Prognostic value of microRNA-203a expression in breast cancer

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Abstract. Tumor heterogeneity and the poor outcome of breast cancer (BC) patients have led researchers to define new markers of this disease. In recent years, microRNA expression patterns have proven to be valuable disease indicators. The level of miR-203a, in particular, was shown to be altered in different types of cancer. The objective of the present study was to assess the relationship between miR-203a expression and clinicopathological features of BC in a Portuguese cohort. The expression levels of miR-203a were analyzed in 109 formalin-fixed paraffin-embedded paired normal and tumor tissue samples. Significant overexpression of miR-203a in the tumor tissues was found (1.7-fold higher) compared to the expression in the normal adjacent tissues (p=0.003). In addition, several clinicopathological characteristics presented an association with higher miR-203a expression levels. Tumors with diameter ≤18.5 mm (1.5-fold; p=0.019), tumors positive for estrogen receptor (fold-change, 1.71; p=0.042), progesterone receptor (fold-change, 1.50; p=0.046) and negative for HER2 (fold-change, 1.50; p=0.016) and high Ki-67 index (fold-change, 2.60; p=0.024) presented a significant difference in miR-203a expression compared with adjacent normal tissues. Tumors without invasion of lymph nodes also presented higher expression of miR-203a (fold-change, 2.40; p=0.004). With regard to histological classification, ductal carcinomas *in situ* (fold-change, 2.20; p=0.028) and invasive carcinoma NOS (fold-change, 1.71; p=0.009) displayed significantly higher expression of miR-203a. Moreover, we found a significant downregulation of miR-203a with increased stage in invasive lobular carcinomas, suggesting that miR-203a could represent a potential marker to discriminate stages in invasive lobular carcinomas.

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Introduction

Breast cancer (BC) remains a worldwide burden with an estimated incidence of more than 1.5 million new cases and approximately half a million deaths per year (1). Due to early detection, improvement in treatment options and changes in lifestyle paradigms, the mortality rates have been decreasing in developed countries. Conversely, developing countries are witnessing an increase in BC incidence and mortality rates, most probably due to the lack of awareness campaigns and changes in daily habits such as sedentary lifestyle, high consumption of sugars and fat that lead to overweight and obesity, known risk factors of BC (1). Moreover the proportion of cases diagnosed in less developed countries is meagre when compared to developed regions thus leading to higher mortality rates (2).

Although the molecular mechanisms that underlie the development of breast cancer have been well investigated, our current knowledge is far from complete. BC is a heterogeneous malignancy. Clinical diagnosis and prescribed therapy relie on the TNM staging system based on tumor (T), node status (N) and metastasis (M). Estrogen and progesterone receptor status, human epidermal growth factor receptor 2 (HER2/neu) status and the Ki-67 proliferative index, in addition to tumor-infiltrating lymphocytes, as well as the age of the patient, are used to classify BC into various subtypes (2-4). Nonetheless, these conventional breast cancer prognostic factors have intrinsic limitations, and their use does not allow an accurate prediction of treatment resistance or relapse. Defining new molecular prognostic factors to refine BC classification could be useful in improving the therapeutic schemes.

Recently, various microRNAs (miRNAs) have been characterized and identified as regulators and/or biomarkers in breast cancer development, including initiation, metastasis and therapeutic resistance (5-11). miRNAs are small 18-22 nucleotide RNA molecules that regulate protein expression by mostly binding to the 3'-UTR (3'-untranslated region) of mRNAs, thus inhibiting translation through repression or degradation of mRNAs (12,13). Due to their size, miRNAs are stable in

human samples and can easily be used as molecular signatures in cancer (14-20).

miR-203 was originally described as a keratinocyte-specific miRNA (21) but was soon shown to play an important role in bladder cancer (22) and to be epigenetically silenced in hematopoietic cancers (23). Several studies have shown an association between miR-203 and chemotherapeutic resistance to cisplatin (24), invasiveness (25,26), proliferation (26-29) and metastases (30,31), and as a biomarker (32,33). Some miR-203 targets have been identified, such as *BMI1*, *SNAI2*, *SOCS3*, *BIRC5* and *LASP1* (24-26,28,34), but a complete picture of the expression of miR-203 in different cohorts of BC, its mechanisms of action and the circuitry of its effects remain to be fully clarified.

