A genetic variant in microRNA-146a is associated with sporadic breast cancer in a Southern Brazilian Population

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Abstract

MicroRNAs (miRNAs) play an essential role in gene expression and affect the development of tumours, including breast cancer (BC). Polymorphisms in miRNA genes can affect the interaction of miRNAs with their target messenger RNA by interfering, creating or disrupting target sites. The single nucleotide polymorphism (SNP) rs2910164, located in the seed region of miR146a, was shown to be associated with BC among different populations. In the present study, we investigated whether rs2910164 is associated with BC in 326 patients and 411 controls from a Brazilian population of predominantly European ancestry. The presence of the allele rs2910164*C was associated with an increased risk of BC (OR=1.4, 95% CI=1.03-1.85, \(p=0.03\)). We also analysed publicly available RNA-seq data to evaluate if miR146a is differentially expressed in different subtypes of BC. Genotyping was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP). By leveraging public data from TCGA database, we analysed 461 patients and found that miR146a is significantly more expressed in BC than in non-tumor tissue (1.47 fold, \(p=0.02\)) and is expressed to a greater degree in aggressive BC subtypes.

Keywords: miRNA polymorphism; miR146a; rs2910164; Breast cancer; Case-control study.

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Introduction

Breast cancer (BC) is the second most frequent type of cancer in Brazilian women (INCA, 2018) and is considered the leading cause of death by malignant disease among women worldwide (Bray et al., 2018). Identifying genetic factors associated with BC can aid in predicting disease risk at the individual and population levels, and provide information about the underlying disease mechanism. Some of the genetic variants implicated in BC are SNPs (single nucleotide polymorphisms) located in microRNA (miRNA) genes (Alshatwi et al., 2012).

miRNAs are a highly conserved class of small (~22 nucleotide) endogenous non-coding RNAs (Bartel, 2004). They play an essential role in cancer development by modulating the expression of oncogenes and tumour suppressor genes (Croce, 2009). Small alterations in the miRNA sequence can lead to substantial phenotypic consequences.

Their function can be modified in three main ways: 1) altered transcription levels of the primary transcript; 2) altered processing and transport of the pri-miRNA and premiRNA; 3) altered interaction of the miRNA with their target mRNAs (Ryan et al., 2010).

Genome-wide association studies (GWAS) previously identified 102 loci associated with BC in Europeans (Michailidou et al., 2017). GWAS showed that 90% of the SNPs associated with complex diseases were located in non-protein coding regions, including the ones transcribed to miRNAs (Hrdlickova et al., 2014; Almeida et al., 2018). Notwithstanding, SNPs have been found in only 10% of the known pre-miRNAs. Within the seed region, Variation occurs in only less than 1% of the known seed regions, which consists of the nucleotides 2-8 from the mature miRNA sequence. The seed region specifies to which miRNAs the particular miRNA will be able to bind and the lack of variation suggests a purifying selection (Saunders et al., 2007).

The mir146a gene is located at the chromosomal region 5q33.3 and encodes the mir146a-3p and the mir146a-5p. The SNP rs2910164 is a G>C alteration at position +60 from the first nucleotide of the pre-miR146a (Jazdzewski et al., 2008), on the seed region of miR146a-3p. The result is a G:U to C:U change leading to a weaker
pairing within the pre-miRNA hairpin structure (Hu et al., 2008), which alters the processing efficiency of pre-miR146a. It has been shown that the amount of mature miR146a is two times lower with the C allele. This difference is due to a less efficient pri-miRNA processing and a reduced transport of pre-miR146a from the nucleus to the cytoplasm (Jazdzewski et al., 2008).

There are few case-controls studies focusing the polymorphism rs2910164 in association with BC. Five studies were described in the European population (Catucci et al., 2010; Pastrello et al., 2010; Garcia et al., 2011; Cardeñosa et al., 2012; McVeigh et al., 2017) and one in an Australian Caucasian population (Uphadhyaya et al., 2015). The majority of these studies have focused on familial BC (Catucci et al., 2010; Pastrello et al., 2010; Garcia et al., 2011; Cardeñosa et al., 2012). The results are conflicting showing positive association in only two studies, with C allele (GC+CC) in Pastrello et al. (2010) and with G allele in sporadic BC in Uphadhyaya et al. (2015).

Comparing patients with triple-negative breast cancer (TNBC) and no TNBC, we recently demonstrated that miR146a-5p presents a higher level of expression in TNBC (Sugita et al., 2016). This result motivated the investigation of whether rs2910164 is associated with BC in a Southern Brazilian population and to evaluate the expression of miR146a-5p among subtypes of BC.

