Med Oncol (2014) 31:255
DOI 10.1007/s12032-014-0255-6

ORIGINAL PAPER

Wild-type genotypes of *BRCA1* gene SNPs combined with micro-RNA over-expression in mammary tissue leading to familial breast cancer with an increased risk of distant metastases' occurrence

Imen Medimegh · Wafa Troudi · Nejla Stambouli · Houssein Khodjet-El-Khil · Olfa Baroudi · Hajer Ayari · Ines Omrane · Nancy Uhrhammer · Maud Privat · Amel Mezlini · Farhat Ben Ayed · Khaled Ben Romdhane · Sylvie Mader · Yve Jean Bignon · Amel Benammar Elgaaied

Received: 30 May 2014 / Accepted: 18 September 2014 / Published online: 2 October 2014

Abstract Germ line deleterious mutations of *BRCA1* gene are not the unique factor that could inactivate *BRCA1* protein which leads to familial breast cancer onset with distant metastases' occurrence. The present research explores the role that could be assigned to *BRCA1* SNPs to inactivate *BRCA1* protein and therefore to the occurrence of familial breast cancer with an increased risk of distant metastases' occurrence. The presence or the absence of *BRCA1* protein was first analyzed by applying the immunohistochemistry technique to the tumors with sporadic and familial breast cancer. Then, a case–control study was conducted including 40 patients with familial breast cancer, 46 ones with sporadic breast cancer and 34 healthy controls based on the genotyping of nine *BRCA1* SNPs (c.442.58delT, c.2082C>T, c.2311T>C, c.2612C>T, c.3113A>G, c.3119G>A, c.3548A>G, c.4308T>C and 4837A>G) via direct sequencing. Finally, the functional role that could be assigned to these SNPs was focused upon. miRbase site was used as a bioinformatics tool to predict potential micro-RNAs (miRs) targeting SNPs that are associated with familial breast cancer according to the results of this research. These predicted miRs were confirmed by Q-PCR analysis and correlated with *BRCA1* protein expression among patients along with potential distant metastases. Clinical outcome showed that distant metastasis concerned 45 % of familial breast cancer patients and 19.5 % with sporadic breast cancer. Analysis of *BRCA1* protein expression revealed a negative staining among 46.6 % of familial breast cancer patients and only 16.6 % within sporadic breast cancer ones. The association of four variants was identified within *BRCA1* gene (c.442.58 delT, c.2311T>C, c.2612C>T and c.4308T>C) to familial breast cancer across their wild genotypes. miR-1179 was selected as potential miR that targets the region of *BRCA1* mRNA containing the c.2311T>C variant within the TT genotype. The expression of miR-1179 was significantly associated with familial breast cancer patients without *BRCA1* deleterious mutations compared to those with sporadic breast cancer according to TT genotype along with *BRCA1* negative staining and according to the occurrence of distant metastases. Combination between TT genotype of c.2311T>C and miR-1179 over-expression could generate a lack of *BRCA1* protein leading to a high risk of familial breast cancer with distant metastases.

Keywords Familial breast cancer · Micro-RNA · SNP · Metastasis · Tunisia

Wafa Troudi and Nejla Stambouli have equally contributed this work.

I. Medimegh (✉) · W. Troudi · N. Stambouli · H. Khodjet-El-Khil · O. Baroudi · H. Ayari · I. Omrane · A. B. Elgaaied
Laboratory of Genetics, Immunology and Human Pathology, Department of Biology, Faculty of Sciences of Tunis, University of Tunis El Manar, Campus Universitaire, 2092 Tunis, Tunisia
e-mail: imen.medimegh@gmail.com

N. Uhrhammer · M. Privat · Y. J. Bignon
Laboratory of Genetics and Molecular Diagnostic, Centre Jean Perrin, Clermont-Ferrand, France

A. Mezlini · F. B. Ayed · K. B. Romdhane
Salah Azaiez Institute of Carcinology of Tunis, Tunis, Tunisia

S. Mader
Laboratory of Molecular Screening in the Treatment of Breast Cancer in Immunology and Cancer Research Institute, Montreal University, Montreal, Canada

Abbreviations

FBC	Familial breast cancer
FFPE	Formalin-fixed, paraffin-embedded
HC	Healthy controls
IHC	Immunohistochemistry
miR	micro-RNA
Q-PCR	Quantitative real-time polymerase chain reaction
SBC	Sporadic breast cancer
SNP	Simple nucleotide polymorphism

Introduction

Breast cancer is the most common type of cancer affecting women worldwide, accounting for 23 % of the total new cancer cases. It is also the second leading cause of cancer death among women after the lung cancer [1]. Breast carcinoma is the most common type of cancer attacking women in Tunisia, with an age standardized rate of 16.7 per 100,000 per year. Average age at diagnosis is 50 [2]. Breast cancer is a sporadic disease in almost 90–95 % of the cases, while the familial form is observed in only 5–10 %. In this latter form, *BRCA1* is the most commonly involved gene, along with *BRCA2* gene [3]. Dysfunction or alteration within these two major genes predisposes to breast cancer with a poor prognosis, and it is often associated with high risk of distant metastases' occurrence and even with lymph node metastases onset [4, 5]. *BRCA1* gene is located on chromosome 17q21, containing 22 coding exons and two noncoding ones with a transcript of 7.8 kb which encodes a protein of 1,863 amino acids. *BRCA1* protein is described as a tumor suppressor molecule which maintains genomic stability through its involvement in homologous recombination and in DNA repairs [6]. *BRCA1* gene mutational spectrum is not entirely depicted. More than one thousand mutations are incorporated in breast cancer information core BIC database, which are classified as deleterious mutations, SNP and UV (unknown value) [7]. Half of the deleterious mutations are frame shift, nonsense or splice-site alterations that lead to premature truncation of the protein upon translation, and approximately 60 % of these deleterious mutations are unique to a family [8]. However, germ line deleterious mutations in *BRCA1* are not always revealed in inherited breast cancer. Previous studies performed by direct sequencing of *BRCA1* gene among Tunisian patients with familial breast cancer showed deleterious mutations in <20 % of the cases [9]. The same study showed that such mutations in *BRCA1* gene are observed in 50 % of the families with breast and ovarian cancer and in only 4 % of the families with breast cancer without any history of ovarian cancer [10]. Since this last situation corresponds to the majority (70 %) of familial breast cancer cases in Tunisian population, the present research is only concerned with the involvement of *BRCA1* gene across families with breast cancer only. Indeed, it could be hypothesized that mutations in other suppressor genes could be associated with this disease. However, other mechanisms involving *BRCA1* gene could be suggested. More recently, several studies have showed that the lack of gene function may be due to epigenetic factors such as micro-RNAs [11]. Many micro-RNAs were described to be involved in cancer and were called "onco-miR" that down-regulate tumor suppressor genes. Others were described to act as "tumor suppressor miR" that down-regulate oncogenes [12]. Micro-RNAs, miRs or miRNAs are small noncoding RNAs ranging from 20 to 24 nucleotides in length that are highly conserved, encoded by genomes of plants and animals [13]. They are posttranscriptional regulators of gene expression by targeting mRNAs, and they also play an essential role in cell signaling pathways. Micro-RNAs precursors are transcribed either from nonprotein coding areas of DNA (RNA genes), or from introns of protein-coding genes as rather long (1,000 nucleotides) primary transcripts (pri-miRNA), which undergo a multistep maturation process to become biologically active miRs [13]. Some micro-RNAs were described in literature as being the target of *BRCA1* gene such as miR182 and miR17 correlated with an increased risk of familial breast cancer [14]. This correlation needs to be more investigated, along with the absence of deleterious mutations in *BRCA1* gene across families with breast cancer.

