ARTICLE

Mutation screening of *MIR146A/B* and *BRCA1/2* 3′-UTRs in the GENESIS study

Amandine I Garcia[1], Monique Buisson[1], Francesca Damiola[1], Chloé Tessereau[1], Laure Barjhoux[1], Carole Verny-Pierre[1], Valérie Sornin[1], Marie-Gabrielle Dondon[2,3,4], Séverine Eon-Marchais[2,3,4], GENESIS investigators, Olivier Caron[5], Marion Gautier-Villars[6], Isabelle Coupier[7,8], Bruno Buecher[6], Philippe Vennin[9,†], Muriel Belotti[6], Alain Lortholary[10], Paul Gesta[11], Catherine Dugast[12], Catherine Noguès[6], Jean-Pierre Fricker[13], Laurence Faivre[14,15], Dominique Stoppa-Lyonnet[6,16], Nadine Andrieu[2,3,4], Olga M Sinilnikova[1,17,†] and Sylvie Mazoyer*,[1]

Although a wide number of breast cancer susceptibility alleles associated with various levels of risk have been identified to date, about 50% of the heritability is still missing. Although the major *BRCA1* and *BRCA2* genes are being extensively screened for truncating and missense variants in breast and/or ovarian cancer families, potential regulatory variants affecting their expression remain largely unexplored. In an attempt to identify such variants, we focused our attention on gene regulation mediated by microRNAs (miRs). We screened two genes, *MIR146A* and *MIR146B*, producing miR-146a and miR-146b-5p, respectively, that regulate *BRCA1*, and the 3′- untranslated regions (3′-UTRs) of *BRCA1* and *BRCA2* in the GENESIS French national case/control study (*BRCA1*- and *BRCA2*-negative breast cancer cases with at least one sister with breast cancer and matched controls). We identified one rare variant in *MIR146A*, four in *MIR146B*, five in *BRCA1* 3′-UTR and one in *BRCA2* 3′-UTR in 716 index cases and 619 controls. Among these 11 rare variants, 7 were identified each in 1 index case. None of the three relevant *MIR146A/MIR146B* variants affected the pre-miR sequences. The potential causality of the four relevant *BRCA1/BRCA2* 3′-UTRs variants was evaluated with luciferase reporter assays and co-segregation studies, as well as with bioinformatics analyses to predict miRs-binding sites, RNA secondary structures and RNA accessibility. This is the first study to report the screening of *miR* genes and of *BRCA2* 3′-UTR in a large series of familial breast cancer cases. None of the variant identified in this study gave convincing evidence of potential pathogenicity.

INTRODUCTION

A new class of epigenetic gene expression regulators has been identified about 20 years ago, microRNAs (miRs). miRs are small, non-coding RNAs of 19 to 22 nucleotides (nt) that bind to the 3′- untranslated regions (3′-UTRs), or less frequently to the coding sequence or 5′-UTR of targeted mRNAs, thereby inducing their degradation or inhibiting their translation, resulting in gene down-regulation. Recognition of an mRNA by a targeting miR is mediated by the seed region (nt 2_7 of a miR) around which partial complementarity extends. Alterations of miR/mRNA interactions are likely to impair the control of gene expression, should they occur through mismatches introduced by SNPs within *miR* genes or miR-binding target sites, or through variations in miR expression levels.

The involvement of miRs in genetic susceptibility to diseases, and particularly to complex disorders such as cancer, is being extensively investigated since the first report of a phenotype-causing variant creating a potential illegitimate miR target site in 2006.[1] However, the focus is principally on finding genetic association with common SNPs located in *miR* genes or miR-binding target sites,[2] and very few studies aimed to identify rarer variants in case–control series even if large scale resequencing approaches focusing on *miR* genes and 3′-UTR are starting to be used.[3] This paucity of mutation screening studies could stem from the difficulty in interpreting rare variants that would be identified by such an approach, miR true targets remaining rather scarce, their genes poorly characterized, not to mention their regulation. Most *miR* genes are transcribed by RNA polymerase II into primary miR transcripts, pri-miR, which are about 1000 nt long and contain one or more stable stem-loop structures. They are subsequently processed first to shorter pre-miRs (70_100 nt), then to mature miRs. Variants falling in pre-miR or mature miR sequences can benefit from the use of computational algorithms to predict their potential effects and be tested in functional assays, as has been done

