The association of a single-nucleotide variant in the microRNA-146a with advanced colorectal cancer prognosis

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Abstract
The aim of this study was to evaluate the association of single-nucleotide variant n.60G>C (rs2910164) of microRNA (miR)-146a, related to suppressing of BRCA1/2 DNA repair protein, with the risk and survival of colorectal cancer patients, as well as miR-146a and BRCA1/2 levels and miR binding efficiency. The genotypes were identified in 125 colorectal cancer patients and 276 controls using TaqMan polymerase chain reaction assay. The miR-146a and BRCA1/2 levels were assessed by quantitative–polymerase chain reaction protocols. Primary precursor of miR-146a containing G (wild-type) and C (variant) allele were cloned into pcDNA.3.3 vector and co-transfected in HT-29 colorectal cancer cell line. Luciferase reporter assay was performed to assess miR-146a binding to BRCA2 3'-untranslated region in HT-29. The differences between groups were calculated using chi-square or Fisher’s exact test, logistic regression, and Mann–Whitney test. The prognostic impact of single-nucleotide variant genotypes on overall survival was evaluated by Kaplan–Meier estimate and Cox regression. The GC or CC genotypes prevalence was similar in patients and controls (50.4% vs 50.7%, p = 0.74). However, patients with tumors in advanced stage with miR-146a GG genotype had 2.41 more chance of dying than GC or CC genotypes. In addition, tumor tissues of patients with GG genotype presented higher miR-146a (p = 0.02) and lower BRCA1 (p = 0.01) and BRCA2 (p < 0.0001) levels when compared to those with GC or CC genotypes. In fact, pcDNA.3.3-miR-146a-G presented increased binding capacity to the 3'-untranslated region of BRCA2 (p = 0.001) compared to pcDNA.3.3-miR-146a-C. In addition, the G allele altered the binding affinity between miR-146a and its BRCA2 3'-untranslated region target (p < 0.001), thus enhancing suppression of BRCA2 expression. Our results suggest that single-nucleotide variant rs2910164 does not influence the colorectal cancer risk in Brazilian patients; however, the GG genotype could act as a factor of worse prognosis in patients with advanced disease due to suppression of BRCA1/2 modulated by miR-146a.

Keywords
Colorectal cancer; single-nucleotide variant, SNV n.60G>C (rs2910164), risk, prognosis, miR-146a, BRCA1/2

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Introduction
Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both men and women worldwide. According to the National Cancer Institute (INCA), there are 40,990 estimated new CRC cases in Brazil for 2020. Therefore, the estimated prevalence of the disease in Brazil is about 20 cases per 100,000 individuals in 2020.

Over expression or silencing of specific microRNAs (miRs) has been described in the carcinogenesis of CRC. Recent studies suggest a potential influence of single-nucleotide variants (SNVs) in miRs for the risk of cancer development. The miR-146a n.60G>C SNV (rs2910164) causes a change from a G:U pair to a C:U mismatch in the stem structure of the miR-146a precursor, leading to process variation and lower expression of the mature sequence. Recent evidence suggests that the SNV rs2910164 is associated with the development and prognosis of various types of cancer, including CRC. However, its association with cancer is controversial and no data are currently available for CRC in the Brazilian population.

Interestingly, miR-146a binds to the same site in the 3’-untranslated region (UTR) of BRCA1 and BRCA2, resulting in downregulating their mRNA expression level in breast cancer. CRCA1/2 are crucial proteins involved in homologous recombination, which is the most effective method of double-stranded breaks (DSBs) repair. Two pathways are specifically dedicated to the repair of DSBs: homologous recombination and non-homologous end joining. The repression of these efficient repair systems permits an accumulation of damage in rapidly dividing cells that can induce apoptosis. The BRCA1 and BRCA2 expression levels are known as lower in CRC patients, indicating repair deficiency favoring damage accumulation in the disease.

The aims of this study were to perform a case-control study evaluating the SNV miR-146a n.60G>C and the risk of development and prognosis of sporadic CRC in a Southwestern-Brazilian population. Furthermore, BRCA2 and SNV miR-146a n.60G>C interaction was evaluated.