This research explores the hypothesis that the combination between genotypes of *BRCA1* SNPs and onco-miR expression levels could generate a high risk of familial breast cancer along with the occurrence of distant metastases. In a previous study, we described at least 18 neutral SNPs in *BRCA1* gene leading to different haplotypes based on 14 SNPs. Nineteen haplotypes were observed in Tunisian population among which only three were in common with American population [10].

In the present research, we conducted a case–control study including patients and healthy controls based on nine SNPs within the *BRCA1* gene among the 14 ones already studied in our previous work, which are as follows: c.442.58delT, c.2082C>T, c.2311T>C, c.2612C>T, c.3113A>G, c.3119G>A, c.3548A>G c.4308T>C and 4837A>G in order to investigate in depth the involvement of *BRCA1* SNPs in familial breast cancer. Moreover, to explain some aspects of this association, we investigated interactions between SNPs associated with familial breast cancer and their targeting of micro-RNAs.

Patients and methods

Patients

A cohort of total 34 subjects were recruited as healthy controls, from Charles Nicolle hospital in Tunis (capital of Tunisia), and the average age was 48.08 ranging from 24 to 72. We ensured that these individuals were unpaired and have no cancer history in their families. Patients with breast cancer were selected from chemotherapy service at Salah Azaiz Institute of Tunis. They were diagnosed between 2002 and 2012. Forty subjects were selected among those who have familial breast cancer based on personal and familial cancer history, and 46 ones were selected among those who have sporadic breast cancer with no cancer history in their families. Familial breast cancer is defined as the index case of family with at least three female relatives affected with breast and/or ovarian cancer. In the cases of families with fewer than three affected relatives, we took into account the presence of one relative diagnosed with breast or ovarian cancer before the age of 45, the presence of one member of the family diagnosed with ovarian cancer and at least one family member on the same side diagnosed with breast cancer (at any age) and the presence of one relative diagnosed with multiple primary breast cancers. Sporadic and familial breast cancer patients' average age was, respectively, 48.24 raging from 22 to 74 and 41.01 raging from 18 to 64 years. Essential information including stage, grade, lymph nodes metastasis and distant metastasis was collected from the Salah Azaiz Institute data base. The peripheral blood was collected into tubes and centrifuged at 3,000 rpm for 15 min; the puffy coat was isolated and stored at −80 °C until use. Return to the archive of anatomopathology department of Salah Azaiz Institute in order to retrieve formalin-fixed, paraffin-embedded (FFPE) tissues of the same patients was a very heavy operation. Finally, we managed to retrieve thirty FFPE tissues for each group of patients with their corresponding normal tissues. Three 10-mm cores were obtained from both the tumor and the normal tissues. A great effort was made to avoid any adjacent normal tissue and to isolate areas of tissue containing more than 70 % of tumor cells. All personal data were concealed to guarantee patients' protection. All patients signed and approved an informed consent. The procedures were in agreement with the regulations for use of human material in research issued by the Medical Ethics Committee of Pasteur Institute of Tunis. An ethical approval was signed by Dr M. Samir BOUBAKER president of the Medical Ethics Committee.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on FFPE tissues. Routine sections of 2 μm thick were cut using DAKO Capillary Gap slides (S2024; DAKO Corp, Carpinteria, CA, USA), fixed in 10 % buffered formalin and dried at 60 °C overnight. Slides were dewaxed in xylene transferred to absolute alcohol and incubated in 3 % hydrogen peroxide in methanol for 10 min to block endogenous peroxidase.

Slides were then transferred to running tap water before being transferred to 3 l of boiling citrate buffer pH 6.0 in a 15-lb pressure cooker, rinsed in Tris-buffered saline (TBS) pH 7.4 and incubated in normal goat serum (1:10) for 10 min.

Serum was tipped off, and the sections were incubated in primary antibody for 60 min at the appropriate dilution. We used monoclonal antibodies anti-*BRCA1* (GLK-2) with dilution 1/50. After incubation, slides were rinsed in TBS and incubated in DAKO Duet (K0492) biotinylated goat anti-mouse/rabbit secondary reagent (1:100) for 35 min. Slides were incubated in DAKO Duet (K0492) streptavidin–biotin–horseradish peroxidase complex for 35 min, rinsed in TBS and treated with DAB (3,3′ diaminobenzidine chromogen 896102, Kem-En-Tec, Copenhagen, Denmark) for 10 min. Finally, slides were rinsed in tap water, counterstained in Mayer's hematoxylin and mounted.

Sequencing

Genomic DNA was first extracted by proteinase K digestion from peripheral blood mononuclear cells isolated from each sample and then purified using the phenol/Chloroform method. Exons of *BRCA1* containing the studied SNPs were PCR amplified in a total reaction volume of 50 μl containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5–4.5 mM MgCl, 50 mM dNTPs, 10 μl of each primer (designed by Centre Jean Perrin; sequences available on demand), 100 ng of genomic DNA and 1 U of either Taq polymerase (Applied Biosystems, Roche). PCR cycling program comprised an initial denaturation at 94 °C for 5 min, followed by 30 cycles including 20 s at of 94 °C, following the annealing step at specific temperatures for each primer pair and the extension at 72 °C for 20 s. Amplicons were purified by solid-phase extraction using QIAquick column gel (QIAGEN). The product was sequenced in forward and reverse reactions using Applied Biosystems Taq DyeDeoxy terminator cycle sequencing kit according to the manufacturer's instructions. Cycle sequencing consisted of 25 cycles at 96 °C for 30 s and 60 °C for 30 s. Sequence analysis was performed using SEQMAN (DNAstar, Madison, WI, USA) and SEQSCAPE V2.5 (Applied Biosystems) software.

Bioinformatics and statistical analysis

Case/control studies were conducted using the Epi-info seven program. *P* value was considered after the Bonferroni correction. Sequences identified relying on the

Table 1 Clinical characteristics among familial breast cancer and sporadic breast cancer patients

Clinical characteristics	Familial breast cancer		Sporadic breast cancer		*P* value	OR
	N = 40	%	*N* = 46	%		
Age					0.3	
<35	18	45	15	32.6		
>35	22	55	31	67.4		
T stage					0.7	
T0–T1	8	20	14	30.4		
T2–T3	15	37.5	19	41.3		
T4	7	17.5	5	10.9		
Unknown	10	25	8	17.4		
Lymph node metastasis						
Yes	12	30	18	39.2		
No	23	57.5	21	45.6		
Unknown	5	12.5	7	15.2		
Grade					0.9	
G1–G2	20	50	26	56.5		
G3	11	27.5	13	28.3		
Unknown	9	22.5	7	15.2		
Distant metastasis					**0.03**	**2.9 (1.17–7.48)**
Yes	18	45	10	19.5		
No	22	55	36	76.5		

Corrected $P < 0.05$ in bold is significant

Ensembl browser's tools http://www.ensembl.org/index.html have served to determine mature micro-RNAs belonging to the class of Homo sapiens which target regions containing the studied SNPs across their wild and mutated alleles. This work was carried out with miRBase, available at http://www.mirbase.org/ [15], which is the central online repository for micro-RNAs nomenclature, sequence data, annotation and target prediction.