With the aim of contributing to a better understanding of the role of miR-203a in breast cancer, this study reports on the assessment of miR-203a expression and clinicopathological features in a Portuguese population with breast carcinoma.

Materials and methods

Sample collection and processing. Patients with breast carcinoma were recruited for the study at the Hospital de São José, from Centro Hospitalar de Lisboa Central, during 2013 and 2014. Each patient signed a written informed consent form and this study was reviewed and approved by the Ethics Committees of the NOVA Medical School and of the Centro Hospitalar de Lisboa Central. All clinical information was gathered by trained and specialized clinicians. All samples originated from surgical sections (mastectomy or tumorectomy). A total of 109 formalin-fixed paraffin-embedded (FFPE) paired normal and tumor tissue samples were collected. Normal tissue was adjacent to the tumor and in all cases was confirmed by the pathology team as being only normal mammary tissue. Diagnosis and common immunohistochemical markers for breast cancer classification such as estrogen and progesterone receptor (ER and PR), HER2 amplification status and Ki-67 proliferative index were evaluated by two highly trained and independent pathologists. Staging was performed by tumor (T), node (N) and metastasis (M) classification (35). The procedure for immunohistochemical detection was carried out according to the recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines (36,37) and the International Ki-67 in Breast Cancer Working Group (38) at the time of sample collection. With the existing canons at the time, the molecular classification of breast tumors was as follows: luminal A: ER-positive or PR-positive and Ki-67 <13%; luminal B: ER-positive or PR-positive and Ki-67 ≥13%; HER2-positive: ER-negative and PR-negative, HER2-positive; triple-negative: ER-negative, PR-negative and HER2-negative.

Total RNA was purified from FFPE tissues using RecoverAll™ Total Nucleic Acid isolation kit (Ambion®) and according to the manufacturer's protocol with slight alterations. Briefly, the samples were deparaffinized with xylol and digested with protease/digestion buffer. Microtube pestles were used to macerate hard samples during digestion. Then, nucleic acid was precipitated with a mixture of absolute ethanol/isolation additive and the supernatant used in the filter cartridges to proceed with DNA digestion using a DNase/DNase buffer mixture. Total RNA containing miRNAs was eluted and preserved at -80˚C for further use.

miR-203a quantification by quantitative RT-PCR (RT-qPCR). Total RNA concentration was quantified using a NanoDrop™ spectrophotometer. miR-203a expression levels were quantified by RT-qPCR using cDNA Synthesis and SYBR®-Green Master Mix kits (Exiqon, Denmark) on an ABI 7300 real-time PCR machine, according to the manufacturer's instructions. For each cDNA synthesis experience a no-enzyme control was used to ensure that there was no contamination during cDNA synthesis, and for each sample a no-template control was used to detect any potential amplification of genomic DNA. U6 snRNA was used as internal control for data normalization. Relative expression of miR-203a in each FFPE sample was determined by $2^{-\Delta Ct}$, where ΔCt is Ct (miR-203a) - median Ct (U6 snRNA). Fold-change was determined by $2^{-\Delta\Delta Ct}$, where ΔΔCt is ΔCt (tumor) - ΔCt (normal). All samples were analyzed in duplicate.

Statistical analysis. All statistical analyses were performed using the SPSS statistical software package version 21.0 (SPSS Inc., Chicago, IL, USA). Non-parametric Wilcoxon signed-rank test was used to analyze the differences between matched samples (normal vs. tumor tissues). The Mann-Whitney U test and Kruskal-Wallis test were used to analyze the differences of miR-203a expression levels in the tumor tissue according to the clinicopathological characteristics. For nominal variables, the relationships between clinicopathological characteristics and miR-203a status were studied using the Chi-square test and Fisher's exact test.