Materials and methods

Study population
The study was conducted according to the Declaration of Helsinki and was approved by Human Ethics Committee of São Francisco University (USF), Bragança Paulista, São Paulo, Brazil, under protocol no. 45723615.0.000.5514. The authors inform that informed consent was obtained from patients and blood donor controls.

The study included patients with diagnosed sporadic CRC treated at USF, between July 2010 and June 2016. The patient cohort consisted of 125 subjects (68 men, 57 women; age (mean ± SD): 66.3 ± 11.0 years; 103 Caucasians and 22 non-Caucasians) with histologically confirmed colorectal adenocarcinoma. The histological grades and tumor stages were established according to the American Joint Committee of Cancer Staging.

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The control cohort included a total of 276 blood donor volunteers recruited from Hematology and Hemotherapy Center of USF (168 men, 108 women; age (mean ± SD): 37.6 ± 11.8 years; 233 Caucasians and 43 non-Caucasians) between August 2015 and August 2016.

For survival analysis, we selected 120 CRC patients; 5 out of 125 patients were sent to other services for treatment and follow-up, and no consistent clinical information could be obtained. The patients were treated according to the institutional protocol. Patients with advanced resectable CRC tumors (n = 44) received neoadjuvant treatment (n = 5), adjuvant treatment (n = 16) or neoadjuvant plus adjuvant (n = 5), with concurrent intravenous 5-Fluoracil associated with different chemotherapies: Leucovorin, Irinotecan, Bevacizumab, Oxaliplatin, or Xelox associated with Capecitabine and Oxaliplatin, before or after surgery, respectively.

DNA isolation and genotyping
Genomic DNA was isolated from tumor tissues using phenol/chloroform-based protocol. Eight milliliters of peripheral venous blood was collected from all controls, and genomic DNA samples for genotyping were isolated using lithium chloride extraction.

For SNP rs2910164 genotyping, real-time polymerase chain reaction (PCR) was performed on StepOne Real-Time PCR (Applied Biosystems, USA) using standard TaqMan® genotyping assay (C__15946974_10) according to the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction
Total RNA from CRC tumor tissues (n = 25) was isolated using Trizol (Invitrogen, USA) according to the manufacturer’s instructions. MiR-146a (assay 000468), BRCA2 (assay 4319979), U6 (assay 001973), and U18S5 (assay 03928990_g1) complementary DNA (cDNA) were synthesized from total RNA according to the TaqMan® real-time assays protocol (Applied Biosystems®). Relative expression of each target was quantified by the delta delta cycle threshold (ΔΔCt)
method. Each sample was examined in triplicate and the raw data were presented as the relative quantity of the target, normalized by U6 and U18S5. The expression value of each gene was represented in arbitrary units (AUs).

For BRCA1 analyses, cDNA conversion from total RNA was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, USA). Each sample was examined in triplicate and the expression of each gene was normalized by control gene (GAPDH) and calculated by applying the 2-ΔΔCt method. The expression value of BRCA1 gene was represented in AUs. Primer sequences used for amplification are as follows: BRCA1 (forward) 5' - TTTTCGTTGCTCTGAACTGA-3' and (reverse) 5' - ATCACTGAGCTTCTGTTG-3'; GAPDH (forward) 5' - CCACCTTGAATTTGGAGGAT-3' and (reverse) 5' - GCACCGTCAAGGCTGAGAC-3'.

SNV rs2910164 cloning, expression, and detection
To study the effect of G and C alleles of the SNV rs2910164 on expression levels of mature miR-146a, DNA fragments from leucocytes of peripheral blood of healthy individuals containing primary precursors of miR-146a with either wild-type genotype GG and variant genotype CC were amplified by PCR and inserted into pcDNA3.3 mammalian expression vector. The pcDNA3.3 empty vector was kindly donated by Professor Ricardo Aguiar. This process generated the pMIR-BRCA2 plasmid.