Selected micro-RNAs are associated with the lowest *E* value which is the most significant to target the analyzed regions. It is a method for comparing pair-wise alignments with different similarities and different lengths. It corresponds to the number of times the database match may have occurred just by chance [15]. Normality of data and micro-RNA fold expression distribution was checked using the Kolmogorov–Smirnov test. The comparison of micro-RNA fold expression among patients according to the clinical outcome, results of *BRCA1* protein expression and genotyping were analyzed using the student *T* test. Statistical significance was set at the 95 % level ($P < 0.05$). These statistical analyses were performed relying on the SPSS 20 (IBM) software.

Quantitative real-time PCR (Q-PCR)

Total micro-RNAs were extracted from FFPE tissues using the miRNeasy FFPE kit GIAGEN and then converted to c.DNA using the miScript II RT Kit QIAGEN. Both miR-4668 5P and miR-1179 expressions were tested by Q-PCR SYBR Green using the miScript SYBR Green PCR Kit QIAGEN and miScript Primer Assays QIAGEN corresponding to these two selected micro-RNAs. Protocol details and cycling conditions were performed according to the manufacturer's instructions. Total RNA concentrations were measured using an ND-1000 spectrophotometer. RNA integrity was confirmed on an Agilent 2100 Bioanalyzer. Micro-RNA expression levels ($2^{-\Delta Ct}$) were normalized to these of Hs_RNU6B_2 as a reference and to the mean expression of the normal samples using the ΔΔCt method.

Table 2 *BRCA1* protein expression in mammary tumors revealed by Immunohistochemistry according to both familial and sporadic breast cancer patients

BRCA1 protein expression	Sporadic breast cancer patients		Familial breast cancer patients		*P* value (OR)
	N = 30	%	*N* = 30	%	
Positive staining	25	83.4	16	53.4	**0.02**
Negative staining	5	16.6	14	46.6	5 (1.3–14.5)

BRCA1 expression was scored as 0 to 3+ and dichotomized into negative (0, 1+) or positive (2+, 3+)

Corrected $P < 0.05$ in bold is significant

Table 3 Case–control study of *BRCA1* variants: c.442.58delT, c.2082C>T, c.2311T>C, c.2612C>T, c.3113A>G, c.3119G>A, c.3548A>G c.4308T>C and 4837A>G across patients groups and healthy controls

SNPs genotypes	Comparison groups					
	Control/sporadic cancer		Control/familial cancer		Sporadic/familial cancer	
	P value	OR	*P* value	OR	*P* value	OR
c.442-58 del T						
TT	**0.02**	0.32 (0.11–0.89)	0.07		**0.0001**	9.67 (3.05–32.2)
T0	**0.02**	3.59 (1.13–11.91)	0.9		**0.008**	0.23 (0.07–0.72)
00	0.94		**0.01**		**0.009**	
Allele T	0.07		**0.003**	4.44 (1.52–13.55)	**0.000001**	8.68 (3.21–24.72)
Allele 0	0.07		**0.003**	0.23 (0.07–0.66)	**0.000001**	0.12 (0.04–0.31)
c.2082C>T						
CC	0.51		0.72		0.4	
CT	0.34		0.87		0.87	
TT	0.9		0.19		0.19	
Allele C	0.99		0.99		0.12	
Allele T						
c.2311T>C						
TT	0.71		0.13		**0.02**	3.16 (1.12–9.05)
TC	0.12		0.93		0.09	
CC	0.39		0.068		0.44	
Allele T	0.9		**0.01**	3.04 (1.22–7.74)	**0.01**	2.76 (1.16–6.67)
Allele C	0.9		**0.01**	0.34 (0.14–0.86)	**0.01**	0.36 (0.15–0.86)
c.2612C>T						
CC	0.43		**0.03**	3.14 (1.09–9.2)	0.18	
CT	0.9		0.33		0.33	
TT	0.44		0.22		0.78	
Allele C	0.24		0.01	2.49 (1.19–5.22)	0.19	
Allele T	0.24		0.01	0.4 (0.19–0.84)	0.19	
c.3113 A>G						
AA	0.41		0.57		0.98	
AG	0.08		0.12		0.92	
GG	0.39		0.33		0.84	
Allele A	0.98		0.9		0.92	
Allele G	0.98		0.9		0.92	
c.3119G>A						
GG	0.21		0.93		0.44	
GA	0.21		0.93		0.44	
AA	–		–		–	
Allele G	0.21		0.93		0.45	
Allele A	0.21		0.93		0.45	
c.3548 A>G						
AA	0.33		0.81		0.3	
AG	0.25		0.86		0.45	
GG	0.81		0.82		0.86	
Allele A	0.48		0.91		0.28	
Allele G	0.48		0.91		0.28	
c.4308T>C						
TT	0.86		0.04	3.3 (1.02–11.01)	0.01	3.96 (1.32–12.24)
TC	0.25		0.97	0 (0–0.58)	0.11	

Table 3 continued

SNPs genotypes	Comparison groups					
	Control/sporadic cancer		Control/familial cancer		Sporadic/familial cancer	
	P value	OR	*P* value	OR	*P* value	OR
CC	0.67		0.008		0.09	
Allele T	0.85		0.001	4.66 (1.7–13.9)	0.002	4.11 (1.56–11.2)
Allele C	0.85		0.001	0.21 (0.08–0.59)	0.002	0.24 (0.09–0.64)
c.4837A>G						
AA	0.98		0.75		0.68	
AG	0.34		0.67		0.78	
GG	0.37		0.19		0.86	
Allele A	0.73		0.25		0.46	
Allele G	0.73		0.25		0.46	

Corrected $P < 0.05$ in bold is significant

Results

Clinical outcome

Clinical data were compared between both sporadic and familial breast cancer patients (Table 1). No significant differences were obtained for the median age under and upper 35 years, along with stage (T0–T4), grade (G1–G3) and lymph node metastasis. However, a significant difference was observed for the distant metastases' occurrence among patients: 45 % of them were categorized as FBC patients, and only 19.5 % of them as SBC ones having distant metastasis, P value = 0.03 and OR = 2.9 (Table 1).

Immunohistochemistry study of *BRCA1* protein

BRCA1 protein expression among sporadic and familial breast cancer patients was assessed according to the presence or the absence of *BRCA1* protein within mammary tissues. Scores ranged from 0 to 3+. Negative staining was scored 0 and 1+, and positive staining was scored 2+ and 3+. We found a significant difference of the *BRCA1* protein staining among the two groups of patients where *BRCA1* protein was absent in (46.6 %) of the FBC cases compared to (16.6 %) of the SBC ones with P value = 0.02 and OR = 5 (Table 2).

