recognized by Argonaut proteins promoting the release of one strand of the mature miR. Argonaut proteins in association with the mature miR and other proteins will constitute the RNA-Induced Silencing Complex (RISC) whose targets will be specific mRNAs.^{12,15,16} Fig 1 outlines the miRs biogenesis.

Fig 1. miRNAs biogenesis and proteins involved in the process.

Recent studies report that miRs regulate cellular growth and apoptosis, therefore their dysregulation could trigger carcinogenesis^{17,18}. MiRs are classified as oncogenic or tumoral suppressor when they inhibit the expression of tumoral suppressor genes or oncogenes respectively. Oncogenic miRs are usually upregulated in the neoplastic process while tumor suppressor miRs are usually downregulated in cancer^{12,18,19}. Consequently, studies have demonstrated by means of analysis of miR expression, that they can be valuable diagnostic and prognostic biomarkers in cancers, including CRC.^{20–23}.

Despite the recent improvements in the diagnostic methods for CRC detection, the overall survival for CRC patients is only 65% while the disease recurrence after

110 tumor resection is approximately 45%, emphasizing the importance of describing
111 new methods in order to guarantee a better prognosis for these patients ^{4,24}.

112 Several studies investigate the importance of miRs expression in CRC
113 prognosis ^{7-9,11,21-23}. However, there is a lack of systematic reviews in the Literature
114 integrating and identifying the main miRs significantly associated with the prognostic
115 aspects of CRC.

116 Given the relationship between the expression of miRs and the prognosis of
117 CRC, the present study aims to highlight this association by means of a systematic
118 review of the Literature, in order to identify the main miRs associated with each
119 prognostic aspect of CRC. This could contribute to develop new prognostic
120 biomarkers and could help to identify new molecular targets useful in CRC treatment.

121 **Methods**

122

123 **Study design and search strategy**

124 This study represents a systematic review of the Literature. For the elaboration
125 of this project a reference review in the PubMed database, Cochrane Library and
126 Web of Science database was performed in order to identify the relevant studies that
127 evaluated miR expression associated with CRC prognosis. The Literature search was
128 performed from February of 2009, year of publication of the first and less recent
129 selected study, to December of 2018, year of publication of the most recent selected
130 study. The following search strategy was used for the identification and selection of
131 the articles: miRNA, colorectal cancer, prognosis. The search was performed in
132 November 2018 and it was limited to English published articles. At the end of the

133 search, all the results were compared and reviewed for the inclusion of relevant
134 publications about the subject.

135

136 **Inclusion criteria**

137 The paper selection was based on criteria related to the study classification
138 and design in order to include the most relevant articles in this review. The relevant
139 criteria related to the classification of the studies were: primary and descriptive
140 studies, studies related to CRC patients and studies completely available online. The
141 relevant criteria related to study design were: articles that evaluate CRC prognosis by
142 overall survival and disease free survival; articles that evaluate miR expression in
143 tumoral tissues compared to normal tissues; articles that evaluate miR expression by
144 Real Time Polymerase Chain Reaction (RT-PCR) using Taqman and/or SYBR
145 Green; articles that evaluate miR expression related to tumor growth/size, lymph
146 nodes metastasis, distant metastasis and TNM stage. Publications belonging to case
147 report, systematic reviews and meta-analysis categories were not included.

148

149 **Data Extration**

150 The data extraction was performed by means of a collection form pre-
151 developed by the authors in which the following data of each publication were
152 collected: name of the first author, year of publication, micro-RNA studied, sample
153 group, origin of cases, type of specimens analyzed, type of methodology used to
154 evaluate the expression of micro-RNAs, target genes and association with
155 pathological and prognostic clinical aspects.

156 This review followed the Preferred Reporting Items for Systematic Reviews
157 and Meta-Analyses (PRISMA) recommendations.

158

159 **Statistical analysis and quality assessment**

160 Due to the heterogeneity of the included studies, it was not possible to perform
161 a weighted analysis of the results extracted from each article. Therefore, the results
162 were analyzed qualitatively and presented narratively. A meta-analysis and the
163 assessment of publication bias were not practically applicable for this study.

164 Problems with the design and execution of individual studies of healthcare
165 interventions raise questions about the validity of their findings, and therefore, the
166 quality of the primary studies was assessed by evaluating eight characteristics
167 extracted from a checklist provided by the STROBE platform: description of the
168 studying setting, description of study participants, definitions of all variables,
169 description of the data sources and measurement tools, justification of sample size,
170 description of statistical methods, description of results and discussion and
171 interpretation of results. After evaluating the studies, each attribute was pointed in a
172 range from 0 to 2 points (0 = not described, 1 = poor described, 2 = well described),
173 with maximum quality score of 16 points in total.

174

175 **Data analysis**

176 The data were reviewed and synthesized in semi-structured tables of
177 Microsoft® Office Excel version 2013. The results were analyzed by descriptive
178 statistics and qualitatively tabulated.