[1]Cancer Research Centre of Lyon, CNRS UMR5286/Inserm U1052/Université Lyon 1, Centre Léon Bérard, Lyon, France; [2]INSERM, U900, Paris, France; [3]Institut Curie, Paris, France; [4]Mines ParisTech, Fontainebleau, France; [5]Département de Médecine Oncologique, Institut de Cancérologie Gustave Roussy, Villejuif, France; [6]Institut Curie, Service de Génétique, Paris, France; [7]Hôpital Arnaud de Villeneuve, CHU Montpellier, Service de Génétique médicale et Oncogénétique, Montpellier, France; [8]ICM Val d'Aurel, Unité d'Oncogénétique, Montpellier, France; [9]Centre Oscar Lambret, Lille, France; [10]Centre Catherine de Sienne, Service d'Oncologie Médicale, Nantes, France; [11]CH Georges Renon, Service Oncogénétique pour la consultation oncogénétique régionale Poitou-Charentes, Niort, France; [12]Centre Eugène Marquis, Service de Génétique, Rennes, France; [13]Centre Paul Strauss, Unité d'Oncologie, Strasbourg, France; [14]Hôpital d'Enfants, Service de Génétique Médicale, Dijon, France; [15]Centre Georges François Leclerc, Oncogénétique, Dijon, France; [16]Inserm, U830, Université Paris-Descartes, Paris, France; [17]Unité Mixte de Génétique Constitutionnelle des Cancers Fréquents, Hospices Civils de Lyon/Centre Léon Bérard, Lyon, France
*Correspondence: Dr S Mazoyer, Centre de Recherche en Cancérologie de Lyon, Centre Léon Bérard, 28 rue Laënnec, Lyon 69373, France. Tel: +33 469 16 66 79; Fax: +33 469 16 66 60; E-mail: sylvie.mazoyer@inserm.fr
†Prematurely passed away.
Received 7 August 2015; revised 11 December 2015; accepted 16 December 2015; published online 20 January 2016

for the eight rare variants identified in precursor or mature miRs during the screening of 59 *miR* genes on the X-chromosome in 193 males with schizophrenia spectrum disorders.[4] For variants identified outside these sequences, prediction of the effect they could have on miR production is nearly impossible, even if evidence indicates that pri-miR processing is regulated during development in order to achieve optimal miRNA expression patterns[5] and can be dysregulated in diseases.[6] As a result, the problematic issue of sorting out the candidate deleterious variants from the more abundant functionally irrelevant ones remains rather perilous.

BRCA1 (MIM# 113705) and *BRCA2* (MIM# 600185) genes are the two major breast cancer susceptibility genes: pathogenic variants in these genes explain nearly 25% of the familial risk for breast cancer.[7] Other high-risk susceptibility genes such as *TP53*, *PTEN*, *CDH1*, *STK11* and *PALB2* have been identified, as well as a number of intermediate-risk genes such as *ATM*, *CHEK2*, *NBN* and *NF1*, and close to 100 low-risk alleles.[8,9] Despite this, all in all, only about 50% of the excess risk is explained today. Evidence is starting to emerge that some breast cancer genes may harbour different types of variants with inversely correlated cancer risks and allele frequencies: for example, *BRCA2* and *TP53*, on top of a myriad of high-risk rare variants, also harbour more frequent variants associated with lower breast cancer risk, p.K3326X[10] and p.R337H,[11] respectively. This has prompted scientists to search in *BRCA1/2*-negative breast cancer families for variants deregulating the expression of the *BRCA1* and *BRCA2* genes, possibly associated with lower cancer risks than inactivating *BRCA1/2* variants. For our part, here, we decided to focus our attention on miRs, as we showed a few years ago that miR-146a and miR146b-5p negatively regulate the expression of *BRCA1*.[12] MiR-146a and miR-146b-5p are well studied miRs with multiple targets, whose gene and processing are rather well known. *MIR146A* (MIM# 610566) has two exons separated by a ~ 16 kb intron[13,14] and is transcribed into a pri-miR of 2329 nt (GenBank accession number: EU147785.1); exon 2 contains the sequence of the pre-miR, which starts 53 nt from its 5′-end. *MIR146B* (MIM# 610567) has only one exon and the transcription start site is located ~ 700 nt upstream of the mature miR-146b-5p sequence,[14] but is not defined precisely, nor is identified the polyadenylation site. We reasoned that variants impacting these genes, hence this regulation, might predispose to breast cancer. We thus screened the *MIR146A* and *MIR146B* genes in a familial breast cancer case–control study, GENESIS, with the hope to find activating mutations that would lead to enhanced expression and/or enhanced *BRCA1* affinity. We also screened *BRCA1* and *BRCA2* 3′-UTRs in GENESIS in order to identify potential 3′-UTR-dependent mechanisms of gene dysregulation.