Subjects and Methods

Study cohort

The study population consisted of 326 Brazilian women diagnosed with sporadic BC, from the Nossa Senhora das Graças Hospital, Curitiba, Brazil. The control group comprised 411 healthy Brazilian women from the bone marrow donor biobank of the Laboratory of Immunogenetics and Histocompatibility, Genetics Department, Federal University of Paraná, Curitiba. Both patients and controls were from the same region in South Brazil and belong to the same ethnic background, which is of predominantly European ancestry (Probst et al., 2000; Sugita et al., 2016). This study was approved by the Brazilian Commission of Ethics in Research (CONEP) under the number CAAE 67400917.3.0000.55 following Brazilian Federal laws. All participants signed informed written consent following the principles of the Declaration of Helsinki. The mean ages of the case and the control groups were 56.23 ± 15 and 47.66 ± 4.69, respectively. Histopathological parameters, immunohistochemical features, and status of regional lymph node invasion from cancer samples are summarized in Table 1. The immunohistochemical classification was based on Goldhirsch et al. (2013).

Genotyping

The SNP rs2910164 was genotyped using polymerase chain reaction with specific sequence primers (PCR-SSP). The primer sequences used were: forward 5’-GGTTGTCAGTGTCAGACCTC-3’, forward 5’-GGTTGTCAGTGTCAGACCTG-3’, reverse 5’-GAGCCTGAGACTCTGCCTTCT-3’ and the product size was 196 bp. PCR amplifications were carried out in a 20 µL reaction containing: 40 ng DNA, 0.2 µM each primer, 0.2 mM dNTPs, KCl 50 mM, Tris-HCl 10 mM and 1.25 U Taq polymerase (Invitrogen, Carlsbad, CA, USA). PCR conditions were: 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, followed by 5 min at 72 °C. After the reaction, the products were analysed by gel electrophoresis in 2% agarose gel stained with GelRed® (Biotium, Fremont, CA, USA). As an internal amplification control, we amplified the galactosylceramide gene (GALC) using the following primers: forward 5’-TTACCCAGAGCCCTATCGTTCT -3’ and reverse 5’-GTCTGCCCATCACCACCTATT -3’. The GALC ampli-

Table 1 - Histopathological and immunohistochemical parameters of breast cancer patients.

| Histology of carcinoma | n | %  | Tumour Grade | n | %  |
|------------------------|---|----|--------------|---|----|
| Invasive ductal         | 233|75%| Grade I      | 37|16%|
| Invasive lobular        | 28 |9% | Grade II     | 125|53%|
| Ductal in situ          | 12 |4% | Grade III    | 75 |32%|
| Invasive mucinous       | 9 |3% | Without Information | 89 | - |
| Others                  | 28 |9% |              |    |    |
| Without information     | 16 | - |              |    |    |

| Immunohistochemical classification | n | %  | Lymph node invasion | n | %  |
|-------------------------------------|---|----|---------------------|---|----|
| Luminal A                           | 84|40%| Present             | 116|47%|
| Luminal B                           | 78|38%| Absent              | 133|53%|
| HER2+                               | 15|7% | Without information | 77 | - |
| Triple-Negative                     | 31|15%|                     |    |    |
| Without information                 | 118| - |                     |    |    |
con (352 bp) was expected in all reactions. We validated the specificity and accuracy of our PCR-SSP method by sequencing five random individuals with Sanger methods and verifying their genotypes.

Expression analysis from publicly available RNA-Seq data

The Cancer Genome Atlas Program (TCGA) is a public database that contains data from thousands of matched cancer and non-cancer samples (TCGA Network, 2012). We analysed RNA-Seq data from 837 BC patients and 105 adjacent tissues to search for possible differential expression levels of miR146a in BC compared to non-tumour tissue and among intrinsic subtypes. Pre-processing and normalization of RNA-seq data were performed by TCGA biolinks package from R/Bioconductor (Colaprico et al., 2016). For expression analysis from molecular subtypes, we analysed 461 patients, being 211 Luminal A, 112 Luminal B, 85 Basal-like, and 53 HER2-Enriched.

Statistical analysis

Association analysis was performed using chi-square test of independence and using odds ratios (OR) and 95% confidence intervals (Mantel and Haenszel, 1959). We used R package Hardy-Weinberg (Graffelman, 2015) to test if genotype distributions were under Hardy Weinberg equilibrium. A chi-square test of independence was used to compare the distribution of rs2910164 genotypes among the several parameters (histopathological, subgroups defined by immunohistochemistry, tumour grade, and lymph node invasion). Bartlett’s test was applied to compare the age of cancer diagnosis in different genotypes (Bartlett, 1937). Analysis of the expression data from TCGA was performed using Kruskal-Wallis test followed by Dunn test (Kruskal and Wallis, 1952; Dunn, 1964). Spearman correlation was used to check the correlation of miR146a and the genes BRCA1, TRAF6, IRAK1, TRAF3IP1.