179

180 **Results**

181 **Articles selection**

182 After searching for the terms: microRNA, colorectal cancer and prognosis in all
183 three data bases, 924 articles were found. By reading the titles, 29 duplicates were
184 removed and 215 abstracts were selected for complete reading. After analyzing the
185 abstracts, 134 articles which evaluate miR expression associated with CRC
186 prognosis were selected for complete reading. After reading, 19 articles that did not
187 achieve the proposed inclusion criteria were excluded. At the end, 115 articles were
188 included in the systematic review. Fig. 2 outlines the study selection process.

189 **Fig 2. Flow diagram outlining the study selection process.**

190 The quality scores of the studies ranged from 14 to 16 with a mean quality rating of
191 15.7 (S1 File).

192

193 **Characteristics of the selected studies**

194 In total, 14.894 CRC patients were studied in the 115 included articles. The
195 number of patients varied from 10 to 765 patients per study. The studies were
196 performed in institutes and universities in Greece, South Korea, United States, Czech
197 Republic, Iran, Sweden, Denmark, Japan and China with most of the research
198 performed in the last two countries. The studies evaluated the expression of 102
199 miRs associated with CRC prognostics aspects. MiR-21 was the most frequent one,
200 being analyzed in six studies^{25–30}. MiR-181a^{31–33}, miR-16^{34–36} and miR-133b^{37–39}
201 were individually investigated in three studies. MiR-126^{8,40}, miR-133a^{41,42}, miR-182

202 ^{43,44}, miR-183 ^{45,46}, miR-199b ^{47,48}, miR-22 ^{49,50}, miR-224 ^{51,52}, miR-24-3p ^{53,54}, miR-7
 203 ^{55,56}, were separately analyzed in two studies. Seven researches studied two miRs
 204 concomitantly ^{30,34,38,39,57–59}, while the others analyzed a single miR. All studies
 205 analyzed miR expression performing RT-PCR by TaqMan and/or SYBR Green
 206 methods in which 64 studies used SYBR Green while 51 studies used Taqman. The
 207 characteristics of the selected studies in this review are available as supplemental
 208 material (S2 Table).

209 Studies that investigated the prognosis of CRC patients by means of overall
 210 survival and disease-free survival demonstrated that upregulated miRs associated
 211 with worse prognosis were more frequent than downregulated miRs. In terms of
 212 pathologic clinical aspects, downregulated miRs associated with advanced TNM
 213 stage, tumor growth and lymph node metastasis were more frequent than
 214 upregulated miRs. Nonetheless, upregulated miRs were more associated with distant
 215 metastasis. The association of the miRs differentially expressed in CRC and their
 216 respective prognostic aspects are summarized in Table 1.

217 **Table 1.** Micro-RNAs differentially expressed in CRC and associated prognostic aspects.