SUBJECTS AND METHODS

Study subjects
The study was conducted on a subgroup of subjects from the GENESIS (GENE SISters) French national study.[15] The index cases are women diagnosed with infiltrating mammary adenocarcinoma who attended a cancer genetics clinic in France and were found negative for *BRCA1/2* pathogenic variants after extensive diagnostic screening (*BRCA1/2* coding sequences and intron–exon boundaries screening and search for large gene rearrangements). The index cases were selected on the basis of a family history of breast cancer consisting of at least one sister with a breast cancer. The recruitment was done through the French national network of cancer genetics clinics ('Genetics and Cancer Group' of UNICANCER). The controls were female friends or colleagues of index cases matched by age (±3 years), unaffected with cancer at the time of ascertainment. Affected and unaffected sisters as well as other family members were included in the study, if available. Inclusions began in April 2007 and ended in December 2012. Information about ethnic origin was self-reported by study subjects. We considered as Caucasians all subjects with two parents reported as 'Caucasian origin'. In the present study, we analysed the subgroup encompassing the first 716 index cases and the first 619 controls included for which blood samples were available and fulfilling the GENESIS study criteria. Their characteristics are shown in Supplementary Table 1.

Ethics statement
All participants gave written informed consent and the GENESIS project was submitted to the appropriate ethics committee (CCP Ile-de-France III) on 18 September 2006 and was approved on 3 October 2006. CNIL authorization: The CNIL (French data protection Authority) has approved this study (22/05/2006).

DNA extraction
Genomic DNA has been extracted from blood samples using the DNA extractor Autopure LS (Qiagen, Courtaboeuf, France), and DNA handling (DNA normalization and aliquoting) has been done using a TECAN EVO instrument. DNA quality and purity has been evaluated by measuring absorbance on a spectrophotometer. For all DNA samples, the A260/A280 ratio has been calculated. Good-quality DNA has been obtained for the vast majority of samples (ratio of 1.7_2.0). Only subjects with good-quality DNAs have been selected for this study.

Mutation screening
MIR146A (99 nt; hg19 coordinates: chr5:159,912,359_159,912,457) and *MIR146B* (73 nt; hg19 coordinates: chr5: chr10:104,196,269_104,196,341) were amplified separately in one fragment containing 274 nt upstream and 50 nt downstream, and 233 nt upstream and 59 nt downstream of the sequence of the primary transcript (pri-miR), respectively.

We designed primers for the amplification by PCR of the 3′-UTR of *BRCA1* (1382 nt; hg19 coordinates: chr17:41,196,313_41,197,694) and of that of *BRCA2* (902 nt; hg19 coordinates: chr13:32,398,771_32,399,672) in 9 and 7 overlapping fragments, respectively.

All primers were tailed in 5′ with M13 universal sequences for subsequent sequencing reactions. All PCR fragments were screened for genetic variants using high-resolution melting (HRM) analysis, except for fragment BRCA1_4F/R that encompasses an Alu sequence (nt 580_881) difficult to analyse by HRM and was therefore sequenced. The primers' sequences, PCR elongation temperatures and size of the fragments are shown in Supplementary Table 2. The reference sequences used for the description of the variants are: NR_029701.1 (*MIR146A*), NR_030169.1 (*MIR146B*), NM_007294.3 (*BRCA1*) and NM_000059.3 (*BRCA2*).

MIR146A/B rare variants have been submitted to the Leiden Open Variation Database (LOVD 3.0 shared installation; http://www.databases.lovd.nl/shared) and those identified in *BRCA1* and *BRCA2* 3′-UTRs to the UMD-BRCA1 and UMD-BRCA2 databases, respectively, (http://www.umd.be/BRCA1/ and http://www.umd.be/BRCA2/).

Vectors
The Luc-*BRCA1* and Luc-*BRCA2* 3′-UTR wild-type (WT) vectors were constructed by cloning the 3′-UTR of *BRCA1*, amplified by PCR using forward primer 5′-<u>TCGCGACGTCC</u>TGCAGCCAGCCACAGG-3′ and reverse primer 5′-<u>GGAATTCCATATGG</u>TTTGCTACCAAAGTTTATTTGCAGTG-3′ or the 3′-UTR of *BRCA2*, amplified by PCR using forward primer 5′-<u>TCGCGACGTC</u>GTCGCATTTGCAAAGGCGAC-3′ and reverse primer 5′-<u>GGAATTCCAT ATG</u>AATCAGTGCCAATTTGAAAGC-3′, respectively. All primers contained a restriction site (underlined) upstream of the specific sequence: forward primers, Aat II; reverse primers, Nde I. The PCR fragments were each cloned between the Aat II and Nde I restriction sites in the pGL3-spacer vector directly downstream the firefly luciferase coding sequence. The integrity of the 3′-UTRs was checked by sequencing. Variants were introduced in the WT vectors using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions.