Case–control study

We performed a case–control study to compare genotypes and alleles frequencies between the three analyzed groups based on the nine selected SNPs (Table 4). We found that c.2082C>T, c.3113A>G, c.3119G>A, c.3548A>G and c.4837A>G SNPs were not associated with breast cancer disease (FBC or SBC) with P value >0.05. However, the comparison of the three aforementioned groups revealed a significant difference for c.442.58 delT, c.2311T>C, c.2612C>T and c.4308T>C SNPs (P value <0.05; Table 3). Comparison of allelic and genotypic frequencies between sporadic and familial breast cancer groups considering c.442.58 del T SNP, showed that TT genotype which is the wild type, seems to be strongly associated with familial breast cancer relative to SBC patients. P value = 0.000001 and OR = 9.67. However, TT genotype appears to be protective against SBC form comparing with HC. P value = 0.02 and OR = 0.32. Considering allele frequency, we showed that T allele which is the wild type increases the risk of familial breast cancer occurrence with P value = 0.000001 and OR = 8.68 (Table 3). The comparison across genotypes' frequencies of c.2311T>C *BRCA1* SNP between sporadic and familial breast cancer patients showed a significant difference in TT wild-type genotype which is associated with the FBC group with P value = 0.02 and OR = 3.16, while CC genotype which is the mutated type was almost entirely absent in the same group. T wild-type allele is associated with familial breast cancer but not to the sporadic form with P value = 0.01 and OR = 2.76 (Table 3). Concerning c.2612C>T *BRCA1* SNP, the association of CC genotype frequency among FBC patients compared to HC is significant with P value = 0.03 and OR = 3.14. C allele frequency is also associated with familial breast cancer cases compared to HC. P value = 0.01 and OR = 2.49 (Table 3). Regarding c.4308 T>C SNP, the TT wild-type genotype frequency is associated with FBC patients relative to both HC and SBC cases, respectively ((P value = 0.04, OR = 3.3) and (P value = 0.01, OR = 4.66)). Similarly, the T wild-type allele is also associated with FBC cases compared to HC and SBC patients, respectively ((P = 0.001, OR = 4.6) and (P value = 0.002, OR = 4.11; Table 3)).

Table 4 Predicted micro-RNAs targeting sequences that contain *BRCA1* SNPs; c.442-58 delT, c.2311T>C, c.2612C>T and c.4308T>C according to their wild- or mutated-type alleles using miRbase tools

SNP of *BRCA1*	Allele	Targeted sequence	Predicted mature mi-RNA (Homo sapiens)	Accession number	*E* value
c.4 42–58 delT	Wild type	TGTTTTTCAACAAGTACATTTTTTT**T**AACCCTTTTAATTAAGAAAACTTTT	hsa-miR-4668–5p [14]	MIMAT0019745	2.3
	Mutated type	TGTTTTTCAACAAGTACATTTTTT-**A**ACCCTTTTAATTAAGAAAACTTTT	–	–	–
c.2311 T>C	Wild type	TAGAGAGTAGCAGTATTTCA**T**TGGTACCTGGTACTGATTAT	hsa-miR-1179 [15]	MIMAT0005824	1.5
	Mutated type	TAGAGAGTAGCAGTATTTCA**C**TGGTACCTGGTACTGATTAT	hsa-miR-1179	MIMAT0005824	8.2
c.2612C>T	Wild type	AAAGCGCCAGTCATTTGCTC**C**GTTTTCAAATCCAGGAAATG	–	–	–
	Mutated type	AAAGCGCCAGTCATTTGCTC**T**GTTTTCAAATCCAGGAAATG	hsa-miR-511 [16]	MIMAT0002808	6.8
			hsa-miR-4782–3p	MIMAT0019945	8.2
c.4308 T>C	Wild type	CCTTCCATCATAAGTGACTC**T**TCTGCCCTTGAGGACCTGCG	–	–	–
	Mutated type	CCTTCCATCATAAGTGACTC**C**TCTGCCCTTGAGGACCTGCG	hsa-miR-3922–3p	MIMAT0018197	6.8

Micro-RNAs were selected according to the lowest *E* value. The underlined nucleotides in blot correspond to the SNPs in their wild and mutated types. Analyzed sequences containing wild or mutated SNPs were considered according to an approximate size of 20–25 pb on either side of each variant

Table 5 Expression level of miR-1179 in both familial and sporadic breast cancer patients

	miR-1179	
	FBC patients	SBC patients
Mean fold expression (Log *N* transformed)	5.83	3.63
SE	0.67	0.45
SD	3.69	2.5
Skewness	0.01	−1.09
Kurtosis	−0.32	3.11
One-way ANOVA(between groups)	*F* ratio = 7.28 *df* = 1	*P* value = 0.009

SE standard error, *SD* standard deviation, *df* degree of freedom, *FBC* familial breast cancer, *SBC* sporadic breast cancer

Corrected $P < 0.05$ is significant

Functional role of the associated *BRCA1* SNPs with familial breast cancer patients

We focused on the eventual functional role that could play the four selected *BRCA1* SNPs c.442.58 delT, c.2311T>C, c.2612C>T and c.4308T>C which are observed to be associated with FBC patients through their wild genotypes and alleles. At first, we used miRbase tools to predict potential micro-RNAs that may target regions within *BRCA1* mRNA containing these SNPs. We selected 2 micro-RNAs: miR-4668-5p and miR-1179, respectively, targeting c.442-58delT and c.2311T>C regions at their wild-type allele based on a low *E* value, 2.3 and 1.5, respectively (Table 4). For c.2612 C>T, miRbase predicts a targeting miR-511 of the wild allele with a high *E* value = 6.8. Consequently, we did not take into account this predictable micro-RNA. Concerning c.4308 T>C SNP, miRbase did not find any predictable micro-RNA that could target its region with the wild-type allele (Table 4). In order to confirm these computing results, we assessed the expression levels of both miR-4668-5p and miR-1179 by Q-PCR SYBER GREEN. This was realized through analyzing 30 mammary tumors of each familial and sporadic breast cancer patients along with their corresponding normal tissues. MiScript Primer of miR-4668-5P did not show any result because of its high concentration in Guanine and Cytosine. We managed to get results only for miR-1179. The experiment was carried out three times for each sample. After normalizing the expression levels with the corresponding mean value of the gene reference RNU6B by the ΔCt method, the samples were checked for outliers to be excluded. The final expression levels of

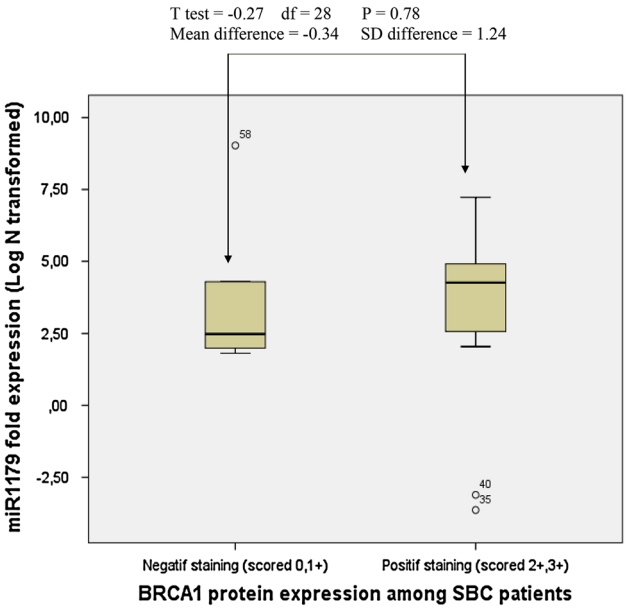

Fig. 1 miR-1179 fold expression among sporadic breast cancer patients according to *BRCA1* protein expression. Student *T* test was calculated considering independent samples method dealing with equal variances assumed. *Arrows* indicate the two analyzed groups by student *T* test. *P* value <0.05 is significant, *df* degree of freedom, *SD* standard deviation, *SBC* sporadic breast cancer