Results

Study population description. Breast tumor and adjacent normal mammary tissues were collected from 109 patients. The study population comprised only Caucasian woman from the greater area of Lisbon and on the day of diagnosis the median age was 62 years (range, 30-85). The median age of menarche and menopause was 13 years (range, 8-17) and 50 years (range, 36-59), respectively, and ~66% of the population was diagnosed with breast carcinoma in post menopause status. Seventy-seven percent of the women had one or more pregnancies and ~73% had one or more children. Forty-five percent claimed to have taken birth control pills. Approximately 50% were overweight or obese. Regarding general tumor characteristics, the median tumor size was 18.5 mm (range, 6-130), ~51% showed no invasion of the lymph nodes, ~80% were ER-positive, 72.5% were PR-positive, and 13.8% showed high amplification of HER2 and 48% showed negative Ki-67 proliferation index. The most common histological type was invasive carcinoma NOS (83.5% of the cases), followed by invasive lobular carcinoma (9.2%), ductal carcinoma *in situ* (6.4%) and invasive lobular and ductal carcinoma (0.9%). The most common molecular subtype was luminal A (47.4% of all cases). Luminal B represented ~40% of the remaining cases. Triple-negative subtype represented ~12% of the cases. The most frequent stage was II (46.8%) followed by I (37.6%) and III (8.3%). All these data, together with the stratification of several variables, is displayed in Tables I-III.

Table I. Association of miR-203a relative expression with clinical characteristics of the breast cancer cases.

		Median relative expression of miR-203			
	n (%)	Normal tissue	Tumor tissue	Tumor tissue/normal tissue	P-value[a]
No. of cases	109 (100)	**0.07**	**0.12**	**1.7**	**0.002**[b]
Age at diagnosis (years), n (%)					
30-39	3 (2.8)	0.04	0.07	1.75	0.593
40-49	17 (15.6)	0.14	0.13	0.93	0.906
50-59	27 (24.8)	0.14	0.15	1.07	0.657
>60	54 (49.5)	**0.04**	**0.11**	**2.75**	**0.001**
Missing	8 (7.3)				
Age at menarche (years), n (%)					
≤13	65 (59.6)	0.06	0.12	2.00	0.066
>13	35 (32.1)	**0.06**	**0.14**	**2.33**	**0.003**
Missing	9 (8.3)				
Age at menopause (years), n (%)					
≤50	44 (52.4)	**0.04**	**0.14**	**3.50**	**<0.001**
>50	29 (34.5)	0.05	0.10	2.00	0.539
Missing	11 (13.1)				
Menopausal status, n (%)					
Pre	25 (22.9)	0.14	0.12	0.86	0.607
Post	72 (66.1)	**0.05**	**0.11**	**2.20**	**0.003**
Peri	1 (0.9)				
Missing	11 (10.1)				
No. of pregnancies, n (%)					
0	15 (13.8)	0.07	0.15	2.14	0.972
1-2	42 (38.5)	**0.06**	**0.11**	**1.83**	**0.046**
3-4	27 (24.8)	0.08	0.11	1.38	0.416
>4	15 (13.8)	**0.04**	**0.16**	**4.00**	**0.001**
Missing	10 (9.2)				
No. of children, n (%)					
0	21 (19.3)	0.04	0.12	3.00	0.295
1-2	64 (58.7)	**0.08**	**0.11**	**1.38**	**0.012**
3-4	12 (11.0)	0.08	0.12	1.50	0.657
>4	4 (3.7)	0.03	0.21	7.00	0.068
Missing	8 (7.3)				
Age at first birth (years), n (%)					
<20	13 (11.9)	**0.05**	**0.17**	**3.40**	**0.033**
20-30	45 (41.3)	0.09	0.12	1.33	0.074
>30	12 (11.0)	0.04	0.09	2.25	0.182
Missing	39 (35.8)				
Breastfeeding, n (%)					
No	32 (29.4)	0.04	0.11	2.75	0.170
Yes	67 (61.5)	**0.08**	**0.13**	**1.62**	**0.002**
Missing	10 (9.2)				
Oral contraceptive, n (%)					
No	48 (44.0)	0.04	0.11	2.75	0.130
Yes	49 (45.0)	**0.08**	**0.12**	**1.50**	**0.004**
Missing	12 (11.0)				

[a]$p<0.05$ was considered significant according to the non-parametric Wilcoxon signed-rank test, unless otherwise specified; [b]$p<0.05$ was considered significant according to the non-parametric Mann-Whitney test. Significant results are indicated in bold.