The pRL-SV40 *Renilla* luciferase vector (Promega, Charbonnières-les-Bains, France) was used as a transfection control.

All the plasmids used for transfections were prepared with the Nucleobond Xtra Midi Plus kit (Macherey-Nagel, Hoerdt, France) following the manufacturer's instructions.

Luciferase assay

HeLa, HBL-100 and MCF7 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and 1% penicillin–streptomycin, and 1 mM sodium pyruvate and non essential amino acids for MCF7 (Gibco, Cergy Pontoise, France), in a 5% CO_2 incubator at 37 °C. They were seeded at 15 000 cells per well in 96-well plates 17 h before being transfected with the plasmids encoding the firefly and *Renilla* luciferase proteins, pGL3-3′-UTR-*BRCA1* WT, pGL3-3′-UTR-*BRCA2* WT or mutant vectors (150 ng of each), using the jetPEI reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instructions. Cells were washed 24 h after transfection. Forty-eight hours post-transfection, cells were washed with 1× PBS and lysed. Firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase Assay (Promega) according to the manufacturer's instructions. Firefly luciferase expression was adjusted to *Renilla* luciferase expression to normalize for transfection efficiency.

Bioinformatics prediction

The following online programs were used to assess miRs-binding sites on the 3′-UTR of *BRCA1* and *BRCA2* and/or the potential effects of variants on miRs binding with the default parameters:

microRNA.org: http://www.microrna.org/microrna/home.do
miRcode: http://www.mircode.org/
miRDB: http://mirdb.org/miRDB/
miRmap: http://mirmap.ezlab.org/
TargetScan: http://www.targetscan.org/
RegRNA 2.0: http://regrna2.mbc.nctu.edu.tw/

The potential effects of variants on mRNA secondary structures were assessed using the following online programs:

RNAfold: http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi
RegRNA 2.0: http://regrna2.mbc.nctu.edu.tw/

RESULTS

The *MIR146A* and *MIR146B* genes and the 3′-UTR of the *BRCA1* and *BRCA2* genes were screened in a total of 1335 individuals of the French national GENESIS study: 716 breast cancer index cases, negative for *BRCA1*/*BRCA2* pathogenic variants in the coding sequence and in intron/exon junctions as well as for large rearrangements, having a sister also diagnosed with breast cancer; and 619 controls without cancer at the time of enrolment. Whenever possible, the presence of the rare variants identified was verified in affected and unaffected relatives of the index cases.

Apart from the well studied frequent variant, n.60C > G (rs2910164; dbSNP MAF not available), only one rare variant was identified in *MIR146A* in an index case, n.-143T > A (Table 1 and Supplementary Table 3). We identified four rare variants in *MIR146B*: two variants, n.-130G > C and n.-9C > T, each found in one index case, were reported for the first time, while the other two, n.-102T > C present in several cases and several controls and n.1C > T present in two controls had already been reported, albeit at different frequencies (Table 1 and Supplementary Table 3). None of the variants identified only in index cases in *MIR146A* nor in *MIR146B* affected the pre-miR sequences. As shown in Supplementary Figure 1, it has not been possible to verify if any of these variants co-segregated with the disease because no affected family member participated in the study.

Three frequent variants were detected in the 3′-UTR of *BRCA1*: c.*421G > T (rs8176318; dbSNP MAF = 0.324); c.*855delA (rs33947868; dbSNP MAF = 0.498); c.*1287C > T (rs12516; dbSNP MAF = 0.342), and three in the 3′-UTR of *BRCA2*: c.*105A > C (rs15869; dbSNP MAF = 0.161); c.*369A > G (rs7334543; dbSNP MAF = 0.222);

Table 1 Rare variants identified during the screening of the *MIR146A/B* gene coding sequences and of the 3'-UTR of the *BRCA1/2* genes in the GENESIS case/control study (grey rows highlight variants found only in screened index cases, whose pedigrees are shown in Supplementary Figure 1)