Prognostic Aspects	Downregulated micro-RNAs	Upregulated micro-RNAs
Advanced TNM stage	miR-302c ⁶⁷ , miR-466 ⁶⁸ , miR-340 ⁶⁹ , miR-196b-5p ⁷⁰ , miR-944 ⁷¹ , miR-30d ⁷² , miR-22 ⁵⁰ , miR-296 ⁷³ , miR-384 ⁷⁴ , miR-199b ⁴⁸ , miR-128 ⁷⁵ , miR-33b ⁷⁶ , miR-155-5p ⁷⁷ , miR-154 ⁷⁸ , miR-149 ⁷⁹ , miR-194 ⁸⁰ , miR-133b ³⁷ , miR-15a ³⁴ , miR-100 ⁸¹ , miR-133a ⁴¹ , miR-335 ⁸² , miR-378a-3p ⁵⁸ , miR-378a-5p ⁵⁸ , miR-218 ⁸³ , miR-16 ³⁴	miR-182 ^{43,44} , miR-1296 ⁸⁴ , miR-888 ⁶⁴ , miR-21 ²⁹ , miR-130a ⁸⁵ , miR-7 ⁵⁵ , miR-96 ⁸⁶ , miR-20a-5p ⁶³ , miR-517a ⁸⁷ , miR-106b ⁸⁸ , miR-320e ⁸⁹ , miR-592 ⁹⁰ , miR-196a ⁵⁷ , miR-196b ⁵⁷ , miR-720 ⁹¹ , miR-133a ⁴² , miR-630 ⁹² , miR-181a ³¹ , miR-210 ⁹³ , miR-25 ⁹⁴ , miR-191 ⁹⁵ , miR-92a ⁹⁶ , miR-31 ⁵⁹ , miR-552 ⁹⁷
Tumor Growth	miR-302c ⁶⁷ , miR-466 ⁶⁸ , let-7a-5p ⁹⁸ , miR-132 ⁹⁹ , miR-206 ¹⁰⁰ , miR-33b ⁷⁶ , miR-154 ⁷⁸ , miR-194 ⁸⁰ , miR-100 ⁸¹ , miR-378 ¹⁰¹ , miR-378a-3p ⁵⁸ , miR-378a-5p ⁵⁸ , miR-148b ¹⁰² , miR-30c ¹⁰³	miR-1296 ⁸⁴ , miR-193a-3p ¹⁰⁴ , miR-592 ⁹⁰ , miR-720 ⁹¹ , miR-210 ⁹³ , miR-182 ⁴⁴ , miR-429 ¹⁰⁵ , miR-125b ⁶⁶ , miR-21 ^{26,28}
Lymph Node Metastasis	miR-6852 ¹⁰⁶ , miR-302c ⁶⁷ , miR-466 ⁶⁸ , miR-340 ⁶⁹ , miR-944 ⁷¹ , miR-30d ⁷² , miR-22 ⁵⁰ , miR-296 ⁷³ , miR-199b ⁴⁷ , let-7a-5p ⁹⁸ , miR-132 ⁹⁹ , miR-497 ¹⁰⁷ , miR-206 ¹⁰⁰ , miR-128 ⁷⁵ , miR-154 ⁷⁸ , miR-149 ⁷⁹ , miR-24-3p ⁵⁴ , miR-15a ³⁴ , miR-16 ^{34,36} , miR-193a-5p ¹⁰⁸ , miR-100 ⁸¹ , miR-133a ⁴¹ , miR-205 ¹⁰⁹ , miR-335 ⁸² , miR-378 ¹⁰¹ , miR-218 ⁸³ , miR-138 ¹¹⁰ , miR-625 ¹¹¹ , miR-212 ¹¹² , miR-195 ¹¹³ , miR-133b ³⁷ , miR-30c ¹⁰³	miR-1296 ⁸⁴ , miR-200c ¹¹⁴ , miR-21 ^{26,29} , miR-130a ⁸⁵ , miR-7 ⁵⁵ , miR-96 ⁸⁶ , miR-1260b ¹¹⁵ , miR-517a ⁸⁷ , miR-106b ⁸⁸ , miR-320e ⁸⁹ , miR-196a ⁵⁷ , miR-196b ⁵⁷ , miR-376a ¹¹⁶ , miR-720 ⁹¹ , miR-494 ⁶¹ , miR-630 ⁹² , miR-181a ³¹ , miR-20a ⁶² , miR-210 ⁹³ , miR-182 ^{43,44} , miR-25 ⁹⁴ , miR-191 ⁹⁵ , miR-183 ⁴⁶ , miR-199a-3p ¹¹⁷ , miR-429 ¹⁰⁵ , miR-92a ⁹⁶ , miR-10b ¹¹⁸ , miR-155 ³⁰ , miR-552 ⁹⁷
Distant Metastasis	miR-466 ⁶⁸ , miR-944 ⁷¹ , miR-30d ⁷² , miR-22 ^{49,50} , miR-296 ⁷³ , miR-140-5p ¹¹⁹ , miR-199b ⁴⁸ , miR-155-5p ⁷⁷ , miR-99b-5p ¹²⁰ , miR-154 ⁷⁸ , miR-149 ⁷⁹ , miR-96-5p ¹²¹ , miR-133b ^{38,39} , miR-126 ⁴⁰ , miR-138 ¹¹⁰ , miR-625 ¹¹¹ , miR-212 ¹¹²	miR-888 ⁶⁴ , miR-20a-5p ⁶³ , miR-517a ⁸⁷ , miR-320e ⁸⁹ , miR-592 ⁹⁰ , miR-720 ⁹¹ , miR-494 ⁶¹ , miR-133a ⁴² , miR-210 ³⁸ , miR-630 ⁹² , miR-17-5p ⁶⁰ , miR-181a ³¹ , miR-25 ⁹⁴ , miR-191 ⁹⁵ , miR-183 ⁴⁶ , miR-224 ⁵² , miR-199a-3p ¹¹⁷ , miR-92a ⁹⁶ , miR-372 ¹²² , miR-185 ³⁹ , miR-21 ^{26,28,30}
Worse Overall Survival	miR-199b ^{47,48} , miR-6852 ¹⁰⁶ , miR-302c ⁶⁷ , miR-466 ⁶⁸ , miR-650 ¹²³ , miR-196b-5p ⁷⁰ , miR-944 ⁷¹ , miR-30d ⁷² , miR-296 ⁷³ , miR-187 ¹²⁴ , let-7a-5p ⁹⁸ , miR-132 ⁹⁹ , miR-206 ¹⁰⁰ , miR-148a ¹²⁵ , miR-33b ⁷⁶ , miR-99b-5p ¹²⁰ , miR-154 ⁷⁸ , miR-217 ¹²⁶ , miR-149 ⁷⁹ , miR-194 ⁸⁰ , miR-7 ⁵⁶ , miR-24-3p ⁵⁴ , miR-29b ¹²⁷ , miR-15a ³⁴ , miR-16 ^{34,36} , miR-204-5p ¹²⁸ , miR-96-5p ¹²¹ , miR-193a-5p ¹⁰⁸ , miR-100 ⁸¹ , miR-133a ⁴¹ , miR-139-5p ¹²⁹ , miR-124-5p ¹³⁰ , miR-335 ⁸² , miR-378 ¹⁰¹ , miR-126 ⁴⁰ , miR-378a-3p ⁵⁸ , miR-378a-5p ⁵⁸ , miR-218 ⁸³ , miR-138 ¹¹⁰ , miR-625 ¹¹¹ , miR-212 ¹¹² , miR-124 ¹³¹ , miR-22 ⁴⁹ , miR-133b ³⁹ , miR-193a-3p ¹⁰⁴ , miR-195 ¹¹³ , miR-30c ¹⁰³	miR-181a ³¹⁻³³ , miR-182 ^{43,44} , miR-1296 ⁸⁴ , miR-888 ⁶⁴ , miR-28-5p ¹³² , miR-183 ^{45,46} , miR-200c ¹¹⁴ , miR-34a ¹³³ , miR-15a-5p ¹³⁴ , miR-181c ¹³⁵ , miR-24-3p ⁵³ , miR-16 ³⁵ , miR-7 ⁵⁵ , miR-96 ⁸⁶ , miR-1260b ¹¹⁵ , miR-20a-5p ⁶³ , miR-517a ⁸⁷ , miR-503 ¹³⁶ , miR-106b ⁸⁸ , miR-211 ¹³⁷ , miR-320e ⁸⁹ , miR-592 ⁹⁰ , miR-196a ⁵⁷ , miR-196b ⁵⁷ , miR-376a ¹¹⁶ , miR-224 ^{51,52} , miR-720 ⁹¹ , miR-494 ⁶¹ , miR-223 ¹³⁸ , miR-133a ⁴² , miR-133b ³⁸ , miR-210 ^{38,93} , miR-630 ⁹² , miR-17-5p ⁶⁰ , miR-20a ⁶² , miR-25 ⁹⁴ , miR-191 ⁹⁵ , miR-183 ^{45,46} , miR-199a-3p ¹¹⁷ , miR-429 ¹⁰⁵ , miR-92a ⁹⁶ , miR-372 ¹²² , miR-10b ¹¹⁸ , miR-185 ³⁹ , miR-21 ^{25-28,30} , miR-155 ³⁰ , miR-125b ⁶⁶ , miR-552 ⁹⁷
Worse Disease-Free Survival	miR-196b-5p ⁷⁰ , miR-944 ⁷¹ , miR-30d ⁷² , miR-22 ⁵⁰ , miR-296 ⁷³ , miR-199b ⁴⁷ , miR-187 ¹²⁴ , let-7a-5p ⁹⁸ , miR-132 ⁹⁹ , miR-29b ¹²⁷ , miR-15a ³⁴ , miR-16 ³⁴ , miR-34a-5p ⁶⁵ , miR-193a-5p ¹⁰⁸ , miR-138 ¹¹⁰ , miR-126 ⁸ , miR-212 ¹¹² , miR-124 ¹³¹	miR-888 ⁶⁴ , miR-28-5p ¹³² , miR-183 ⁴⁵ , miR-200c ¹¹⁴ , miR-34a ¹³³ , miR-15a-5p ¹³⁴ , miR-181c ¹³⁵ , miR-24-3p ⁵³ , miR-16 ³⁵ , miR-96 ⁸⁶ , miR-1260b ¹¹⁵ , miR-20a-5p ⁶³ , miR-517a ⁸⁷ , miR-503 ¹³⁶ , miR-106b ⁸⁸ , miR-320e ⁸⁹ , miR-196a ⁵⁷ , miR-196b ⁵⁷ , miR-224 ⁵¹ , miR-494 ⁶¹ , miR-181a ³² , miR-21 ^{25,26,30} , miR-155 ³⁰