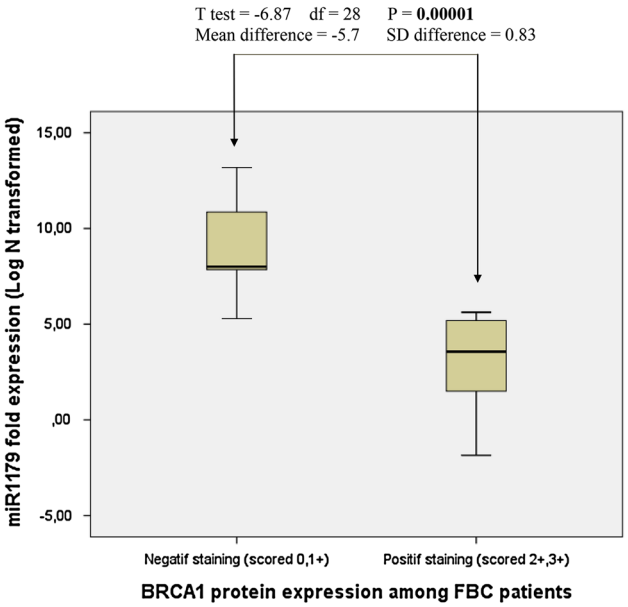

Fig. 2 miR-1179 fold expression among familial breast cancer patients according to *BRCA1* protein expression. Student *T* test was calculated considering independent samples method dealing with equal variances assumed. *Arrows* indicate the two analyzed groups by student *T* test. *P* value <0.05 is significant, *df* degree of freedom. *SD* standard deviation, *FBC* familial breast cancer

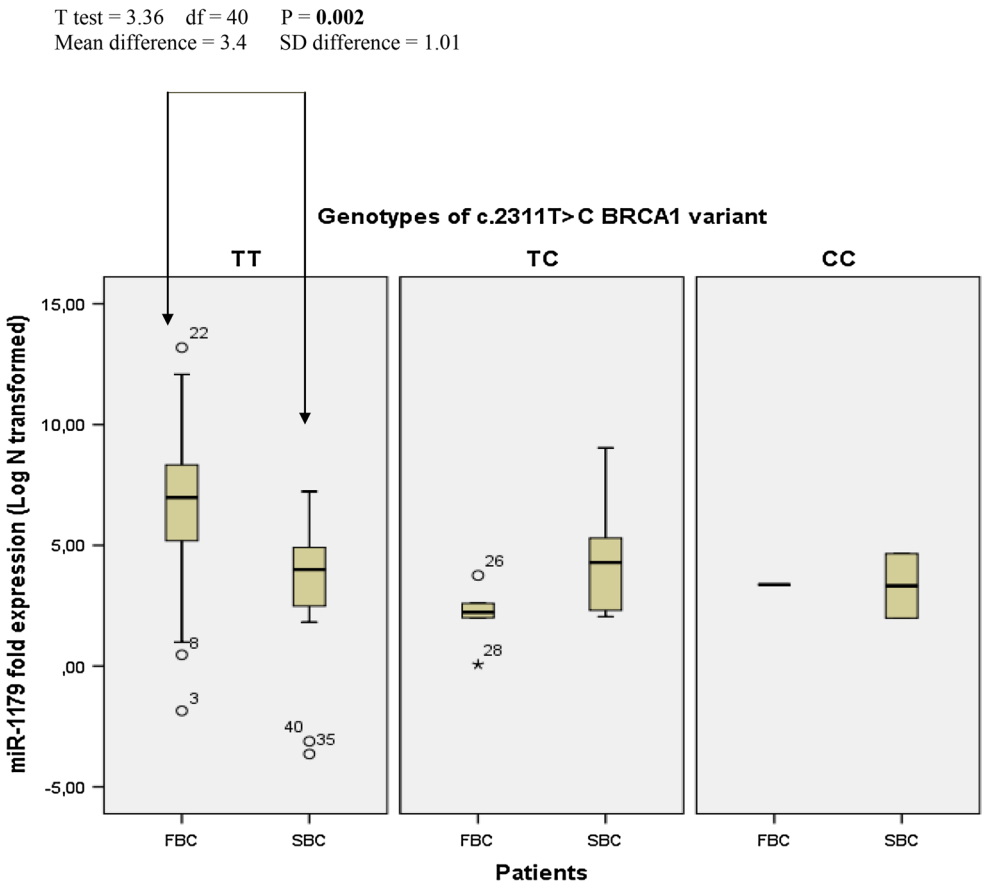

Fig. 3 miR-1179 fold expression among familial and sporadic breast cancer patients according to TT, TC and CC genotypes of c.2311T>C *BRCA1* variant student *T* test was calculated considering independent samples method dealing with equal variances assumed. *Arrows* indicate the two analyzed groups by student *T* test. *P* value <0.05 is significant, *df* degree of freedom, *SD* standard deviation, *FBC* familial breast cancer, *SBC* sporadic breast cancer

miR-1179 were considered after normalizing tumors mean expression levels with those of their corresponding normal tissues according to ΔΔCt method. The expression levels of miR-1179 across FBC and SBC patients were standardized (Log N transformed) and analyzed by descriptive analyses method. Thus, the expression levels were approximately normally distributed among FBC patients with skewness of 0.01 and Kurtosis of −0.32, the mean fold expression =5.83 with a standard deviation (SD) = 3.69. However, in SBC patients the mean fold expression =3.63 with SD = 2.5, the expression levels across those patients were also approximately normally distributed with skewness of −1.09 and Kurtosis of 3.11 (Table 5). The difference of the means was assessed among the two groups of patients using the one-way ANOVA method and was revealed statistically significant with *F* ratio = 7.28 and *P* value = 0.009 (Table 5).