The constructs were transfected into HT-29 colorectal carcinoma cell line using Lipofectamine® 2000 reagent (Invitrogen®) according to the manufacturer’s recommendations. In summary, the cells grown in a 48-well plate were co-transfected with 10 ng of pcDNA3.3-empty vector, pcDNA3.3-miR-146a-G or pcDNA3.3-miR-146a-C; 10 ng of the pMIR-BRCA2; and 5 ng of pRL-TK. After 48 h, the cells were collected, and the luciferase reporter assay was performed in Glomar®-Multi Detection System luminometer (Promega®, USA).

Statistical analysis
The HWE was tested using the chi-square test ($\chi^2$). Differences between groups were analyzed by $\chi^2$, and when sample size was more than 10 individuals or by Fisher’s exact test, when sample size was less than 10. Multivariate analysis using the logistic regression model served to obtain age-status adjusted crude odds ratios (ORs) with 95% confidence intervals (CIs) and to assess the associations between genotypes and CRC. The gender was not included in the OR adjustment because no difference in the patient’s (male: 57.0% and female: 43.0%) or controls (male: 60.9% and female: 39.1%; $p = 0.52$) gender frequencies were observed.

To evaluate the robustness of analysis, we computed the false discovery rate (FDR), which reflects the expected ratio of false-positive findings to the total number of significant findings. Power of analysis was used to calculate the minimum effect size that is likely to be detected in a given study using a sample size, using a statistical power calculator available on the website http://www.dssresearch.com/resources/calculators/statistical-power-calculator-percentage.

The miR-146a and BRCA1/2 expressions in the tumor tissues were probes for normality using Shapiro–Wilk’s test. Because the data sets did not assume normal distribution, Mann–Whitney test performed the comparison of groups. For primary precursor of miR-146a containing G (wild-type) and C (variant) allele expression levels and luciferase assay
analyses, *t*-test was used to compare the groups, since the data assumed normal distribution.

Overall survival (OS) was calculated from the date of diagnosis until the date of death, resulting of any cause. OS times were calculated using Kaplan–Meier estimate probabilities, and differences between survival curves were analyzed by the log-rank test. The prognostic impact of age, gender, ethnic origin, histological type (well, moderately, and poorly differentiated), tumor localization (rectum, colon, and rectum sigmoid), TNM stage (I, II, III, and IV), and each genotype in survival of CRC patients was examined using Cox proportional hazard ratio (HR) regression (backward conditional step-wise selection).

For all statistical tests, significance is two-sided and achieved when *p* values were less than 0.05. All tests were performed using the SPSS 21.0 software (SPSS Incorporation, USA).

**Results**

**Study population**

The control individuals were younger than CRC patients (*p* < 0.001), but the differences in age of individuals of each group were corrected in all comparisons of genotype frequencies by pertinent statistical analyses.

A total of 125 patients were included in the study of which CRC was well differentiated in 6 patients, moderately differentiated in 103, and poorly differentiated in 4; mucinous adenocarcinoma in four patients, adenocarcinoma in tubulovillous adenoma in two patients, and not specified types in 5 patients. Tumors of stages I, II, III, and IV were identified in 12, 29, 44, and 39 patients, respectively. The total number of individuals with tumor histological type and stage (n = 124) differed from the total quoted in study (n = 125) because it was not possible to obtain consistent information about these criteria in some patients.

### SNV rs2910164 genotypes and CRC risk

The samples of CRC patients and controls groups were in Hardy–Weinberg equilibrium (HWE) for SNV rs2910164 (*χ² = 0.08, *p* = 0.77 and *χ² = 0.26, *p* = 0.61, respectively). Similar frequencies of genotypes were seen in patients and controls (Table 1). In addition, GC or CC genotypes’ prevalence was similar in patients and controls (50.4% vs 50.7%, *p* = 0.74), as well GG or GC genotypes (92.0% vs 92.0%, *p* = 0.44; Table 1). Moreover, individuals with distinct genotypes had similar risks of CRC (Table 1).

### SNV rs2910164 genotypes and clinical–pathological characteristics

No associations between SNV rs2910164 genotypes were observed in CRC patients stratified by age, gender, ethnical origin, histological type, and CRC localization (Table 2). However, the frequency of GC or CC genotype was significantly higher in CRC patients with advanced tumors (stage IV) than those with GG genotype (69.2% vs 30.8%, *p* = 0.006, *p* = 0.03 after FDR analysis (Table 2)).

