

A Genetic Variant Located in miR-423 is Associated with Reduced Breast Cancer Risk

ROBERT A. SMITH¹, DOMINIK J. JEDLINSKI^{1,2}, PLAMENA N. GABROVSKA¹,
STEPHEN R. WEINSTEIN³, LARISA HAUPT¹ and LYN R. GRIFFITHS¹

¹Genomics Research Centre, Griffith Health Institute, Griffith University, Gold Coast, QLD, Australia;

²Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland;

³Department of Pathology, Gold Coast Hospital, Southport, QLD, Australia

Abstract. *Background/Aim:* Since microRNAs (miRNAs) act as translational regulators of multiple genes, single nucleotide polymorphisms (SNP) in them can have potentially wide-ranging effects. Using an association approach, this research examined the effects of the rs6505162 SNP, an A>C polymorphism located in the pre-miRNA region of miR-423, on breast cancer development. *Materials and Methods:* Caucasian Australian women with breast cancer and controls matched for age and ethnicity were genotyped for rs6505162 and their genotypic and allelic frequencies analysed for significant differences. *Results:* Analysis indicated that there were significant differences between the case and control populations ($\chi^2=6.70$, $p=0.035$), with the CC genotype conferring reduced risk of breast cancer development (odds ratio=0.50 95% confidence interval=0.27-0.92, $p=0.03$). *Conclusion:* Further functional research is required to determine the mechanism of action of this SNP on miRNA function.

Breast cancer is currently the most common type of cancer in women in developed countries, with the lifetime risk of developing the disease at approximately 1 in 9 (1). Survival rates have been improving, but this type of cancer causes the highest number of deaths of any specific cancer in women in the developed world, with lung cancer coming second, although this may vary by region (2). Improvements in patient survival have been contributed to not only by improved treatments, but also by improved surveillance and imaging for early detection (3).

Correspondence to: Professor Lyn Griffiths, Griffith Health Institute, Gold Coast Campus, Griffith University, 4222, QLD, Australia. Tel: +61 0755528664, Fax: +61 0755529081, e-mail: L.Griffiths@griffith.edu.au

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MicroRNAs (miRNAs) are small, single-stranded RNAs of approximately 21 base pairs in length. They bind to mRNAs in the cytoplasm and in conjunction with the RNA-induced silencing complex (RISC), suppress mRNA translation (4). The suppression of protein translation by miRNAs is mediated by their degree of complementarity to target mRNAs, with higher complementarity leading to greater repression (4). This binding is usually in the 3' untranslated region (UTR) of target mRNA leading to the usual translational repression, but can also occur in the open reading frame (ORF) of the mRNA, where binding is typically perfect and results in mRNA cleavage. Because miRNA specificity is based on sequence recognition, one miRNA can potentially affect the expression of many genes to various degrees, providing control of potentially several gene pathways at once, depending on the relative expression of the miRNA and its targets (4). Indeed, miRNAs have been known to participate in the control of numerous metabolic pathways, including cellular growth and differentiation (4). Their participation in these pathways combined with their ability to target multiple genes means that single nucleotide polymorphisms located within miRNAs can have extremely far reaching effects and may affect the development of multiple diseases, including breast cancer.

miR-423 is a miRNA whose pre-miRNA contains two mature transcripts, named miR-423-3p and miR-423-5p, which are at the 3' and 5' ends of the pre-miRNA, respectively (5). miR-423 is located on chromosome 17 and lies within the first intron of the gene nuclear speckle splicing regulatory protein (*NSRPI*), which is involved in alternate splicing of mRNAs (6). miR-423-3p was originally detected in 2004 in untreated HL-60 leukaemia cells during 2-O-tetradecanoylphorbol-13-acetate induced differentiation experiments, indicating that the mature miRNA is involved in differentiation processes (7). miR-423-5p was first identified as a mature miRNA in 2008 in neuroblastoma tissues and cell lines following RNA cloning and confirmed by northern blot (8). Altered expression of both of the mature

miRNAs produced from miR-423 has been reported in multiple cancer types, including mesothelioma, head and neck cancer, and breast cancer, as well as in psoriasis lesions (9-12). Not all researchers have specified which mature miRNA they discussed, but miR-423 was found to be reduced in expression in mesothelioma and psoriasis lesions, and increased in head and neck squamous cell carcinomas. In breast cancer, miR-423 was characterized as being of low expression by Farazi *et al.*, but their work also indicated that both mature forms of miR-423 were highly expressed in infiltrating ductal carcinomas of women who later went on to develop metastasis, being also an independent prognostic indicator of disease (12).

It is because of the relationship between miR-423 expression and cancer as well as with breast cancer specifically, that we undertook this study into the rs6505162 SNP, which is located within the pre-miR-423. The goal of this research was to determine if the rs6505162 SNP affects the risk of breast cancer development.