Gene name	Variant name	Variant ID	Consequences	RNA secondary structure consequences[a]	Total number of carriers in GENESIS	Frequency in GENESIS (%)	Identification in other studies
MIR146A	n.-143T>A	rs192681491	Unknown	Not applicable	1 (1 case)	0.07	Frequency of 0.02% in 1000 Genomes (0.53% in AMR)
	n.-130G>C	None	Unknown	Not applicable	1 (1 case)	0.07	Not identified
	n.-102T>C	rs77128363	Unknown	Not applicable	19 (11 cases + 8 controls)	1.42	Frequency of 0.10% in 1000 Genomes (0.5% in EUR, 0% in AFR)
MIR146B	n.-9C>T	None	Unknown	Not applicable	1 (1 case)	0.07	Not identified
	n.1C>T	rs76149940	Unknown	Not applicable	2 (2 controls)	0.07	Frequency of 1.13% in Exome Variant Server (0.01% in EURAM, 4.67% in AFAM); Frequency of 1.26% in 1000 Genomes (0% in EUR, 4% in AFR)
BRCA1 (3'-UTR)	c.*291C>T	None	Increase of luciferase activity and decrease of the HuR-mediated repression (Brewster *et al*)[21]	None	1 (1 control)	0.07	Identified in 2 cases (Brewster *et al*)[21]
	c.*713C>T	None	Slight increase of luciferase activity (our study)	Major effect	1 (1 case)	0.07	Identified in 4 cases and 2 controls (Brewster *et al*)[21]
	c.*750A>G	rs138782023	No effect on luciferase activity (Lheureux *et al*[16] and our study)	Small effect	11 (6 cases + 5 controls)	0.82	Identified in 4 cases and 2 controls (Lheureux *et al*)[16]; Identified in 8 cases and 16 controls (Brewster *et al*)[21]; Frequency of 0.22% in 1000 Genomes
	c.*780C>T	None	Slight increase of luciferase activity (our study)	Effect	1 (1 case)	0.07	Not identified
	c.*1013A>G	None	No effect on luciferase activity (our study); prediction of enhanced binding of miR-543	Effect	1 (1 case)	0.07	Not identified
BRCA2 (3'-UTR)	c.*172G>A	None	No effect on luciferase activity (our study); prediction of decreased binding of miR-323b-5p (our study)	Small effect	1 (1 case)	0.07	Not identified

Abbreviations: AMR, American ancestry; EUR, European ancestry; AFR, African ancestry; AFAM, African-American ancestry; EURAM, European–American ancestry.
[a]Predicted by RNAfold.

c.*532A>G (rs11571836; dbSNP MAF=0.197). On top of these, we also detected five rare variants in *BRCA1* 3'-UTR and one in *BRCA2* 3'-UTR, all with a MAF<0.001 in our samples (Table 1 and Supplementary Table 3). Among the five rare variants detected in the 3'-UTR of *BRCA1*, three had been detected in previous targeted screening and/or in the 1000 Genomes Project: c.*291C>T, found in one control, c.*713C>T, present in one index case and c.*750A>G, found in several cases and several controls. One index case (GE0614) carried both *BRCA1* c.*750A>G and *MIR146B* n.-102T>C. The two *BRCA1* 3'-UTR new variants identified each in one index case were c.*780C>T and c.*1013A>G. No variant was identified within or in close proximity to the miR-146a- and miR-146b-5p-binding site (nt c.*489_507). The only rare variant identified in the 3'-UTR of *BRCA2*, c.*172G>A, was identified in an index case and has never been reported before (Table 1 and Supplementary Table 3).

To evaluate the potential causality of the variants identified in index cases in *BRCA1/BRCA2* 3'-UTRs, we performed luciferase reporter assays in three human cell lines: HeLa (cervical carcinoma cells), HBL-100 (normal breast epithelial cells) and MCF7 (breast carcinoma cells). After transient transfection of luciferase reporter vectors containing either WT or mutated UTRs, we observe in some cases significant effect of the variants analysed on luciferase activity, that is, a slight increase or decrease of luciferase expression in the presence of some variants (Figure 1). However, none of these effects is seen in all the cell lines analysed and it is, therefore, difficult to extrapolate from these data to the situation in carriers, even if the results of the luciferase assays seem to be consistent from one study to another (for

Figure 1 Relative luciferase activity after cotransfection of either the Luc-*BRCA1* 3'-UTR or Luc-*BRCA2* 3'-UTR reporter vectors into HeLa, HBL-100 or MCF7 cells carrying WT or mutated sequences, as indicated. Error bars represent SEM for four (HeLa) or three (HBL-100 and MCF7) independent experiments. *P<0.05; **P<0.01; ***P<0.001 (Student's *t*-test with respect to cells transfected with WT sequences).

example, the absence of alteration of effect of luciferase expression on *BRCA1* c.*750A>G in MCF7 cells has already been observed[16]).