218 Some of the selected studies investigated the target genes of the miRs
219 associated with the prognostic aspects of CRC. In total, 65 target genes were
220 analyzed in which the most frequent ones were: PTEN, investigated in six studies
221 ^{29,32,33,50,60,61}, SMAD4, investigated in three studies ⁶²⁻⁶⁴, and TP53 ^{65,66} investigated
222 in two studies.

223

224

225 **Micro-RNAs and the prognosis of colorectal cancer**

226 **Micro-RNAs associated with advanced TNM stage**

227 Twenty-four studies demonstrated the downregulation of twenty-five miRs
228 associated with advanced TNM stage: miR-302c ⁶⁷, miR-466 ⁶⁸, miR-340 ⁶⁹, miR-
229 196b-5p ⁷⁰, miR-944 ⁷¹, miR-30d ⁷², miR-22 ⁵⁰, miR-296 ⁷³, miR-384 ⁷⁴, miR-199b ⁴⁸,
230 miR-128 ⁷⁵, miR-33b ⁷⁶, miR-155-5p ⁷⁷, miR-154 ⁷⁸, miR-149 ⁷⁹, miR-194 ⁸⁰,
231 miR-133b ³⁷, miR-15a ³⁴, miR-100 ⁸¹, miR-133a ⁴¹, miR-335 ⁸², miR-378a-3p ⁵⁸, miR-
232 378a-5p ⁵⁸, miR-218 ⁸³ and miR-16 ³⁴. Two studies demonstrated the upregulation of
233 miR-182 ^{43,44} associated with advanced TNM stage and others 22 studies pointed 23
234 different upregulated miRs associated with advanced TNM stage. In detail, miR-1296
235 ⁸⁴, miR-888 ⁶⁴, miR-21 ²⁹, miR-130a ⁸⁵, miR-7 ⁵⁵, miR-96 ⁸⁶, miR-20a-5p ⁶³, miR-517a
236 ⁸⁷, miR-106b ⁸⁸, miR-320e ⁸⁹, miR-592 ⁹⁰, miR-196a ⁵⁷, miR-196b ⁵⁷, miR-720 ⁹¹, miR-
237 133a ⁴², miR-630 ⁹², miR-181a ³¹, miR-210 ⁹³, miR-25 ⁹⁴, miR-191 ⁹⁵, miR-92a ⁹⁶,
238 miR-31 ⁵⁹ and miR-552 ⁹⁷ have been reported.