Table II. Association of miR-203a relative expression with the lifestyle habits of the breast cancer cases.

		Median relative expression of miR-203			
	n (%)	Normal tissue	Tumor tissue	Tumor tissue/normal tissue	P-value[a]
Body mass index, n (%)					
Underweight	2 (1.8)	0.22	0.31	1.41	0.655
Normal	42 (38.5)	0.08	0.13	1.63	0.252
Overweight	30 (27.5)	**0.04**	**0.11**	**2.75**	**0.006**
Obese	23 (21.1)	0.06	0.11	1.83	0.073
Missing	12 (11.1)				
Smoking habit, n (%)					
No	73 (67.0)	**0.05**	**0.13**	**2.60**	**0.001**
Yes	22 (20.2)	0.09	0.11	1.22	0.465
Missing	14 (12.8)				
Alcohol habits, n (%)					
No	54 (49.5)	0.07	0.13	1.86	0.059
Sporadically	24 (22.0)	**0.04**	**0.12**	**3.00**	**0.037**
Daily	17 (15.6)	0.14	0.12	0.86	0.607
Missing	14 (12.8)				

[a]p<0.05 was considered significant according to non-parametric Wilcoxon signed-rank test. Significant results are indicated in bold.

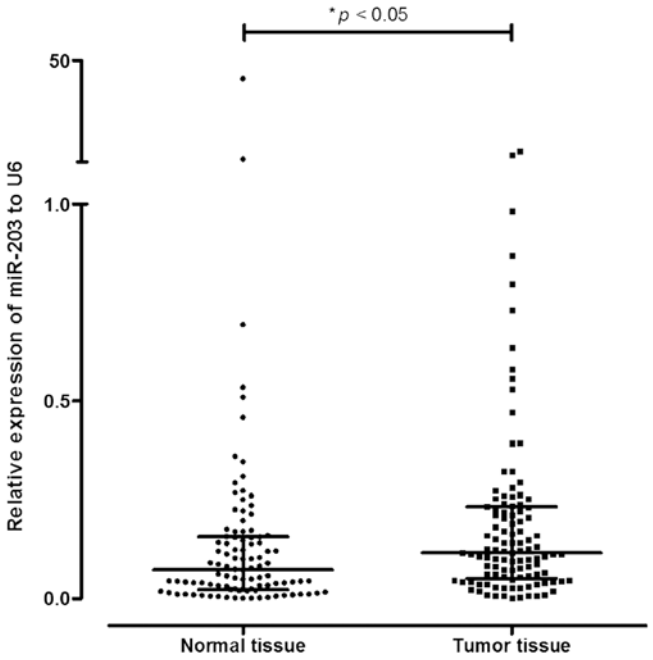

Figure 1. Differences in miR-203a relative expression in tumor and adjacent normal tissues. The values of miR-203a expression levels are shown in arbitrary units as determined by $2^{-\Delta Ct}$ method [ΔCt = Ct (miR-203a) - median Ct (U6 snRNA)]. Lines represent median with interquartile range. p<0.05 was considered significant according to the non-parametric Mann-Whitney test.

miR-203a is overexpressed in tumor tissues compared to normal tissues. There was a significant overexpression of miR-203a in the tumor tissues (1.7-fold higher) compared to the normal adjacent tissues from the 109 patients (p=0.003; Wilcoxon signed-rank test to matched samples) (Table I and Fig. 1).