Potential miR-binding sites were predicted using the microRNA. org, miRcode, miRDB, miRmap and TargetScan programmes available online, as well as the interface provided by the integrated web server RegRNA 2.0 (Supplementary Figure 2). One miR-binding site was identified in the proximity of the *BRCA1* variant c.*1013A>G, which precedes the sequence complementary to the seed region of miR-543 (microRNA.org prediction, Supplementary Figure 3). The variant increases miR-543/*BRCA1* 3'-UTR complementarity, but it should be noted that the other prediction programmes failed to identify a miR-543-binding site on WT *BRCA1* 3'-UTR (the test of the mutant 3'-UTR sequence was not allowed in the programmes' design). TargetScan predicted a seed match for miR-323b-5p at position c.*172_178 of *BRCA2* 3'-UTR; c.*172G>A decreases miR-323-5p/*BRCA2* 3'-UTR complementarity, but as before, this finding was not confirmed with other algorithms (and again, it was not possible to test the mutant 3'-UTR).

Secondary structures of mRNA are important for post-transcriptional regulation as they affect binding of trans-acting factors. We, therefore, used the RNAfold secondary structure prediction programme available online to try to predict the consequences of the variants identified in the 3'-UTRs (Table 1 and Supplementary Figure 4). The *BRCA1* variant c.*291C>T (identified in a control) or c.*750A>G (identified both in breast cancer cases and controls) had no or little effect on *BRCA1* 3'-UTR predicted conformation, respectively (Supplementary Figure 4A). By contrast, the three variants identified exclusively in cases in our study, c.*713C>T, c.*780C>T and c.*1013A>G induced larger effects, particularly c.*713C>T (Supplementary Figure 4B). The *BRCA2* c.*172G>A variant had a small impact on *BRCA2* 3'-UTR predicted conformation (Supplementary Figure 4C). RNA accessibility, assessed through the integrated web server RegRNA 2.0, displayed no major change in the presence of *BRCA1* and *BRCA2* variants (Supplementary Figure 2). A small decrease of RNA accessibility was observed, though, for *BRCA1* c.*1013A>G.

The pedigrees of the families of the patients carrying the *BRCA1/BRCA2* 3'-UTR variants identified only in index cases are shown in Supplementary Figure 1. In two out of four cases, an affected sister was available and the variants, *BRCA1* c.*780C>T and c.*1013A>G, did not segregate with the disease.

DISCUSSION

Numerous studies aim to identify altered miR expression profiles in pathological *versus* normal tissues, and to analyse the impacts of *miR* gene frequent variants on the susceptibility of diseases. *MiR* gene screening, on the contrary, is seldom performed, although evidence has been provided that genetic variation in this class of genes could be linked to diseases, as for protein-coding genes.[17,18] Here, we chose to screen in familial breast cancer cases from the GENESIS study, the coding region of two *miR* genes, *MIR146A* and *MIR146B*, producing miR-146a and miR-146b-5p, respectively, as it has been shown that they regulate the expression of *BRCA1*.[12,13,19] A common *MIR146A* variant, rs2910164, which resides in the region encoding the sequence complementary to the mature miR in the stem-loop structure of the pre-miR, is one of the most extensively studied miR-related genetic variant. In the most recent meta-analysis published to date, no evidence of association between rs2910164 and breast cancer risk was obtained, although association was observed with bladder, cervical, liver and lung cancers, as well as with oral squamous cell carcinoma.[20]
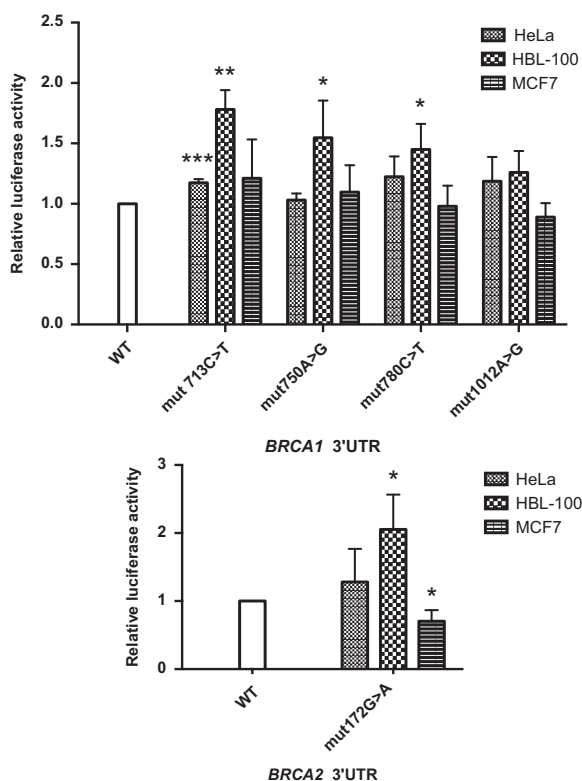
The only rare *MIR146A* variant we identified in our screened region in one index case has already been found in the 1000 Genomes Project at a comparable frequency. It falls within the intron, 90 nt upstream of the intron/exon boundary, and is, therefore, unlikely to affect *MIR146A* expression or function. The two novel rare *MIR146B* variants that we found in index cases only fall within the sequence of pri-miR-146b-5p, but we were unable to predict their potential significance, and neither were we able to study co-segregation. Search for variation in miRs expression in cases *versus* controls was unfortunately not an option, as we have not only access solely to DNA (and not to RNA), but also the only available biological specimen in the GENESIS study is blood. As miR expression is highly tissue specific, expression analyses in lymphocytes would probably not be relevant to breast and ovarian cancers.