Second, results were further explored by comparing the different means of miR-1179 fold expression according to our previous results, *BRCA1* protein expression (negative or positive staining) and genotypes of c.2311T>C *BRCA1* SNP (TT, TC and CC), having or not distant metastasis and according to the presence or absence of *BRCA1* deleterious mutations. This last criterion concerns only FBC patients. Results of the deleterious mutations were already established on the same FBC patients by Troudi et al. [7]; our concern is to compare the mean fold expression on FBC patients having or not deleterious mutations to explain the role that could be assigned to miR-1179 in the absence of *BRCA1* protein independently of deleterious mutations. miR-1179 fold expression among SBC patients according to *BRCA1* protein status (negative and positive staining) did not show any significant difference, *T* test = −0.27, *df* = 28 and *P* value = 0.78 (Fig. 1). However, miR-1179 fold expression across FBC patients considering the negative staining of *BRCA1* protein is clearly higher than the one having *BRCA1* positive staining, *T* test = −6.87, *df* = 28 and *P* value = 0.00001 (Fig. 2). Then, we compared the miR-1179 fold expression between the two groups of patients (FBC and SBC) according to the different genotypes (TT, TC and CC) of c.2311T>C *BRCA1* variant. We found that miR-1179 fold expression among FBC patients with TT genotype is higher than this among SBC patients with the same genotype, *T* test = 3.36, *df* = 40 and *P* value = 0.002; however, the expression between patients according to the other genotypes TC and CC did not show any significant difference (Fig. 3). In addition, we compared the mean fold expression of the

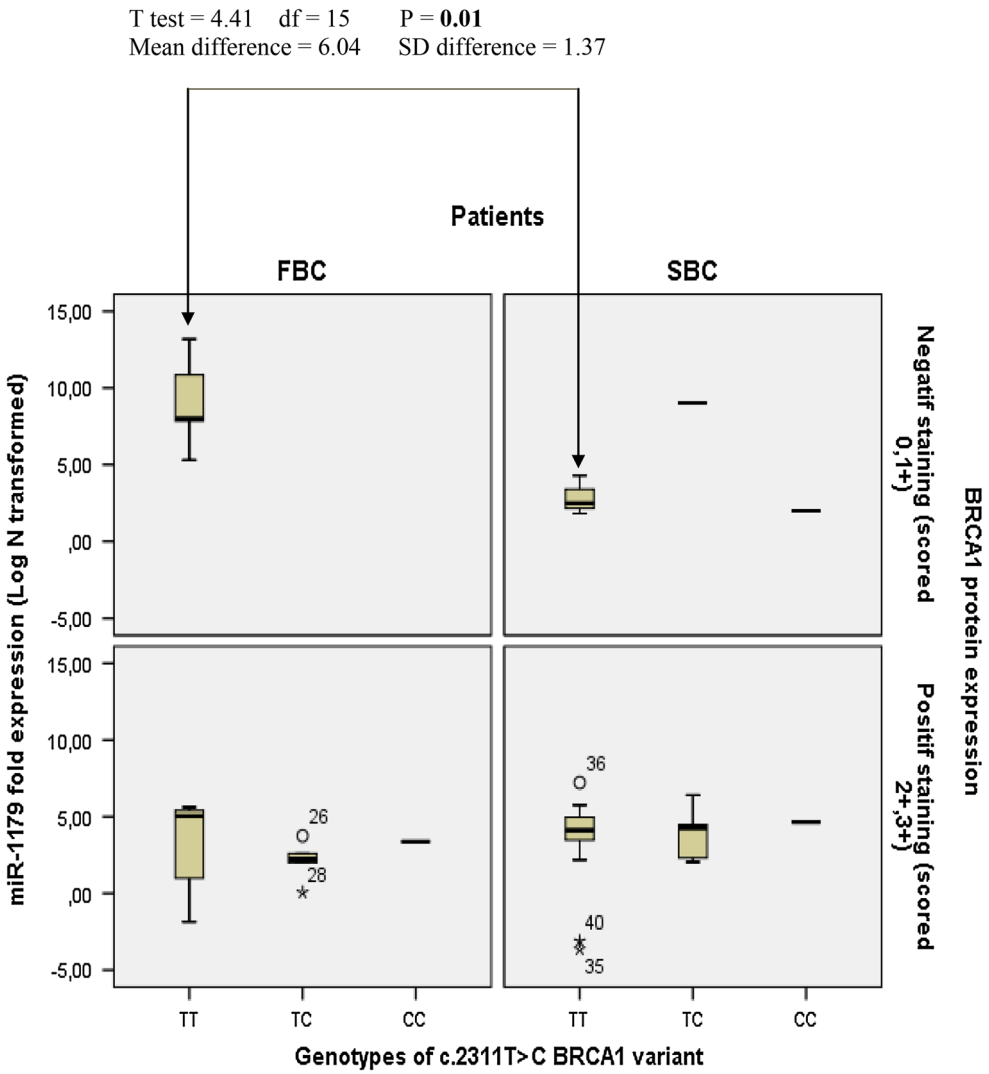

Fig. 4 miR-1179 fold expression according to TT, TC and CC genotypes of c.2311T>C *BRCA1* variant among familial and sporadic breast cancer patients considering *BRCA1* protein expression. Student *T* test was calculated considering independent samples method dealing with equal variances assumed. *Arrows* indicate the two analyzed groups by student *T* test. *P* value <0.05 is significant, *df* degree of freedom, *SD* standard deviation, *FBC* familial breast cancer, *SBC* sporadic breast cancer

analyzed micro-RNA between FBC and SBC patients according to both *BRCA1* protein expression and c.2311 T>C genotypes, and combined together, we found that the mean fold expression of miR-1179 according to both TT genotypes and negative staining of *BRCA1* protein is much higher among FBC patients than those with SBC form, T test = 4.41, $df = 15$ and P value = 0.01. Nevertheless, we did not record any significant difference of the fold expression within FBC and SBC patients according to TC and CC genotypes combined with positive or negative staining of the protein (Fig. 4). To further confirm the involvement of miR-1179 in FBC with *BRCA1* negative staining, we considered previous results about deleterious mutations of *BRCA1* gene that were already established by Troudi et al. [7] on the same FBC patients. We found that the mean fold expression of miR-1179 among FBC patients without *BRCA1* deleterious mutation is absolutely higher than the fold expression in those with deleterious mutations, T test = 2.7, $df = 22$ and P value = 0.01 (Fig. 5). Finally, we assessed the mean fold expression levels of the analyzed micro-RNA to the occurrence of distant metastasis.

Our results show well that miR-1179 expression levels according to distant metastases' occurrence are higher within FBC patients compared to those with SBC form, T test = 3.43, $df = 15$ and P value = 0.04 (Fig. 6).

General discussion

BRCA1 gene is commonly the most characterized gene in familial breast cancer. Previous studies failed to demonstrate in most hereditary breast cancer cases, the role assigned to *BRCA1* germ line deleterious mutations in the dysfunction of *BRCA1* protein. The involvement of *BRCA1* gene in mammary tumors through epigenetic mechanisms