Alpha (α) was set at 0.05 for all tests and all analyses were performed with R Software (R core team, 2017) with the packages Nortest and readxl (Gross and Ligges, 2015; Wickham and Bryan, 2017).

Results

The presence of the allele rs2910164C is associated with breast cancer risk

The genotype GC was more frequent in patients compared to controls (Table 2). Considering that the difference in the distribution of the three genotypes was not significant (p = 0.10), we determined the magnitude of the effect on BC susceptibility testing three models by odds ratio tests: G vs. C for additive, CC + GC vs. GG for dominant and CC vs. GC + GG for recessive inheritance. The dominant model (presence of allele C) was associated with an increased risk of BC (OR = 1.4, p = 0.03). The C allele was observed 4% more frequently in the case than in the control population. The distribution of genotypes followed Hardy-Weinberg equilibrium in the control population (p = 0.70).

We observed no differences in distribution of lymph node invasion (p = 0.25), tumour grade (p = 0.30) and immunohistochemical subgroups (p = 0.90) comparing risk group GC+CC vs. GG group from BC patients. Applying Bartlett test for homogeneity, no difference was observed in the variance analysis for assessing the risk of developing BC in younger ages for the risk group GC+CC vs. the GG group.

miR146a is differentially expressed in breast cancer

Accessing publicly available RNA-Seq data from TCGA database, we found miR146a significantly more expressed in tumour tissue compared to non-tumour adjacent tissue.

![Figure 1](image-url)
Kruskal-Wallis analysis and Dunn’s test indicated significant differences in the expression of miR146a between each molecular subtype (Figure 2).

Spearman correlation analysis was performed to analyse the expression of miR146a-5p and predicted target genes, BRCA1, IRAK1, TRAF3IP1, and TRAF6 using TCGA RNA-seq data. No correlation with BRCA1 was found (rho = -0.02, p=0.58) However, we observed a negative correlation between miR146a-5p expression levels with TRAF6 (rho = -0.13, p=0.0002) and TRAF3IP1 (rho =-0.27, p=8.5e-14), and a positive correlation of miR146a-5p with IRAK1 expression (rho= 0.27, p=3.5x10^{-14}). For BRCA1 we tested the correlation with miR146a-5p within each molecular subtype but did not find correlation in any of them. Also, rs2910164 was not found in linkage disequilibrium with any other variant described at ENSEMBL database and 1000genomes database in any population (The 1000 Genomes Project Consortium, 2015; Zerbino et al., 2018).

Discussion

The allelic frequencies of rs2910164 vary between populations; as a result, some alleles that may confer risk in a particular population may be the same that are protective in others (Upadhyaya et al., 2016). The allelic frequencies of rs2910164 observed in the control group of the present study were similar to the Europeans from Hapmap (CEU G-76% and C-24%) (International HapMap Consortium, 2003), as well as the Europeans from NHLBI Exome Sequencing Project (NHLBI E_A G-77% and C-23%) (The 1000 Genomes Project Consortium, 2015). This result is following the previously reported predominantly European ancestry of the population from Southern Brazil (Probst et al., 2000; Sugita et al., 2016).

The association of rs2910164 and BC was studied in several studies from different populations (Xu et al., 2013; Dai et al., 2015; Uppadhyaya et al., 2015; Barjui et al., 2017; Zhang et al., 2017). Dai et al. (2015) showed that the effect of rs2910164 on BC susceptibility is different across populations. The authors suggest that the allele C is associated with BC risk in Europeans (2777 cases and 2898 controls) but not in Asians (1537 cases and 1587 controls). This discrepancy could be explained by the interaction of rs2910164 with different genetic backgrounds, by different exposure to carcinogens, or due to linkage disequilibrium with a different causal variant. However, we have not found significant LD between rs2910164 with any other polymorphism described in either ENSEMBL or the 1000genomes database. Our results, therefore, suggest that LD is not the primary factor influencing the different associations observed for rs2910164 with BC. Also evidencing the differences of the effect of rs2910164 on the risk of BC in diverse populations, the allele rs2910164G was associated with increased risk of sporadic BC in Australians (OR =1.77, p < 10^{-4}; Uppadhyaya et al., 2015) and Iranians (OR = 1.91, p = 0.03; Barjui et al., 2017). Other studies, on the other hand, reported no association (Zhang et al., 2016; Mu et al., 2017).