### Survival analysis

The median follow-up time of 120 CRC patients was 35.5 months (range: 0.1–99.0 months). The final status of patients was established in January 2020. At this date, 65 patients were alive and 55 patients died. The 36 months overall survival (OS) was 56.1%.

At 36 months of follow-up, lower OS was observed in patients with tumor stage TNM IV (39.2% vs 63.5%, *p* = 0.001; Kaplan–Meier estimates). The significance of differences between groups remained the same in Cox analysis (HR: 2.44, 95% CI: 1.43–4.16). The miR-146a n.60G>C genotypes did not influence the OS of our CRC patients (Table 3).

Considering only the CRC patients with advanced tumor stage (IV; n = 36), at 36 months of follow-up, OS was shorter in patients with miR-146a GG genotype (10.0% vs 55.6%, *p* = 0.03; Figure 1(e); Kaplan–Meier estimate). The significance of difference between groups remained the same in Cox analysis (HR: 2.41, 95% CI: 1.06–5.48).

### miR-146a and BRCA2 expressions at G and C-allele-pcDNA.3.3 constructs

The sequence alignment of mature MIR146A and it complementary sites in the BRCA1 3’-UTR at position 606-613 and BRCA2 3’-UTR at position 584-590 is schematically represented in Figure 1(a). Also, the
Table 2. MiR-146a n.60G>C (rs2910164) genotypes among 125 sporadic colorectal cancer patients stratified by the clinical features and biological aspects of the tumor.

|                            | GG or GC | CC  | GG  | GC or CC |
|-----------------------------|----------|-----|-----|----------|
| Age, n (%)                  |          |     |     |          |
| Younger than 67 years       | 52 (88.1)| 7 (11.9)| 24 (40.7)| 35 (59.3) |
| 67 years and older          | 63 (95.5)| 3 (4.5) | 38 (57.6)| 28 (42.4) |
| p value                     | 0.18     | 0.06 |     |          |
| Gender, n (%)               |          |     |     |          |
| Male                        | 64 (94.1)| 4 (5.9) | 33 (48.5)| 35 (51.5) |
| Female                      | 51 (89.5)| 6 (10.5)| 29 (50.9)| 28 (49.1) |
| p value                     | 0.51     | 0.79 |     |          |
| Ethnical origin, n (%)      |          |     |     |          |
| Caucasians                  | 95 (92.2)| 8 (7.8) | 53 (51.5)| 50 (48.5) |
| Non-Caucasians              | 20 (90.9)| 2 (9.1) | 9 (40.9) | 13 (59.1) |
| p value                     | 0.68     | 0.48 |     |          |
| TNM, n (%)                  |          |     |     |          |
| I–III                       | 78 (91.8)| 7 (8.2) | 50 (58.8)| 35 (41.2) |
| IV                          | 36 (92.3)| 3 (7.7) | 12 (30.8)| 27 (69.2) |
| p value                     | 1.00     | 0.006 |     |          |
| Histological type, n (%)    |          |     |     |          |
| Well* or moderately differentiatedb | 99 (90.8) | 10 (9.2) | 51 (46.8) | 58 (53.2) |
| Poorly differentiatedc      | 16 (100.0)| 0 (0.0) | 11 (68.8)| 5 (31.3) |
| p value                     | 0.35     | 0.10 |     |          |
| CRC localization (%)        |          |     |     |          |
| Rectum                      | 25 (96.2)| 1 (3.8) | 11 (42.3)| 15 (57.7) |
| Colon or rectum sigmoid     | 90 (90.9)| 9 (9.1) | 51 (51.5)| 48 (48.5) |
| p value                     | 0.68     | 0.40 |     |          |

CRC: colorectal cancer.

*Well differentiated (low-grade malignancy).

bModerately differentiated (average grade malignancy).

cPoorly differentiated (high-grade malignancy).

d,e p = 0.03 after false discovery rate analysis.