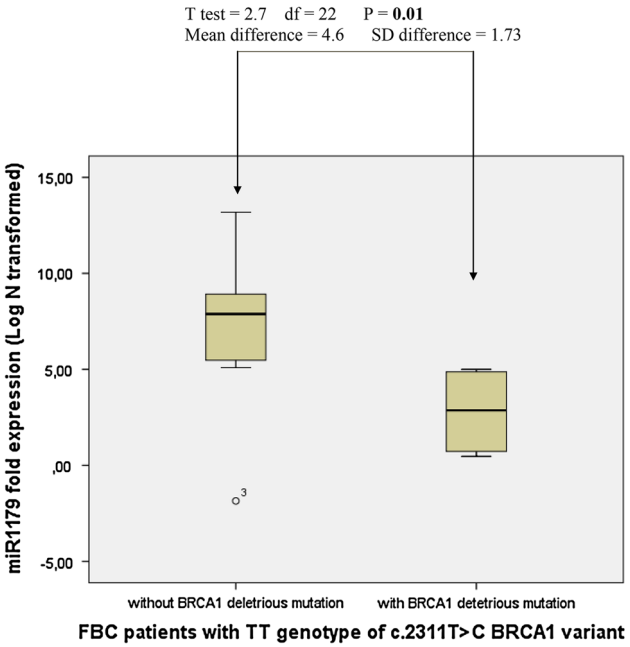

Fig. 5 miR-1179 fold expression among familial breast cancer patients with TT genotype of c.2311T>C *BRCA1* variant depending on whether or not to have deleterious mutation in *BRCA1* gene. Student *T* test was calculated considering independent samples method dealing with equal variances assumed. *Arrows* indicate the two analyzed groups by student *T* test. *P* value <0.05 is significant, *df* degree of freedom, standard deviation, *FBC* familial breast cancer

was proven by several studies. Methylation of promoters as well as over-expression of oncogenic micro-RNAs targeting tumor suppressor genes is well documented [16, 17]. Recently, it has been suggested that deregulation of *BRCA1* transcription could contribute to a sporadic form of breast cancer, in relationship with microenvironment effect [18]. Several research works demonstrated that *BRCA1* gene dysfunction plays a crucial role in distant metastases' occurrence especially in brain and pulmonary metastases [19]. This could explain our clinical outcome where we observed high frequency of patients having distant metastases' occurrence within FBC group compared to those with SBC form. This observation suggests that distant metastases' occurrence among FBC patients may be due to the dysfunction of *BRCA1* protein. Our investigation was performed on nine SNPs within *BRCA1* gene. We analyzed their frequencies in three groups of unrelated women from Northern Tunisia: healthy subjects, patients with sporadic and familial breast cancer. Sample sizes were adapted to the number of familial breast cancer diagnosed in this region where 1,000 cases of breast cancer are observed per year among which only 5 % took the family form. According to our case–control study, four among nine SNPs revealed significant differences in genotypes and alleles frequencies among the studied groups. Wild-type alleles and genotypes of c.442-58 delT, c.2311T>C, c.2612 C>T and c.4308T>C SNPs are clearly associated with familial breast cancer with an odds ratio ranging from 2.49 to 4.66. Two questions are raised in the light of these results: The first is the unexpected feature related to the risk associated with wild-type genotypes of *BRCA1* SNPs, and the second is related to the functional role that these SNPs could play across their wild-type genotypes in familial breast cancer onset.

Considering *BRCA1* wild-type allele as an ancestral form that was selected during evolution and then adapted to the different ways of life for thousands of years, we notice that in the last 50 years, women lifestyle has changed dramatically.

Wild allelic form of *BRCA1* gene adapted to the ancient lifestyle became at risk of breast cancer in the new environmental conditions. This observation is not unique for breast cancer, and it was also observed for other diseases such as obesity and high blood pressure (HBP) [20]. Indeed, genes selected in conditions of lack of food and salt became at risk, respectively, for obesity and HBP due to over nutrition and excessive salt consumption [21]. During the last decades, the Tunisian population just like others was subject to epidemiological transition wrapping an increasing number of breast cancer cases, including familial cases [22]. This could be explained by changes in lifestyle which became part of modern life societies including social and environmental factors such as stress, fatty food, hormonal contraception, lack of exercise, pregnancy history and breast-feeding and by the interaction between genetic and epigenetic features.

Among the four associated SNPs to familial breast cancer, the c.2612 C>T variant could have an effect on the protein sequence with an amino acid change (Proline to Leucine) at position 871, suggesting an alteration on the protein function and an ambivalent role of wild allele to familial breast cancer susceptibility. However, the c.4304T>C variant was not associated with any amino acid change. Its association with breast cancer might be indirect, due to linkage disequilibrium with other SNPs. However, its direct involvement in breast cancer could not be excluded. For c.442-58delT *BRCA1* SNP, we found that it is complementary to mature miR-4668-5p. This micro-RNA was identified in normal and tumor breast tissues, suggesting its role as an onco-miR [23]. We tried to check the validity extent of this result by evaluating its expression levels in mammary tumors using Q-PCR, but we did not find any amplification because of the high G and C concentration in miR-4668-5P miScript primer assay. Concerning c.2311T>C *BRCA1* SNP, our computing findings showed that the wild-type allele is targeted by miR-1179. The over-expression of this micro-RNA was already demonstrated in breast cancer and human sarcoma, and it was up-regulated in colorectal cancers with distant

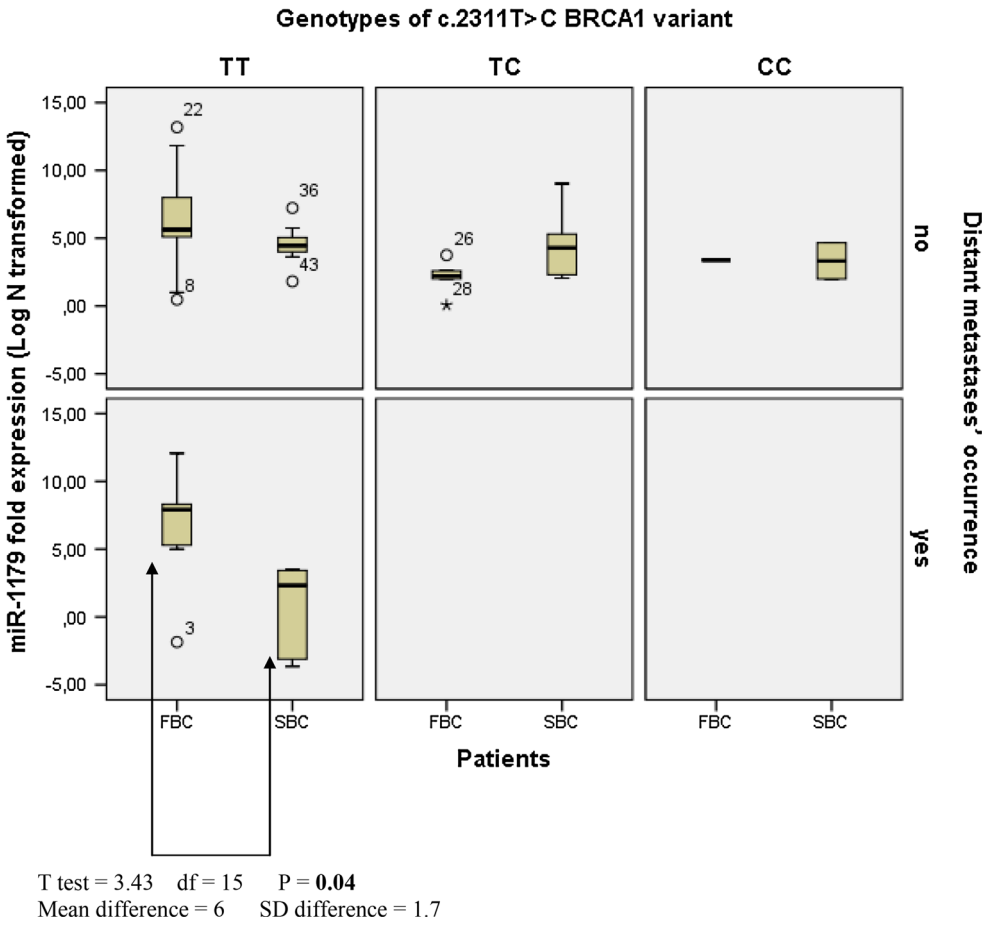

Fig. 6 miR-1179 fold expression according to TT, TC and CC genotypes of c.2311T>C *BRCA1* variant among familial and sporadic breast cancer patients considering the occurrence or not of distant metastasis. Student *T* test was calculated considering independent samples method dealing with equal variances assumed. *Arrows* indicate the two analyzed groups by student *T* test. *P* value <0.05 is significant, *df* degree of freedom, *SD* standard deviation, *FBC* familial breast cancer, *SBC* sporadic breast cancer