In order to identify potential 3′-UTR-dependent mechanisms of gene dysregulation, we also screened the 1382 nt- and 902 nt-long *BRCA1* and *BRCA2* 3′-UTRs, respectively. This led to the identification of six rare variants, of which three are novel. When compiling bioinformatic predictions, the results of a functional assay and genetic data, we did not find evidence that any of these variants could dramatically impact *BRCA1* or *BRCA2* expression. It should be noted that the strong impact predicted by RNAfold of c.*713C>T on *BRCA1* 3′-UTR secondary structure is accompanied by an increase of luciferase activity in two out of the three cell lines tested; however, this latter effect is opposite to what would be expected from a variant associated with increased breast cancer risk.

A few studies examined the possibility that variants in the 3′-UTR of the *BRCA1* gene might be associated with breast cancer risk and reported the screening of this region in familial breast cancer cases and sometimes in controls as well.[16,21–25] The first published studies reported two variants: c.*36C>G, identified while screening 211 breast cancer cases,[22] and c.*372_387del16 while screening 78 breast cancer cases belonging to high-risk breast and ovarian cancer families.[25] Pietschmann *et al* screened *BRCA1/BRCA2* coding sequences and 5′- and 3′-UTRs in 10 Iranian high-risk breast cancer families.[23] They identified one rare variant in *BRCA1* 3′-UTR, c.*381_389del9ins29, which was not considered as causal as it was not present in the other four breast cancer cases in the family of the proband. Lheureux *et al* identified two novel rare variants, c.*750A>G and c.*1286C>A in 70 *BRCA1/2*-negative high-risk breast or ovarian cancer cases, with clear evidence of neutrality for the former and little evidence of causality for the latter based on luciferase reporter assays and bioinformatics predictions.[16] Brewster *et al* reported the largest study to date, with the screening of 1612 breast cancer cases and 1554 controls, sourced from five collections, and identified 23 rare variants, of which 15 were novel.[21] Using luciferase reporter assays and bioinformatics predictions, they were able to show that c.*1340_1342delTGT introduces a functional miR-103 target site and might be therefore pathogenic.[21]

Data are much scarcer concerning the 3′-UTR of *BRCA2*, as we are aware of only one small study published so far in 10 Iranian high-risk breast cancer families,[23] and 1 study performed on 100 Turkish early-onset or familial breast cancer,[26] which both did not lead to the identification of any rare variant. Our study is thus the first to report the screening of this region in a large series, which showed that 3′-UTR variants are extremely rare in *BRCA2* (only one variant found, c.*172G>A). No evidence of pathogenicity was found for this variant.

In conclusion, although several studies reported the association of *BRCA1* 3′-UTR, *miR* gene or miR-related frequent variants with breast cancer risk,[26–28] the clear involvement of rare variants has not been evidenced so far.

1 Clop A, Marcq F, Takeda H *et al*: A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet* 2006; **38**: 813–818.
2 Wojcicka A, de la Chapelle A, Jazdzewski K: MicroRNA-related sequence variations in human cancers. *Hum Genet* 2014; **133**: 463–469.
3 Chen X, Paranjape T, Stahlhut C *et al*: Targeted resequencing of the microRNAome and 3'UTRome reveals functional germline DNA variants with altered prevalence in epithelial ovarian cancer. *Oncogene* 2015; **34**: 2125–2137.
4 Feng J, Sun G, Yan J *et al*: Evidence for X-chromosomal schizophrenia associated with microRNA alterations. *PLoS ONE* 2009; **4**: e6121.
5 Blahna MT, Hata A: Regulation of miRNA biogenesis as an integrated component of growth factor signaling. *Curr Opin Cell Biol* 2013; **25**: 233–240.
6 Adams BD, Kasinski AL, Slack FJ: Aberrant regulation and function of microRNAs in cancer. *Curr Biol* 2014; **24**: R762–R776.
7 Mavaddat N, Antoniou AC, Easton DF, Garcia-Closas M: Genetic susceptibility to breast cancer. *Mol Oncol* 2010; **4**: 174–191.
8 Economopoulou P, Dimitriadis G, Psyrri A: Beyond BRCA: new hereditary breast cancer susceptibility genes. *Cancer Treat Rev* 2015; **41**: 1–8.
9 Michailidou K, Beesley J, Lindstrom S *et al*: Genome-wide association analysis of more than 120,000 individuals identifies 15 new susceptibility loci for breast cancer. *Nat Genet* 2015; **47**: 373–380.
10 Meeks HD, Song H, Michailidou K *et al*: BRCA2 polymorphic stop codon K3326X and the risk of breast, prostate and ovarian cancers. *J Natl Cancer Inst* 2015; **108**: djv315.