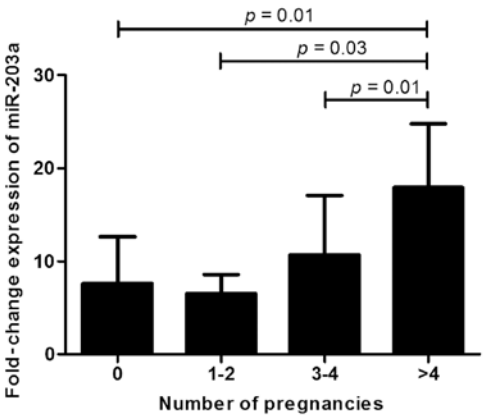

Figure 2. Differences in the fold-change in miR-203a expression between the numbers of pregnancies. The values of miR-203a expression levels are shown in arbitrary units as determined by $2^{-\Delta\Delta Ct}$ method [ΔΔCt = ΔΔCt (tumor) - ΔCt (adjacent normal)]. Bars represents mean and error bars, SEM. p<0.05 was considered significant according to the non-parametric Kruskal-Wallis test.

Association between miR-203a expression and reproductive characteristics. The evaluation of clinical variables (Table I) revealed a significantly different distribution in the fold-change of expression of miR-203a when considering the number of pregnancies (Kruskal-Wallis p=0.006; Fig. 2). Specifically, there was a higher fold-change of expression in woman with four or more pregnancies compared to the other classes (no pregnancies vs. >4, p=0.01; 1-2 vs. >4, p=0.03; 3-4 vs. >4, p=0.01; Fig. 2). Significant differences in age were also found (Fig. 3).

Using Wilcoxon signed-rank test to matched samples with the same variables, patients >60 years of age on the day of diagnosis (fold-change, 2.75; p=0.001), with menarche

Table III. Association of miR-203a relative expression with the pathological characteristics of the breast cancer patients.

		Median relative expression of miR-203			
	n (%)	Normal tissue	Tumor tissue	Tumor tissue/normal tissue	P-value[a]
Size of the tumor (mm), n (%)					
≤18.5	54 (49.5)	**0.08**	**0.12**	**1.50**	**0.019**
>18.5	54 (49.5)	0.06	0.12	2.00	0.076
Missing	1 (1.0)				
Lymph node invasion, n (%)					
No	52 (51.4)	**0.05**	**0.12**	**2.40**	**0.013**
Yes	53 (47.7)	0.09	0.11	1.22	0.137
Missing	4 (0.9)				
Estrogen receptor status, n (%)					
Negative	16 (14.7)	0.07	0.15	2.14	0.074
Positive	87 (79.8)	**0.07**	**0.12**	**1.71**	**0.042**
Missing	6 (5.5)				
Progesterone receptor status, n (%)					
Negative	22 (20.2)	0.04	0.12	3.00	0.091
Positive	79 (72.5)	**0.08**	**0.12**	**1.50**	**0.046**
Missing	8 (7.3)				
HER2 status, n (%)					
Negative	87 (79.8)	**0.08**	**0.12**	**1.50**	**0.016**
Positive	15 (13.8)	0.72	0.73	1.01	0.609
Missing	7 (6.4)				
Ki-67 index status, n (%)					
Negative	53 (48.6)	**0.05**	**0.13**	**2.60**	**0.024**
Positive	48 (44.0)	0.08	0.10	1.25	0.253
Missing	8 (7.3)				
Histological type, n (%)					
Ductal carcinoma *in situ*	7 (6.4)	**0.05**	**0.11**	**2.20**	**0.028**
Invasive carcinoma NOS	91 (83.5)	**0.07**	**0.12**	**1.71**	**0.009**
Invasive lobular carcinoma	10 (9.2)	0.13	0.11	0.84	0.575
Invasive lobular and ductal carcinoma	1 (0.9)				
Molecular type, n (%)					
Luminal A	48 (47.5)	0.07	0.13	1.86	0.054
Luminal B ($HER2^-$)	27 (26.7)	0.08	0.11	1.38	0.527
Luminal B ($HER2^+$)	13 (12.9)	0.07	0.08	1.14	0.221
Triple-negative	12 (11.9)	0.06	0.15	2.50	0.139
$HER2^+$	1 (1.0)				
Stage, n (%)					
0	7 (6.4)	**0.05**	**0.11**	**2.20**	**0.028**
I	41 (37.6)	0.08	0.12	1.50	0.126
II	51 (46.8)	**0.06**	**0.13**	**2.17**	**0.009**
III	9 (8.3)	0.10	0.11	1.10	0.678
Missing	1 (0.9)				