Materials and Methods

Study population. Our study population consisted of 193 Caucasian women with breast cancer, and an equal number of controls, matched for age (within 5 years), sex and ethnicity. Breast cancer cases had no known family history of breast cancer to eliminate any effect of familial mutations. Average ages of the affected and control populations were 58 ± 12 and 57.5 ± 11.6 years, respectively. The study population was recruited in collaboration with the Gold Coast Hospital and all cases and controls underwent informed consent. The study was approved by the Ethics Committees of the Gold Coast Hospital and Griffith University.

Detection of rs6505162 SNP genotypes. Allelic detection for rs6505162 was undertaken using high resolution melt (HRM). HRM for this research was performed using basic polymerase chain reaction (PCR) with SYTO-9 (Invitrogen, Melbourne, Australia) as the detection dye. The PCR reaction mix used consisted of 1 unit of Go-Taq DNA polymerase (Promega, Madison, WI, USA), 1X Go-Taq PCR Buffer, 1.5 mM of $MgCl_2$, 0.3 μM each of the forward and reverse primers, 0.4 mM dNTPs, 1.5 μM SYTO-9 (Invitrogen, Melbourne, Australia) and 40 μg of template DNA in a 25 μl reaction volume. The amplicon produced for this PCR was 137 bp long; the forward primer sequence was 5'-GGGCAGAGAGCGAGACTTT-3' and the reverse primer sequence was 5'-GGAAGCCAGGAAGTGTCTCT-3'.

Thermal cycling took place in a Qiagen RotorGene PCR instrument (QIAGEN, Doncaster, VIC, Australia) and consisted of an initial denaturing at 95°C for 3 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds. Melting took place from 79°C to 89°C in 0.1°C increments, with a 2 second hold at each melt step. Each experimental set included a negative template control, as well as positive control samples of each genotype to allow more accurate genotyping. Genotypes were determined by variation in melting profiles as demonstrated in Figure 1. Samples with ambiguous genotypes were re-tested and those which could not be clearly genotyped from retesting were discarded. A selection of

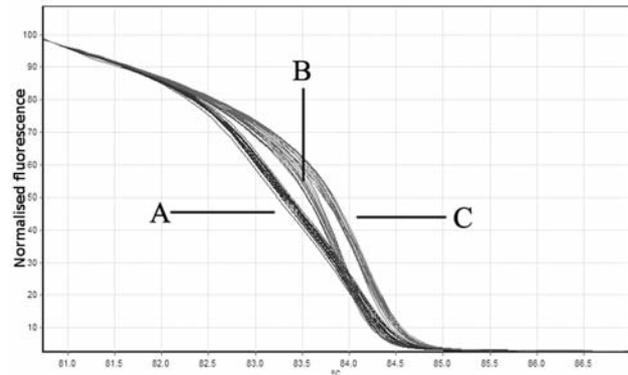


Figure 1. Example of normalised melt curve for rs6505162. This figure shows the variation in the melting profile of each genotype of the rs6505162 single nucleotide polymorphism obtained in this study. Following normalisation of fluorescence, genotypes can be determined by variation in the shape of the melting curve produced. Samples labelled A are AC genotype, samples labelled B are AA genotype and samples labelled C are CC genotype.

randomly selected samples from each genotype underwent Sanger sequencing to verify the results of the HRM.

Analysis. Analysis of data obtained from HRM was carried out using a Chi-square test. In addition, both cancer and control populations were tested for Hardy Weinberg equilibrium, and the relative effect of each genotype on breast cancer risk was determined using and odds ratio calculation.

Results

Following completion of all PCRs, HRM genotyping and sequencing verification, 179 cases and 174 controls were accurately genotyped. The results obtained for our population along with the initial Chi-square analysis are summarised in Table I. Both populations proved to be in Hardy Weinberg equilibrium ($p=0.155$ and 0.307 for cases and controls, respectively).

Our results indicated that the breast cancer population showed an increased incidence of the A allele for rs6505162 compared to the control population. Our results also indicated a shift in genotypic frequencies in the breast cancer population compared to the controls, showing an increase in AA and AC genotypes and a decrease in CC genotype. This difference was determined by Chi-square analysis to be significant ($p=0.035$). Following this determination, we performed an odds ratio calculation to determine which genotype was exerting the effect on breast cancer risk. The results of the tested odds ratio models can be seen in Table II.

Odds ratio calculations determined that, compared to the AA genotype, the CC genotype conferred a reduced risk of breast cancer development. Heterozygotes for rs6505162 bore no significant change to their breast cancer risk.

Table I. Genotypes and Chi-square analysis results for rs6505162 single nucleotide polymorphism.