239

240 **Micro-RNAs associated with tumor growth**

241 Tumor growth was associated with the downregulation of 14 miRs reported in 13
242 studies: miR-302c⁶⁷, miR-466⁶⁸, let-7a-5p⁹⁸, miR-132⁹⁹, miR-206¹⁰⁰, miR-33b⁷⁶,
243 miR-154⁷⁸, miR-194⁸⁰, miR-100⁸¹, miR-378¹⁰¹, miR-378a-3p⁵⁸, miR-378a-5p⁵⁸,
244 miR-148b¹⁰² and miR-30c¹⁰³. Ten studies verified the upregulation of miR-1296⁸⁴,
245 miR-193a-3p¹⁰⁴, miR-592⁹⁰, miR-720⁹¹, miR-210⁹³, miR-182⁴⁴, miR-429¹⁰⁵, miR-
246 125b⁶⁶ and miR-21^{26,28} associated with tumor growth as well.

247

248 **Micro-RNAs associated with the presence of lymph node** 249 **metastasis**

250 A total of 32 studies demonstrated the association between the downregulation of
251 32 miRs and the presence of lymph node metastasis such as miR-6852¹⁰⁶, miR-
252 302c⁶⁷, miR-466⁶⁸, miR-340⁶⁹, miR-944⁷¹, miR-30d⁷², miR-22⁵⁰, miR-296⁷³, miR-
253 199b⁴⁷, let-7a-5p⁹⁸, miR-132⁹⁹, miR-497¹⁰⁷, miR-206¹⁰⁰, miR-128⁷⁵, miR-154⁷⁸,
254 miR-149⁷⁹, miR-24-3p⁵⁴, miR-15a³⁴, miR-16^{34,36}, miR-193a-5p¹⁰⁸, miR-100⁸¹,
255 miR-133a⁴¹, miR-205¹⁰⁹, miR-335⁸², miR-378¹⁰¹, miR-218⁸³, miR-138¹¹⁰, miR-625¹¹¹,
256 miR-212¹¹², miR-195¹¹³, miR-133b³⁷ and miR-30c¹⁰³. Other 30 studies verified
257 the upregulation of miR-1296⁸⁴, miR-200c¹¹⁴, miR-21^{26,29}, miR-130a⁸⁵, miR-7⁵⁵,
258 miR-96⁸⁶, miR-1260b¹¹⁵, miR-517a⁸⁷, miR-106b⁸⁸, miR-320e⁸⁹, miR-196a⁵⁷, miR-
259 196b⁵⁷, miR-376a¹¹⁶, miR-720⁹¹, miR-494⁶¹, miR-630⁹², miR-181a³¹, miR-20a⁶²,
260 miR-210⁹³, miR-182^{43,44}, miR-25⁹⁴, miR-191⁹⁵, miR-183⁴⁶, miR-199a-3p¹¹⁷, miR-
261 429¹⁰⁵, miR-92a⁹⁶, miR-10b¹¹⁸, miR-155³⁰ and miR-552⁹⁷ associated with the
262 presence of lymph node metastasis.

263

264 **Micro-RNAs associated with the presence of distant metastasis**

The downregulation of miR-466⁶⁸, miR-944⁷¹, miR-30d⁷², miR-22^{49,50}, miR-296⁷³, miR-140-5p¹¹⁹, miR-199b⁴⁸, miR-155-5p⁷⁷, miR-99b-5p¹²⁰, miR-154⁷⁸, miR-149⁷⁹, miR-96-5p¹²¹, miR-133b^{38,39}, miR-126⁴⁰, miR-138¹¹⁰, miR-625¹¹¹ and miR-212¹¹² was associated with the presence of distant metastasis in 19 studies. The upregulation of miR-888⁶⁴, miR-20a-5p⁶³, miR-517a⁸⁷, miR-320e⁸⁹, miR-592⁹⁰, miR-720⁹¹, miR-494⁶¹, miR-133a⁴², miR-210³⁸, miR-630⁹², miR-17-5p⁶⁰, miR-181a³¹, miR-25⁹⁴, miR-191⁹⁵, miR-183⁴⁶, miR-224⁵², miR-199a-3p¹¹⁷, miR-92a⁹⁶, miR-372¹²², miR-185³⁹ and miR-21^{26,28,30} was associated with the presence of distant metastasis in 23 studies.