[a]$p<0.05$ was considered significant according to non-parametric Wilcoxon signed-rank test. Significant results are indicated in bold.

age >13 years (fold-change, 2.33; p=0.003) and menopause <50 years (fold-change, 3.50; p<0.001), showed a significant overexpression of miR-203a when comparing tumor tissues with normal adjacent tissues. Patients diagnosed in

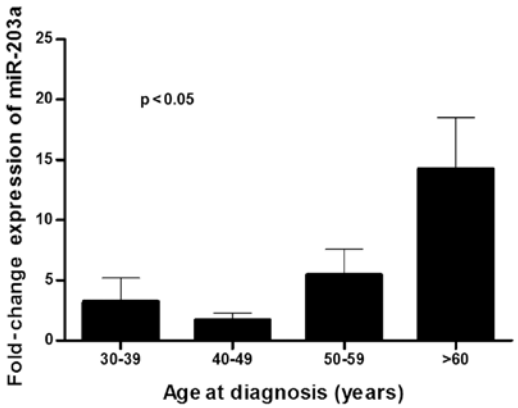

Figure 3. Differences in the fold-change in miR-203a expression between categories of age at diagnosis. The values of miR-203a expression levels are shown in arbitrary units as determined by $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta Ct = \Delta Ct$ (tumor) - ΔCt (adjacent normal)]. Bars represents mean and error bars, SEM. $p<0.05$ was considered significant according to the non-parametric Kruskal-Wallis test.

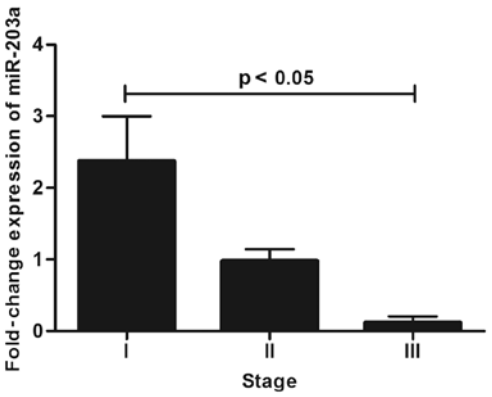

Figure 4. Differences in the fold-change in miR-203a expression between tumor stages in invasive lobular carcinoma. The values of miR-203a expression levels are shown in arbitrary units as determined by $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta Ct = \Delta Ct$ (tumor) - ΔCt (adjacent normal)]. Bars represents mean and error bars, SEM. $p<0.05$ was considered significant according to the non-parametric Kruskal-Wallis test.

post-menopause status (fold-change, 2.20; p=0.003) and who had <40 years of fertile status (<30 years: fold-change, 3.75; p=0.041; 30-40 years: fold-change, 2.40; p=0.005) also presented increased expression of miR-203a in tumor tissues compared to adjacent normal tissues. Regarding the number of pregnancies, patients with >4 pregnancies showed a significantly increased expression of miR-203a in tumor tissues (fold-change, 4.00; p=0.001). These results are in accordance with the ones described above where Kruskal-Wallis test was applied. In accordance, although not significantly, women with >4 children showed an increased expression of miR-203a. Patients with first childbirth before 20 years of age also showed an increased expression of miR-203a (fold-change, 3.40; p=0.033). Breastfeeding status and oral contraceptive consumption also showed statistically significant results (Table I).

Association between miR-203a expression and lifestyle characteristics. It is known that various lifestyle habits can be a risk factor for cancer. In our series we included body mass index and smoking and alcohol habits. Overweight patients showed an increase in miR-203a expression in tumor tissues (fold-change, 2.75; p=0.006) and those who did not smoke (fold-change, 2.60; p=0.001) or sporadically drank alcoholic beverages (fold-change, 3.00; p=0.037) also showed an increased expression of miR-203a (Table II).