metastases' occurrence [24]. In this research, using Q-PCR analysis, we intend to show that expression levels of miR-1179 within FBC tumors are significantly higher than those in SBC ones and this according to both TT genotype of c.2311T>C SNP and the absence of germ line *BRCA1* deleterious mutations. In addition, the mean fold expression of the studied micro-RNA was associated with distant metastases' occurrence with TT genotype among FBC patients compared to those with SBC form. This observation supports the functional role that could be assigned to *BRCA1* SNPs with its wild-type alleles on FBC onset and therefore on distant metastasis.

In light of these results, we can propose the following model: Combination between the wild-type TT genotype of c.2311T>C and miR-1179 over-expression generates a lack of *BRCA1* protein expression leading to a higher risk of developing a familial breast cancer with distant metastases' occurrence.

We suggest that micro-RNAs are mostly directed against wild-type allele sequences. In a way of life, endogenous factors such as hormones and inflammatory cytokines could be influenced and therefore could have an effect on oncogenic micro-RNAs such as miR-4668-5p and miR-1179 by up-regulation mechanism. Therefore, a subject carrying wild-homozygous-type genotypes within the *BRCA1* gene could be threatened by breast cancer with the onset of distant metastasis.

Conclusion

This study is in agreement with genetic end epigenetic interactions in breast cancer across micro-RNAs expression which could be considered as a mechanism rather than deleterious mutations, responsible for familial breast cancer onset with distant metastases' occurrence. Onco-miRs targeting *BRCA1* gene and other suppressor genes have to be better studied. Transduction ways leading to their expression in relation to environmental and endogenous factors remain to be further explained. This research gives the hope to prevent familial breast cancer inspecting *BRCA1* germ line deleterious mutations along with the high risk of distant metastases' occurrence.

Acknowledgments The authors would like to thank all those who have contributed to the realization of this work in Salah Azaiz

Institute of Tunis, Laboratory of Genetic Immunology and Human Pathology, Laboratory of Genetics and Molecular Diagnostic "LOM" in Clermont-Ferrand and in Laboratory of Molecular screening in breast cancer in Montreal for their technical assistance.

Conflict of interest The authors declare that they have no competing interests.

References

1. Saika K, Sobue T. Cancer statistics in the world. Gan To Kagaku Ryoho. 2013;40(13):2475–80.
2. Charef-Hamza S, et al. Loss of heterozygosity at the *BRCA1* locus in Tunisian women with sporadic breast cancer. Cancer Lett. 2005;224(2):185–91.
3. Guaragnella N, et al. The expanding role of yeast in cancer research and diagnosis: insights into the function of the oncosuppressors p53 and *BRCA1/2*. FEMS Yeast Res. 2013;111(10):548–62.
4. Albiges L, et al. Spectrum of breast cancer metastasis in *BRCA1* mutation carriers: highly increased incidence of brain metastases. Ann Oncol. 2005;16(11):1846–7.
5. Egawa C, et al. Quantitative analysis of *BRCA1* and *BRCA2* mRNA expression in sporadic breast carcinomas and its relationship with clinicopathological characteristics. Jpn J Cancer Res. 2001;92(6):624–30.
6. Wu J, Lu LY, Yu X. The role of *BRCA1* in DNA damage response. Protein Cell. 2010;1(2):117–23.
7. Troudi W, et al. Contribution of the *BRCA1* and *BRCA2* mutations to breast cancer in Tunisia. J Hum Genet. 2007;52(11):915–20.
8. Im KM, et al. Haplotype structure in Ashkenazi Jewish *BRCA1* and *BRCA2* mutation carriers. Hum Genet. 2011;130(5):685–99.
9. Cao W, et al. *BRCA1* germ-line mutations and tumor characteristics in eastern Chinese women with familial breast cancer. Anat Rec (Hoboken). 2013;296(2):273–8.
10. Troudi W, et al. Complete mutation screening and haplotype characterization of *BRCA1* gene in Tunisian patients with familial breast cancer. Cancer Biomark. 2008;4(1):11–8.
11. Chang S, et al. Tumor suppressor *BRCA1* epigenetically controls oncogenic microRNA-155. Nat Med. 2011;17(10):1275–82.
12. Folini M, et al. miR-21: an oncomir on strike in prostate cancer. Mol Cancer. 2010;9:12.
13. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004;5(7):522–31.
14. Moskwa P, et al. miR-182-mediated downregulation of *BRCA1* impacts DNA repair and sensitivity to PARP inhibitors. Mol Cell. 2011;41(2):210–20.
15. Griffiths-Jones S, et al. miRBase: tools for microRNA genomics. Nucleic Acids Res. 2008;36(Database issue):D154–8.
16. Olive V, et al. A component of the mir-17-92 polycistronic oncomir promotes oncogene-dependent apoptosis. Elife. 2013;2:e00822.
17. Filipponi D, et al. Wip1 controls global heterochromatin silencing via atm/*BRCA1*-dependent dna methylation. Cancer Cell. 2013;24(4):528–41.
18. Di LJ, et al. Transcriptional regulation of *BRCA1* expression by a metabolic switch. Nat Struct Mol Biol. 2010;17(12):1406–13.
19. McMullin RP, et al. A *BRCA1* deficient-like signature is enriched in breast cancer brain metastases and predicts DNA damage-induced poly (ADP-ribose) polymerase inhibitor sensitivity. Breast Cancer Res. 2014;16(2):R25.
20. Bouhaha R, et al. Study of TNFalpha-308G/A and IL6-174G/C polymorphisms in type 2 diabetes and obesity risk in the Tunisian population. Clin Biochem. 2010;43(6):549–52.
21. Bouhaha R, et al. TCF7L2 is associated with type 2 diabetes in nonobese individuals from Tunisia. Pathol Biol (Paris). 2010;58(6):426–9.
22. Binns C, Low WY, Lee MK. Breast cancer: an increasing public health problem in the Asia pacific region. Asia Pac J Public Health. 2013;25(5):364–7.
23. Subramanian S, et al. MicroRNA expression signature of human sarcomas. Oncogene. 2008;27(14):2015–26.
24. Lin M, et al. MicroRNA expression profiles in human colorectal cancers with liver metastases. Oncol Rep. 2011;25(3):739–47.