Micro-RNAs associated with worse overall survival

Among the evaluated studies, 49 demonstrated the downregulation of miR-199b^{47,48}, miR-6852¹⁰⁶, miR-302c⁶⁷, miR-466⁶⁸, miR-650¹²³, miR-196b-5p⁷⁰, miR-944⁷¹, miR-30d⁷², miR-296⁷³, miR-187¹²⁴, let-7a-5p⁹⁸, miR-132⁹⁹, miR-206¹⁰⁰, miR-148a¹²⁵, miR-33b⁷⁶, miR-99b-5p¹²⁰, miR-154⁷⁸, miR-217¹²⁶, miR-149⁷⁹, miR-194⁸⁰, miR-7⁵⁶, miR-24-3p⁵⁴, miR-29b¹²⁷, miR-15a³⁴, miR-16^{34,36}, miR-204-5p¹²⁸, miR-96-5p¹²¹, miR-193a-5p¹⁰⁸, miR-100⁸¹, miR-133a⁴¹, miR-139-5p¹²⁹, miR-124-5p¹³⁰, miR-335⁸², miR-378¹⁰¹, miR-126⁴⁰, miR-378a-3p⁵⁸, miR-378a-5p⁵⁸, miR-218⁸³, miR-138¹¹⁰, miR-625¹¹¹, miR-212¹¹², miR-124¹³¹, miR-22⁴⁹, miR-133b³⁹, miR-193a-3p¹⁰⁴, miR-195¹¹³ and miR-30c¹⁰³ associated with worse prognosis (lower overall survival) of CRC patients. Fifty-six studies identified the upregulation of miR-181a³¹⁻³³, miR-182^{43,44}, miR-1296⁸⁴, miR-888⁶⁴, miR-28-5p¹³², miR-183^{45,46}, miR-200c¹¹⁴, miR-34a¹³³, miR-15a-5p¹³⁴, miR-181c¹³⁵, miR-24-3p⁵³, miR-16³⁵, miR-7⁵⁵, miR-96⁸⁶, miR-1260b¹¹⁵, miR-20a-5p⁶³, miR-517a⁸⁷, miR-503¹³⁶, miR-

289 106b⁸⁸, miR-211¹³⁷, miR-320e⁸⁹, miR-592⁹⁰, miR-196a⁵⁷, miR-196b⁵⁷, miR-376a
290 ¹¹⁶, miR-224^{51,52}, miR-720⁹¹, miR-494⁶¹, miR-223¹³⁸, miR-133a⁴², miR-133b³⁸,
291 miR-210^{38,93}, miR-630⁹², miR-17-5p⁶⁰, miR-20a⁶², miR-25⁹⁴, miR-191⁹⁵, miR-183
292 ^{45,46}, miR-199a-3p¹¹⁷, miR-429¹⁰⁵, miR-92a⁹⁶, miR-372¹²², miR-10b¹¹⁸, miR-185³⁹,
293 miR-21^{25-28,30}, miR-155³⁰, miR-125b⁶⁶ and miR-552⁹⁷ was associated with worse
294 overall survival of CRC patients.

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296 **Micro-RNAs associated with worse disease free-survival**

297 In total, 17 studies demonstrated the downregulation of 18 miRs associated with
298 worse disease free-survival such as miR-196b-5p⁷⁰, miR-944⁷¹, miR-30d⁷², miR-22
299 ⁵⁰, miR-296⁷³, miR-199b⁴⁷, miR-187¹²⁴, let-7a-5p⁹⁸, miR-132⁹⁹, miR-29b¹²⁷, miR-
300 15a³⁴, miR-16³⁴, miR-34a-5p⁶⁵, miR-193a-5p¹⁰⁸, miR-138¹¹⁰, miR-126⁸, miR-212
301 ¹¹² and miR-124¹³¹. Twenty-one studies associated the upregulation of miR-888⁶⁴,
302 miR-28-5p¹³², miR-183⁴⁵, miR-200c¹¹⁴, miR-34a¹³³, miR-15a-5p¹³⁴, miR-181c¹³⁵,
303 miR-24-3p⁵³, miR-16³⁵, miR-96⁸⁶, miR-1260b¹¹⁵, miR-20a-5p⁶³, miR-517a⁸⁷, miR-
304 503¹³⁶, miR-106b⁸⁸, miR-320e⁸⁹, miR-196a⁵⁷, miR-196b⁵⁷, miR-224⁵¹, miR-494⁶¹,
305 miR-181a³², miR-21^{25,26,30} and miR-155³⁰ with worse disease free-survival.

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Target genes

In order to demonstrate the association among miRs and the clinical and prognostic aspects of CRC, 60 studies investigated also the putative target genes for these miRs. Sixty-five target genes were identified and, among these genes PTEN, SMAD4, P53 and KRAS were the most frequent ones. The downregulation of PTEN was associated with the upregulation of miR-181a^{32,33}, miR-17-5p⁶⁰, miR-494⁶¹, miR-22⁵⁰ and miR-21²⁹. The downregulation of SMAD4 was associated with the upregulation of miR-20a⁶², miR-20a-5p⁶³ and miR-888⁶⁴. The downregulation of P53 was related to the upregulation of miR-125b⁶⁶ and to the downregulation of de miR-34a-5p⁶⁵. The upregulation of KRAS was associated with the downregulation of miR-96-5p¹²¹ and miR-384⁷⁴.