Association between miR-146a and BRCA2 expression and miR-146a n.60G>C genotypes

In accordance with in vitro assays, the mean mRNA expression level was significantly higher in tumors of patients with miR-146a GG wild-type genotype (n = 12) when compared to tumors of patients with GC or CC genotypes (n = 13; 1.38 AUs (SD: 1.10) vs 0.53 AUs (SD: 0.38); p = 0.02; Figure 1(d), left side). However, BRCA1/2 expression levels were lower in GG genotype (n = 12) when compared with GC or CC genotypes (n = 13) patients (1.27 AUs (SD: 0.73) vs 2.61 AUs (SD: 1.68); p = 0.01; 1.26 AUs (SD: 0.63) vs 4.87 AUs (SD: 3.30); p < 0.0001; Figure 1(d), right side).

Luciferase target in vitro assay

A significant reduction of luciferase activity in the cells transfected with both pcDNA.3.3-miR-146a-C and pMIR-BRCA2 report when compared to pcDNA.3.3-miR-146a-G report (10% increased, p = 0.03; Figure 1(c)). These results indicated that the G allele altered the binding affinity between miR-146a and BRCA2 3'-UTR target, enhancing suppression of BRCA2 expression.

SNV n.60G>C, located at miR-146a mature sequence, is given in bold and indicated by arrow in Figure 1(a). We found that the expression level of mature miR-146a in G wild-type allele was significantly higher than those in C variant allele (5.75 AUs (standard deviation (SD): 1.04) vs 1.39 AUs (SD: 0.15); p = 0.0002; Figure 1(b)). In fact, the expression level of mature miR-146a was fivefold higher in G allele than in C allele. In contrast, expression levels of BRCA1/2 in G-allele-specific pcDNA.3.3 construct were similar than those in C allele (0.41 AUs (SD: 0.03) vs 0.41 AUs (SD: 0.09); p = 0.96; 0.63 AUs (SD: 0.21) vs 1.34 AUs (SD: 0.58); p = 0.06; Figure 1(b)).

Established in the cells transfected with both pcDNA.3.3-miR-146a-C and pMIR-BRCA2 report compared to the cells transfected with pcDNA.3.3-EV and pMIR-BRCA2 report, indicating the binding between miR-146a and 3'-UTR of BRCA2 in vitro (30% decreased, p < 0.0001; Figure 1(c)). Interestingly, the luciferase activity was re-established in the cells transfected with both pcDNA.3.3-miR-146a-C and pMIR-BRCA2 report when compared to pcDNA.3.3-miR-146a-G report (10% increased, p = 0.03; Figure 1(c)). These results indicated that the G allele altered the binding affinity between miR-146a and BRCA2 3'-UTR target, enhancing suppression of BRCA2 expression.

Table 2. MiR-146a n.60G>C (rs2910164) genotypes among 125 sporadic colorectal cancer patients stratified by the clinical features and biological aspects of the tumor.
Table 3. Clinical and tumor aspects, and miR146a n.60G>C polymorphism in overall survival of 120 colorectal cancer patients.

| Variables                       | Overall survival |        |        |
|---------------------------------|------------------|--------|--------|
|                                 | N total/ N event | HR (95% CI) | P value |
| Median age (years)              |                  |        |        |
| ≤67                             | 58/23            | Reference | 0.11  |
| >67                             | 62/32            | 1.54 (0.90–2.64) |        |
| Gender                          |                  |        |        |
| Male                            | 67/36            | 1.68 (0.96–2.95) | 0.06  |
| Female                          | 53/19            | Reference |        |
| Histological grade              |                  |        |        |
| Well or moderately differentiated| 105/48           | Reference | 0.62  |
| Poorly differentiated           | 15/7             | 1.21 (0.55–2.69) |        |
| Tumor localization              |                  |        |        |
| Colon or rectum sigmoid         | 94/45            | 1.46 (0.73–2.90) | 0.27  |
| Rectum                          | 26/10            | Reference |        |
| TNM                             |                  |        |        |
| I–III                           | 36/25            | 2.44 (1.43–4.16) | **0.001** |
| miR146a n.60G>C                 |                  |        |        |
| GG                              | 59/29            | 1.19 (0.70–2.03) | 0.50  |
| GC or CC                        | 61/26            | Reference |        |
| GG or GC                        | 110/51           | 1.30 (0.47–3.59) | 0.61  |
| CC                              | 10/4             | Reference |        |

N: number of patients, HR: hazard ratio, CI: confidence interval.
*p values < 0.05 are presented in bold letters.