Group	Frequency	Allelic frequency		Genotypic frequency			Total
		A	C	AA	AC	CC	
Breast cancer	N	215	143	60	95	24	179
	%	60.06	39.94	33.52	53.07	13.41	
Control	N	184	164	52	80	42	174
	%	52.87	47.13	29.89	45.98	24.14	
		$(\chi^2=3.70, p=0.054)$		$(\chi^2=6.70, p=0.035)$			

Discussion

Our results indicate that the CC genotype of the rs6505162 SNP, located in the pre-miR-423 offers a reduced risk of breast cancer development. Most research to date on miR-423 has consisted of expression analyses, where altered expression of both mature forms of the miRNA has been seen in cancer, as well as during cellular differentiation and psoriasis (7-12). The potential for a SNP in the microRNA to affect the development of cancer is thus plausible, however, rs6505162 is not located in either of the mature miRNAs produced by miR-423, being located in the pre-miRNA only, 12 base pairs 5' of miR-423-5p. This would indicate that the SNP does not affect mature miRNA binding to target mRNAs, and thus does not affect the expression of these genes. Research on other SNPs in pre-miRNA sequences has shown that polymorphisms in both pri- and pre-miRNAs that are not present in the mature sequence can influence the expression of mature forms, as well as the binding of nuclear factors involved in miRNA processing (13-16). Based on the effects of pre-miRNA SNPs from other miRNAs, it is possible that rs6505162 affects the expression or processing of miR-423. There are, however, no studies examining the effect of this SNP in miRNA functionality, so we cannot be sure of its true significance.

Despite this lack of research into the effects of the SNP, there has been previous research into the effect of the SNP in cancer susceptibility, specifically in esophageal, breast and ovarian cancer. The first of these studies, published in 2009 by Ye *et al.*, was conducted on esophageal cancer using a population of 346 Caucasian esophageal cancer patients and produced data similar to our own, with the C allele of rs6505162 being significantly lower in controls compared to cancer patients (17). No specific genotype effects were reported by Ye *et al.* but their analysis indicated that the most likely model for the effect of the SNP was additive. In addition to producing similar allele frequencies to those

Table II. Odds ratio (OR) calculation for effects of rs6505162 genotype.

Cases/controls	OR (95% confidence interval)		
179/174	AC versus AA 1.03 (0.64-1.66) $p=0.91$	CC versus AA 0.50 (0.27-0.92) $p=0.03$	AC/CC versus AA 0.85 (0.54-1.32) $p=0.46$

obtained in our research, Ye *et al.* also found a similar risk reduction to that in our research, with the C allele offering an odds ratio of 0.57 (95% CI 0.44-0.73).

In contrast to this, the only other study on rs6505162 risk in cancer, undertaken in 2009 by Kontorovitch *et al.*, indicated that the AA genotype of rs6505162 offered a reduced risk of developing both ovarian and breast cancer in Breast Cancer Associated 2 (*BRCA2*) mutation carriers (18). They also noted that the AA genotype resulted in a significantly later age at presentation for patients with ovarian cancer. The population used by Kontorovitch and colleagues was significantly different to those used by our research and that of Ye *et al.*, consisting of populations of Jewish carriers of *BRCA1* and *BRCA2* mutations, the majority of the latter being Ashkenazi Jews. The different genotype effects noted by Kontorovitch *et al.* may reflect interactions between *BRCA2* mutations and miR-423, or may reflect the relative effects of underlying variation in Ashkenazi Jews.

Our research indicated that the CC genotype of the rs6505162 SNP reduces the risk of sporadic breast cancer development. Our population is relatively small, however, and it is possible that we lacked the population size necessary to detect a similar effect for the C allele in general. This is made somewhat more likely by the results of Ye *et al.*, who not only detected a role for the C allele alone in esophageal cancer risk, but determined that an additive model is the most plausible mode of action for the SNP. While we have no known functional effect for the SNP as yet, there is the potential for the C allele of the SNP affecting the expression or processing of the mature miR-423 sequences. Future research should examine this possibility. Based on the results of Kontorovitch *et al.*, it may also be a fruitful line of investigation to examine how rs6505162 interacts with *BRCA2* mutations, as this may also have implications for the effects of miR-423 in breast cancer progression, particularly in the development of metastatic disease.

As a final aside, there is another miRNA present on the DNA complementary to miR-423. This complementary miRNA is miR-3184, which has been detected as being differentially expressed in an miRNA profile of melanomas (19). The miR-3184 pre-miRNA is slightly smaller than that of miR-423 (by 12 base pairs 3' and 7 bases 5'), but perfectly overlaps it. This would place rs6505162 in the pri-

miR-3184, and it is possible that some of the effects for this SNP may be mediated through miR-3184 instead of miR-423, hence future research should simultaneously examine both miRNAs.

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