Association between miR-203a expression and clinicopathological characteristics. Several clinicopathological characteristics showed an association with miR-203a expression (Table III). Tumors with diameter ≤18.5 mm, showed significant difference, albeit with a slight fold-change of 1.5 compared with adjacent normal tissue (p=0.019), together with tumors positive for ER (fold-change, 1.71; p=0.042), PR (fold-change, 1.50; p=0.046), negative for HER2 (fold-change, 1.50; p=0.016) and Ki-67 index (fold-change, 2.60; p=0.024). Tumors that did not invade the lymph nodes also presented higher expression of miR-203a (fold-change, 2.40; p=0.013). With regard to histological classification, ductal carcinomas *in situ* (fold-change, 2.20; p=0.028) and invasive carcinoma NOS (fold-change, 1.71; p=0.009) showed a significantly higher expression of miR-203a. Stage 0 and II also showed significantly increased expression (fold-change, 2.20; p=0.028; fold-change, 2.17; p=0.009, respectively). When considering only invasive lobular tumors significant differences were found in staging, mainly when comparing stage III with stage I (Fig. 4).

Discussion

Several studies have established that specific miRNA expression patterns can be correlated with biological and clinical features. Studies of miRNA expression patterns in different populations are of utmost importance in order to unveil the significance of these molecules in the diagnosis and prognosis of breast cancer. In the present study, we showed that miR-203a was overexpressed in tumor tissues when compared to adjacent normal tissues in a Portuguese cohort. To our knowledge this is the first study to report miR-203a expression in a Portuguese breast cancer population. Our results are in accordance with another study by Ru *et al* (24). However, they did not compare adjacent normal tissues with tumor tissues but an independent disease-free population. The same pattern of overexpression was also observed in ovarian cancer (39), cervical cancer (40), kidney and bladder cancers (22), colon adenocarcinoma (41) and head and neck squamous cell carcinoma (42). Conversely, miR-203 expression levels were decreased in hepatocellular carcinoma (43). Altogether these data support the notion that miR-203a plays an important role in the development of cancer in a tissue-specific manner.

In the present study, we compared miR-203a expression levels in ductal carcinoma *in situ* (n=7), invasive carcinoma NOS (n=91) and invasive lobular carcinoma (n=10). Our sample population also comprised one mixed tumor (invasive ductal and lobular carcinoma) but this was not considered in the statistical analysis of the histological subtypes. Comparing matched samples, we observed significant differences between miR-203a levels in the tumor and adjacent normal tissues in

the ductal carcinoma *in situ* and invasive carcinoma NOS. However, there were no significant differences between the two groups. Due to the fact that two different types of breast tumors were presented, ductal and lobular, we analyzed ductal carcinoma *in situ* and invasive carcinoma NOS separately. However, we did not find significant differences either. Nevertheless, we highlight the fact that there was a decrease in the miR-203a expression level in invasive carcinoma NOS when compared with ductal carcinoma *in situ*. These results suggest that during tumor development, miR-203a may be downregulated, thus suggesting that miR-203a may be implicated in early stages of tumor development. Indeed, this involvement of miR-203a in invasiveness through inhibition of the Polycomb group gene *BMI1* has alreadybeen reported in melanoma (34) and non-small cell lung cancer (26), in which miR-203a expression levels are inversely correlated with *BMI1* expression levels according to the cell type. Zhang *et al* (25) also reported an increased expression of miR-203a in breast tumors compared to matched adjacent normal tissue, even though their cohort was smaller. Additionally, the authors determined miR-203a expression levels in several non-tumorigenic, non-metastatic and metastatic breast cell lines and showed an increased expression of miR-203a in non-metastatic compared to non-tumorigenic and metastatic lines. These results led the authors to speculate that miR-203a is overexpressed in a protective mechanism to deal with cell proliferation and invasiveness, and thereafter, most probably through epigenetic mechanisms, the tumor cells repress miR-203a expression to enable proliferation, invasion and metastasis through increased expression of the pro-metastatic gene *SNAI2*. In fact, our data are in accordance with this report, since when we stratified the tumors according to lymph node invasion, the tumors that metastasized had a decreased expression of miR-203a (fold-change expression, 1.22; n=53; Table III) compared to those that did not metastasize to the lymph node (fold-change expression, 2.40; n=52; Table III), although the result was not statistically different. Additionally, we also found that miR-203a had decreased expression in tumors positive for HER2 and a high level for the proliferation index Ki-67 (Table III). Altogether these data are in accordance with the fact that miR-203a may act as a tumor suppressor, and in early stages of cancer development miR-203a may play a protective role. Yet, throughout tumor development, miR-203a may be repressed in order to enable tumor cells to proliferate, invade and metastasize.