Discussion

Genetic variations in miR genes and/or their target mRNAs might represent a new mechanism in CRC susceptibility.26 We investigated here whether the SNV rs2910164 alters the risk of CRC, biological aspects of the tumor, and survival of patients.

We initially observed that SNV rs2910164 did not alter CRC risk. Our results are in agreement with previous CRC studies.12,15,17 Studies have been demonstrated that patients who possessed the GC or CC genotypes had a higher risk of CRC compared to those with the GG genotype.13,16 In contrast, one study has showed significantly decreased risk for CRC in those GC or CC genotype patients.14 The divergent results in our study and previous studies could be attributed to our highly heterogeneous population.27

Our finding supports the evidence that the C allele of the SNV rs2910164 is a factor for the aggressiveness of CRC since the frequency of GG or CC genotypes was significantly higher than those at GG genotype in the patients with advanced stage and/or metastasis. Our results are in accordance with a Japanese study that observed GC or CC genotypes were associated with significantly more synchronous liver metastasis.15

Numerous miRs are involved in tumor formation and progression by regulating the expression of many oncogenes and tumor suppressor genes.28 A recent study provided evidence that miR-146a acts as a modulator of the innate immune response and of the adaptive immune response as well.29 Additional studies showed that miR-146a expression is upregulated in papillary thyroid carcinoma,6,30 breast cancer,31 cervical,32 and anaplastic thyroid carcinomas33 in contrast to pancreatic cancer34 and gastric cancer35,36 in which the expression of this miR is downregulated. MiR-146a has been associated with CRC risk, which is overexpressed in cancer tissues compared to healthy tissues.37 In our study, miR-146a expression level was significantly higher in patients harboring GG genotype in comparison to GC or CC in tumor tissues. A previous study has compared the miR-146a expression level and SNV rs2910164 genotypes in thyroid carcinoma tissues,6 and the authors showed that the GC heterozygote state, but not the homozygote CC state, is associated with an increased risk of acquiring papillary thyroid cancer. However, there are no previous studies comparing miR-146a expression level and SNV rs2910164 genotypes in CRC patients.

The evidence that miR-146a can inhibit the expression of BRCA1/2 has been demonstrated in breast cancer.7,8 Here, we provide the first evidence that miR-146a can inhibit BRCA2 in a CRC cell line and patient tumor tissues, indicating the involvement of miR-146a and DNA damage repair genes in CRC. BRCA1 and BRCA2 play an integral role in response to cellular stress, the localization to sites of damaged DNA, and the activation of DNA repair processes. In particular, BRCA1 and BRCA2 usually repair DSB through the conservative mechanism of homologous recombination.18,19,38,39 The BRCA proteins bind an essential recombinant, RAD51, and mutations affecting the binding ability of BRCA1 or BRCA2 to RAD51, which lead to genomic instability.18,19,38,39 We showed in the present study a higher miR-146a expression level in CRC patients with G-allele for the SNV rs2910164, which can lead to a low-expression level of BRCA1/2.

In addition, we observed that CRC patients with stage IV tumors and GG wild-type genotype for the SNV rs2910164 had worst OS when compared to carriers of variant allele. In contrast, one Korean study showed in Kaplan–Meier survival analysis that the CC variant genotype was associated with a worse survival outcome in CRC patients when compared to GG or GC genotypes.13 However, the authors have not analyzed the miR-146a expression level according to the SNV rs2910164 genotypes or stage of the disease. Our findings indicate that the individual carriers of GG genotype for SNV rs2910164 presented BRCA1/2 downregulated and might be more likely to develop CRC in advanced stage. Meanwhile, our results need to be confirmed in a larger patient cohort.
Conclusion

Our results suggest that SNV rs2910164 did not influence the CRC risk in Brazilian patients. However, the GG wild-type genotype seems to influence the prognosis of the disease in advanced stage, probably due to the miR-146a overexpression, which may downregulate the BRCA2 gene expression in tumor tissues.