11 Giacomazzi J, Graudenz MS, Osorio CA *et al*: Prevalence of the TP53 p.R337H mutation in breast cancer patients in Brazil. *PLoS ONE* 2014; **9** (e99893).

12 Garcia AI, Buisson M, Bertrand P *et al*: Down-regulation of BRCA1 expression by miR-146a and miR-146b-5p in triple negative sporadic breast cancers. *EMBO Mol Med* 2011; **3**: 279–290.

13 Kumaraswamy E, Wendt KL, Augustine LA *et al*: BRCA1 regulation of epidermal growth factor receptor (EGFR) expression in human breast cancer cells involves microRNA-146a and is critical for its tumor suppressor function. *Oncogene* 2014; **34**: 4333–4346.

14 Taganov KD, Boldin MP, Chang KJ, Baltimore D: NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA* 2006; **103**: 12481–12486.

15 Sinilnikova OM, Dondon MG, Eon-Marchais S *et al*: A French national resource to study the missing heritability of breast cancer. *BMC Cancer*. (in press).

16 Lheureux S, Lambert B, Krieger S *et al*: Two novel variants in the 3'UTR of the BRCA1 gene in familial breast and/or ovarian cancer. *Breast Cancer Res Treat* 2011; **125**: 885–891.

17 Abelson JF, Kwan KY, O'Roak BJ *et al*: Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science* 2005; **310**: 317–320.

18 Dusl M, Senderek J, Muller JS *et al*: A 3′-UTR mutation creates a microRNA target site in the GFPT1 gene of patients with congenital myasthenic syndrome. *Hum Mol Genet* 2015; **24**: 3418–3426.

19 Shen J, Ambrosone CB, DiCioccio RA, Odunsi K, Lele SB, Zhao H: A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis. *Carcinogenesis* 2008; **29**: 1963–1966.

20 Nikolic ZZ, Savic Pavicevic DL, Vucic NL, Romac SP, Brajuskovic GN: Association between a genetic variant in the hsa-miR-146a gene and cancer risk: an updated meta-analysis. *Public Health Genomics* 2015; **18**: 283–298.

21 Brewster BL, Rossiello F, French JD *et al*: Identification of fifteen novel germline variants in the BRCA1 3′UTR reveals a variant in a breast cancer case that introduces a functional miR-103 target site. *Hum Mutat* 2012; **33**: 1665–1675.

22 Newman B, Mu H, Butler LM, Millikan RC, Moorman PG, King MC: Frequency of breast cancer attributable to BRCA1 in a population-based series of American women. *JAMA* 1998; **279**: 915–921.

23 Pietschmann A, Mehdipour P, Atri M *et al*: Mutation analysis of BRCA1 and BRCA2 genes in Iranian high risk breast cancer families. *J Cancer Res Clin Oncol* 2005; **131**: 552–558.

24 Pongsavee M, Yamkamon V, Dakeng S *et al*: The BRCA1 3′-UTR: 5711+421T/T_5711 +1286T/T genotype is a possible breast and ovarian cancer risk factor. *Genet Test Mol Biomarkers* 2009; **13**: 307–317.

25 Puget N, Stoppa-Lyonnet D, Sinilnikova OM *et al*: Screening for germ-line rearrangements and regulatory mutations in BRCA1 led to the identification of four new deletions. *Cancer Res* 1999; **59**: 455–461.

26 Erturk E, Cecener G, Polatkan V *et al*: Evaluation of genetic variations in miRNA-binding sites of BRCA1 and BRCA2 genes as risk factors for the development of early-onset and/or familial breast cancer. *Asian Pac J Cancer Prev* 2014; **15**: 8319–8324.

27 Khan S, Greco D, Michailidou K *et al*: MicroRNA related polymorphisms and breast cancer risk. *PLoS ONE* 2014; **9**: e109973.

28 Pelletier C, Speed WC, Paranjape T *et al*: Rare BRCA1 haplotypes including 3′UTR SNPs associated with breast cancer risk. *Cell Cycle* 2011; **10**: 90–99.

Supplementary Information accompanies this paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)