Notably, miR-203a expression decreased from stage 0 to stage I, then increased in stage II, and again decreased in stage III. This upregulation and downregulation across stages was unexpected, since as a putative tumor suppressor, miR-203a expression should decrease with increasing stages. Petrovic *et al* (44) observed a similar pattern in invasive breast carcinomas but with miR-21. miRNA levels are dependent on cell differentiation, thus displaying differently expression levels according to stage. Analyzing only invasive lobular carcinoma tumors, there were significant differences between stage I (n=2), II (n=6) and III (n=2). Although the number of samples was small, we observed a pronounced decrease in miR-203a expression within the stages (Fig. 4). Thus, miR-203a expression levels in invasive lobular carcinoma can be used as a marker to distinguish different stages.

Breast cancer risk increases with age. However, individual risk depends on other factors, including reproductive history, lifestyle habits and family history, among others. Our data showed significant differences with age stratification. Indeed, women over 60 years at diagnosis presented an increased expression of miR-203a when tumor tissue was compared with adjacent normal tissue. As estimated by The Surveillance, Epidemiology, and End Results (SEER) program of the National Cancer Institute (45), there is a risk increment for developing breast cancer with age. Our stratification was carried out using the same criteria, and we observed a higher expression of miR-203a in patients above 60 years of age. Regarding age at menarche and age at menopause, known players for breast cancer, the expression levels were higher for matched samples for age at menarche >13 years and for age at menopause <50 years. Although we observed significant differences in matched samples for the classes referred, there were no differences for age at menarche and menopause classes indicating that miR-203a expression levels were not influenced by these factors.

Furthermore, it is known that female hormones, such as 17-β-estradiol (E_2), regulate gene expression by binding to estrogen receptors (46). Indeed, Yu *et al* (27), showed that E_2 can regulate miRNA expression and thus control cell proliferation. The authors showed that miR-16, miR-143 and miR-203a expression was suppressed after E_2 stimulation hence upregulating bcl-2, cyclin D1 and survivin. Thus, the authors proposed a mechanism whereby cells that undergo stimulation by E_2 have increased proliferation by inhibiting tumor suppressor miRNAs involved in cell proliferation and survival. Additionally, the authors ascertained the expression levels of these miRNAs in triple-positive and triple-negative breast tumors and showed that they had increased levels of expression in triple-positive tumors, indicating that these miRNAs may function as tumor suppressors in triple-positive breast tumors. In contrast, our data showed that triple-positive samples had lower expression of miR-203a than triple-negative tumors (data not shown). Indeed, when we stratified our data according to hormone receptor status, individually, we obtained always an increased expression level of miR-203a in tumors with negative receptor status. When sample matching was analyzed we found significant differences between tumor tissues and adjacent normal tissues with positive status. To confirm these data, when we analyzed the samples by stratifying them by molecular subtype, we observed that basal-like tumors had higher expression of miR-203a. Although the terms basal-like tumor and triple-negative tumors are not used interchangeably (3), in this case we can consider that all basal-like were triple-negative tumors. Interestingly, women who had used oral contraceptives had lower expression of miR-203a in these tissues. Thus, miR-203a expression might be influenced by estrogen and progesterone (27).

In summary, miR-203a appears to be involved in breast cancer development, mainly in the early stages of development. Early-stage tumor cells might upregulate miR-203a in a self-protective manner in order to manage the augmented cell proliferation and then, most probably, through epigenetic mechanisms or E_2 mediated suppression, miR-203a might be downregulated and its targets upregulated. Accordingly, miR-203a could represent a potential marker for invasiveness.

In the present study, we also showed that miR-203a may be a potential marker to discriminate stages in invasive lobular carcinoma. Further studies with larger populations of invasive lobular carcinoma cases must be performed in order to validate these results.

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