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Author contributions

M.M.O. contributed to the conception and design. C.A.R.M. helped in collection of tissue samples from patients who went to surgery. J.S.S., G.L.Z., A.B.N., and M.S.G.R. contributed to acquisition of data. M.M.O. and G.J.L. involved in analyses and interpretation of data. G.J.L. helped in statistical analyses. M.L.R. provided important reagents. M.M.O. helped in drafting of the manuscript. M.M.O. involved in study supervision. All authors were involved in revision of the manuscript and have approved the final version.

Availability of data and materials

The data generated or analyzed during this study are included in this published article.

Declaration of conflicting interests

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This study was approved by Ethics Committee of USF.

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Informed consent
Written consent was obtained from patients and controls.

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References
1. American Cancer Society: Cancer Facts Figures 2017. Atlanta, GA: American Cancer Society, 2017, https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html
2. Ministry of Health National Cancer Institute (INCA). Cancer Incidence in Brazil 2019. https://www.inca.gov.br/estimativa/regiao/brasil
3. Ye JJ and Cao J. MicroRNAs in colorectal cancer as markers and targets: recent advances. World J Gastroenterol 2014; 20(15): 4288–4299.
4. Srivastava K and Srivastava A. Comprehensive review of genetic association studies and meta-analyses on miRNA polymorphisms and cancer risk. Plos One 2012; 7(11): e50966.
5. Hu Y, Yu CY, Wang JL, et al. MicroRNA sequence polymorphisms and the risk of different types of cancer. Sci Rep 2014; 4: 3648.
6. Jadzewska K, Murray EL, Franssila K, et al. Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2008; 105(20): 7269–7274.
7. Shen J, Ambrosone CB, DiCioccio RA, et al. A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis. Carcinogenesis 2008; 29(10): 1963–1966.
8. Garcia AI, Buisson M, Bertrand P, et al. Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers. EMBO Mol Med 2011; 3(5): 279–290.
9. Permutt-Wey J, Thompson RC, Burton Nabors L, et al. A functional polymorphism in the pre-miR-146a gene is associated with risk and prognosis in adult glioma. J Neurooncol 2011; 105(3): 639–646.
10. Wu MY, Huang SJ, Yang F, et al. Detection of nasopharyngeal carcinoma susceptibility with single nucleotide polymorphism analysis using next-generation sequencing technology. Oncotarget 2017; 8(32): 52708–52723.
29. Rusca N and Monticelli S. miR-146a in immunity and disease. Mol Biol Int 2011; 2011: 437301.
30. He H, Jazdzewski K, Li W, et al. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci U S A 2005; 102(52): 19075–19080.
31. Bhamik D, Scott GK, Schokrpur S, et al. Expression of microRNA-146 suppresses NF-κB activity with reduction of metastatic potential in breast cancer cells. Oncogene 2008; 27: 5643–5647.
32. Wang X, Tang S, Le SY, et al. Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. Plos One 2008; 3(7): e2557.
33. Pacífico F, Crescenzi E, Mellone S, et al. Nuclear factor-κB contributes to anaplastic thyroid carcinomas through up-regulation of miR-146a. J Clin Endocrinol Metab 2010; 95(3): 1421–1430.
34. Yu J, Li A, Hong SM, et al. MicroRNA alterations of pancreatic intraepithelial neoplasias. Clin Cancer Res 2012; 18(4): 981–992.
35. Hou Z, Xie L, Yu L, et al. MicroRNA-146a is down-regulated in gastric cancer and regulates cell proliferation and apoptosis. Med Oncol 2012; 29(2): 886–892.
36. Kogo R, Mimori K, Tanaka F, et al. Clinical significance of MIR-146a in gastric cancer cases. Clin Cancer Res 2011; 17(13): 4277–4284.
37. Omrane I, Kourda N, Stambouli N, et al. MicroRNAs 146a and 147b biomarkers for colorectal tumor’s localization. Biomed Res Int 2014; 2014.
38. Patel KJ, Yu VP, Lee H, et al. Involvement of Brca2 in DNA repair. Mol Cell 1998; 1(3): 347–357.
39. Scully R, Chen J, Plug A, et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell 1997; 88(2): 265–275.