Discussion

Colorectal cancer is one of the most prevalent neoplasms in the world and it is estimated that the five-year overall survival for CRC patients is only 65%²⁴. Several factors associated with CRC prognosis have been already studied, however sensible and specific prognostic biomarkers capable of predicting survival and recurrence of the disease are still unknown¹³⁹.

Recent reviews have demonstrated strong correlations between the prognostic aspects of breast cancer¹⁴⁰ and leukemia¹⁴¹ with miR expression, however there are few systematic reviews integrating the main miRs associated with the prognostic aspects of CRC. The present study evaluated 113 articles that investigated the

differential expression of 100 tissue miRs associated with the main clinicopathological and prognostic aspects of CRC, demonstrating that such molecules may be useful as valuable prognostic biomarkers in CRC.

MiRNAs expression can be analyzed in different biological samples such as tumor tissue⁴⁶, blood (serum or plasma)¹⁴², feces¹⁴³, or urine¹⁴⁴. In the present study, articles that evaluated the expression of miRs in tumor tissues were prioritized and selected. Tumor tissues can be stored in liquid nitrogen or paraffin, allowing greater stability to the miRs molecules for later analysis in retrospective studies that aim to evaluate patients' survival. The analysis in the tumor tissues confers greater specificity to the results since circulating miRs (present in serum or plasma) may come from different anatomical sites. In addition, tumor samples allow the analysis of miRs expression in comparison to the adjacent normal tissue of the same patient providing greater reliability to the results. In addition, studies indicate that factors associated to the tumor microenvironment influence the expression of miRs making the analysis in tumors more adequate for this type of study^{145,146}.

The expression of miRs can be evaluated by different methods such as northern blotting, immunohistochemistry, real-time PCR (RT-PCR), microarray, and others¹⁴⁷. Each method has its own particularities and limitations. In the present study, only articles that evaluated the expression of miRs in the CRC by means of RT-PCR using TaqMan or SYBR Green were selected, because these techniques often present high sensitivity and specificity, besides allowing the comparison of a specific miR in relation to an endogenous control.

The miRs are classified as oncogenic or tumor suppressors according to their respective target genes. MiRs that inhibit the expression of tumor suppressor genes are considered oncogenic and are generally upregulated in neoplasms. The most frequent oncogenic miRs in this study were miR-21, miR-181a, miR-182, miR-183, miR-210 and miR-224 and the upregulation of most of these miRs was associated with distant metastasis, lymph node metastasis and poorer prognosis in patients with CRC.

Conversely, miRs that inhibit the expression of oncogenes are considered tumor suppressors and are generally downregulated in the carcinogenic processes. The most frequent tumor suppressor miRs in this study were miR-126, miR-199b and miR-22 and their downregulation was associated with the presence of distant metastasis, worse prognosis and a greater risk of disease relapse (worse disease-free survival). In detail, the downregulation of miR-199b and miR-22 was also associated with more advanced TNM stage and the presence of lymph node metastasis. Other miRs such as miR-133a, miR-133b, miR-16, miR-24-3p and miR-7 were also relatively frequent in the present study, however, investigations analyzing the expression of these miRs have shown controversial results. The downregulation of these five miRs was associated with a worse prognosis and with the exception of miR-7, all the other miRs were downregulated and associated with the presence of lymph node metastasis.

In addition, other 84 miRs were also identified in the present review and their differential expression was associated with distinct clinicopathological and prognostic aspects of CRC, however, these miRs were evaluated in a single study. In general, it was observed that down and upregulated miRs associated with lymph node metastasis and worse prognosis (overall survival) in CRC were the most frequently studied.

Among the miRs identified, miR-21 was the most frequent one being analyzed in six studies. In these studies, an upregulation of miR-21 in the tumor compared to the adjacent normal tissue was found in addition to a strong correlation with more aggressive CRC characteristics such as tumor size, lymph node metastasis, distant metastasis and worse prognosis. A systematic review with meta-analysis conducted by Peng et al. selected studies that analyzed the role of miR-21 as diagnostic biomarker and prognostic of CRC, and statistically demonstrated that miR-21 upregulation in the tumor was associated with worse overall survival and worse disease-free survival, as in the present review ¹⁴⁸. The findings in both studies suggest the potential of miR-21 to predict survival and relapse of the disease making it promising biomarker for CRC prognosis.

Another systematic review with meta-analysis conducted by Gao et al. evaluated the prognostic value of miRs in colorectal cancer as in the present review. However, in that review, studies that analyzed the expression of miRs in different types of samples such as serum, plasma and tissue and performing other methods of detection, as immunohistochemistry, were considered, so they presented miRs not identified in the present study. Nevertheless, the findings of the review by Gao et al. and the present review demonstrate similar results regarding the analysis of miRs expression in tumor tissues associated with the prognostic aspects of CRC, that is, the upregulation of miR-181a, miR-224, miR-21 and miR-126 downregulation were also present associated with worse overall survival in CRC patients ²¹.

The molecular mechanisms by which polyps and adenomas develop to CRC are diverse. However, the major altered signal pathways responsible for this progression in

CRC are: MAPK / EGFR pathway, TGF β pathway and via P13K / AKT pathway ¹⁴⁹.