We observed a higher frequency of patients carrying the allele rs2910164C in comparison to controls. Although

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**Figure 2** - A. Comparison of hsa-mir-146a expression in breast cancer intrinsic subtypes; B. Dunn’s test results summarized.

|        | Luminal A | Luminal B | Basal-Like | HER2-enriched |
|--------|-----------|-----------|------------|--------------|
| Luminal B | -2.341    | -9.761    | -3.607     | -3.998        |
| p      | 0.096     | < 10^{-4} | 0.002      | < 10^{-4}    |
| Basal-Like | -9.761    | -6.814    | -1.682     | 3.998         |
| p      | < 10^{-4} | < 10^{-4} | 0.463      | < 10^{-4}    |

alpha = 0.05
the rates of the genotype CC were similar in both groups, this genotype was observed in relatively low frequency (5%). It is plausible to expect that studying larger cohorts could reveal differences between rates of the genotype CC in cases and controls. In fact, increased risk for CC genotype was observed by different studies that analysed Europeans (Armaou et al., 2009). In a study involving 40 American women with hereditary BC, the presence of the allele rs2910164C was increased in women diagnosed at younger ages, with a median age of 45 versus 56 (p=0.029; Shen et al., 2008). A study of 348 Italian women with hereditary BC showed similar results with a median age at diagnosis of 35 versus 52; (p=0.028; Pastrello et al., 2010). On the other hand, a third study reported a lack of association with younger age (Cutucu et al., 2010). Studying sporadic BC cases, we did not find any association of rs2910164 with the age of diagnosis. These findings were similar to those observed in other studies with sporadic BC, and this possible difference in the association between sporadic and hereditary BC with rs2910164 is still not understood (Cardenosa et al., 2012; Qi et al., 2015; Barjui et al., 2017; McVeigh et al., 2017).

We also did not find any association between genotypes of rs2910164 and regional lymph node invasion, immunohistochemical subgroups or tumour grade, which suggests a lack of association of rs2910164 with tumour progression. This result is similar to studies in the Irish population (McVeigh et al., 2017) and the Chinese (Qi et al., 2015).

We found that miR146a was significantly more expressed in BC than in healthy tissue. The highest expression was observed in basal-like tumours, which have partial overlap with TNBC in immunohistochemical classification. Among the BC subgroups defined by immunohistochemistry, triple-negative is recognized to inactivate BRCA1 tumour suppressor gene (Turner et al., 2007). A reduced expression level of BRCA1 is observed in a significant proportion of sporadic BC cases, despite the absence of somatic mutations in BRCA1 (Mueller and Roskelley, 2003). Interestingly, miR146a-5p was predicted to target BRCA1 and thereby reduce its expression (Shen et al., 2008; Garcia et al., 2011). Also, the expression levels of miR146a-5p were observed three times higher in triple-negative tumours compared to other subgroups of mammary tumours (M'Hamed et al., 2015). The hypothesis of regulation of BRCA1 expression by miR146a-5p was not corroborated in our analysis using RNA-Seq data. This analysis did not show a correlation between expression levels of miR146a and BRCA1 in BC in general, neither in basal-like patients.

Studies suggest that TRAF6, IRAK1 (Bhaumik et al., 2008) and EGFR, a protein associated with tumour progression and metastasis (Kumaraswamy et al., 2016), are targets for mir146a-5p. Our analysis showed a negative correlation between the expression of mir146a-5p with TRAF6 as well as with TRAF3IP1. However, we found a positive correlation of miR146a-5p with IRAK1. It has been shown that reduced TRAF6 and IRAK1 levels lessen the activity of NF-kB, a potential inducer of proliferation, survival, angiogenesis, and metastasis (Bhaumik et al., 2008). In summary, the studies on miR146a and their targets suggest that rs2910164 can exert their effects on cancer mainly by changing the binding to the target rather than by modulating the expression levels of miR146a-5p. However, further studies are needed to validate these targets and to provide direct evidence of the regulation by miR146a.

In conclusion, our study provides insights into the role of miR146a in a Brazilian population of predominantly European ancestry. We show the presence of rs2910164C increasing the susceptibility to sporadic BC among women despite its apparent lack of influence in disease progression. We observed significantly higher expression levels of miR146a-5p associated with the severity of BC, the highest expression observed being in patients with basal-like tumours. Our results point to the importance of miRNAs in the susceptibility to cancer and suggest that functional studies will further elucidate the impact of miR146a in breast cancer.

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Conflicts of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicial to the impartiality of the reported research.

Authors Contribution

HMB, RAC, DGA, DFG, JCO and EMSFR conceived the study, HMB, CM, DGA, DFG and JCO conducted and supervised the experiments; HMB, CM, DGA, RCA, IJC, EMSFR analysed the data, HMB, RAC, EMSFR wrote the draft manuscript, RSL, FK and CAU collected the samples and revised all clinical data; all authors read and approved the final version.
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