Coincidentally, the most frequent target genes for the miRNAs identified in the present study were also directly associated with these pathways.

The phosphatase and tensin homolog (PTEN) protein is encoded by a tumor suppressor gene responsible for the inactivation of pathways associated with cell proliferation. PTEN negatively regulates the PI3K / AKT pathway by hydrolyzing the Phosphatidylinositol-3,4,5-trisphosphate (PIP3) into Phosphatidylinositol 4,5-bisphosphate (PIP2) inactivating AKT, a protein that has antiapoptotic action. Similarly, PTEN inactivates the MAPK pathway by ERK (Extracellular Regulated Kinase) dephosphorylation, inactivating the transmission of signals through this pathway and consequently inhibiting cell proliferation. In addition, PTEN activates RAD51, a DNA-repairing protein, and CENPC (Centromere Protein C), a protein responsible for the stability of centromere and chromosomal integrity ¹⁵⁰. Situations in which PTEN is downregulated result in dysregulation in the cell cycle and destabilization of the cellular DNA and can trigger carcinogenic processes like CRC. In the present study, the upregulation of miR-181a, miR-17-5p, miR494, miR-22 and miR-21 was correlated with the downregulation of PTEN gene and worse prognosis of CRC patients.

The transforming growth factor β (TGF β) is a tumor suppressor cytokinin protein known to exert important functions in the regulation of apoptosis, differentiation, migration and cell proliferation. Through the binding of TGF β to its TGFR2 receptor, numerous proteins of this pathway are phosphorylated and activated, including the SMAD4 protein. However, studies indicate that mutations or downregulation of SMAD4, can hyperactivate TGF β pathway conferring it the potential to promote tumor growth,

invasion and metastasis^{149,151}. In the present study, upregulation of miR-20a, miR-20a-5p and miR-888 was associated with the downregulation of SMAD4 and the presence of distant metastasis, advanced TNM stage, worse overall survival and worse disease-free survival in patients with CRC.

The TP53 gene encodes the tumor suppressor protein P53 that plays important roles in cell cycle control and apoptosis. MiRs belonging to the miR-34 family can modulate the antiproliferative and pro-apoptotic activity of P53 by inhibiting oncogenes and cell cycle associated genes. Thus, downregulation of miR-34 or overexpression of oncogenic miRs that negatively regulate p53 results in a dysregulation of the cell cycle leading to a carcinogenic process¹⁵². In the present study, the downregulation of P53 was correlated with the upregulation of miR-125b and downregulation of miR-34a-5p, resulting in a worse prognosis for patients with CRC.

KRAS is a protein belonging to the RAS family of proteins that control the activation of several pathways related to cell proliferation, such as the MAPK pathway. MiRs considered to be tumor suppressors may act as regulators of KRAS expression. Thus, the downregulation of these miRs may increase the activation of KRAS resulting in a dysregulated cellular proliferation¹⁵³. The present review demonstrated that upregulation of KRAS was associated with downregulation of miR-96-5p and miR-384, which in turn was associated with an advanced TNM stage, presence of distant metastasis and worse overall survival of patients with CRC. Fig 3 outlines the differential expression of the miRs and their respective target genes.

Fig 3. Upregulation and downregulation of miRs associated with their respective target genes and cell functions.

By analyzing the results of the present review fully, it was possible to observe the large number of miRs associated with the different prognostic aspects of CRC, making them eligible as possible prognostic markers. However, few articles have analyzed the expression of two or more miRs concomitantly. Thus, the development of studies that analyze these miRs synergistically, would allow the identification of network interactions, making them more sensitive and specific in predicting the prognosis of patients with CRC and assisting in their therapeutic behavior.

Some limitations were observed during the elaboration of the present study. The large number of studies and miRs investigated made it difficult to conduct a quantitative and qualitative analysis capable of demonstrating the most sensitive and specific differentially expressed miRs associated with each clinical-pathological and prognostic aspect of the CRC. In addition, the study was limited to articles published in English, making it harder to obtain data from studies published in other languages.

Conclusion

After analyzing the results of the present systematic review, specific tissue miRs demonstrate to be promising CRC prognostic biomarkers due to their strong correlation with prognostic aspects of this disease, including advanced TNM stage, presence of distant metastasis and lymph node metastasis, tumor growth, prediction of worse

prognosis (worse overall survival) and recurrence of the disease (worse disease-free survival).

The miR-21 was the most frequent studied miR in the present review among the miRs identified and its upregulation was associated with tumor size, lymph node metastasis, distant metastasis and worse prognosis making it a promising biomarker of CRC prognosis. However, new studies are needed to systematize and identify the most sensitive and specific miRs associated with each prognostic aspect of CRC in order to choose the most relevant ones and apply them into clinical practice.

In addition, most studies have shown strong correlations between the expression of miRs and their respective target genes, so the development of functional studies aiming to elucidate these associations becomes important as a way to help in the description of new therapeutic targets of this disease.

The vast number of miRs identified in the present study also translates the complexity and heterogeneity of CRC, emphasizing the importance of new studies that clarify the regulatory mechanisms of the expression of these miRs in this tumor, allowing a better understanding of the molecular biology of this disease.

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