Single Nucleotide Polymorphism in *hsa-mir-196a-2* and Breast Cancer Risk: A Case Control Study

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microRNAs are small, non-coding RNAs that influence gene expression on a post-transcriptional level. They participate in diverse biological pathways and may act as either tumor suppressor genes or oncogenes. As they may have an effect on thousands of target mRNAs, single-nucleotide polymorphisms in microRNA genes might have major functional consequences, because the microRNA's properties and/or maturation may change. *miR-196a* has been reported to be aberrantly expressed in breast cancer tissue. Additionally, the SNP rs11614913 in *hsa-mir-196a-2* has been found to be associated with breast cancer risk in some studies although not in others. This study evaluated the association between rs11614913 and breast cancer risk in a Caucasian case-control cohort in Queensland, Australia. Results do not support an association of the tested *hsa-mir-196a-2* polymorphism with breast cancer susceptibility in this cohort. As there is a discrepancy between our results and previous findings, it is important to assess the role of rs11614913 in breast cancer by further larger studies investigating different ethnic groups.

∎ **Keywords:** breast cancer, micro-RNA, cancer risk, SNP, rs11614913

Background

Worldwide, breast cancer is the leading cause of cancer-related deaths in women (American Cancer Society, 2007). Several genes, including the well-characterized BRCA1 and BRCA2, are known to be involved in breast carcinogenesis (Campeau et al., 2008; Loman et al., 2001). Although research continues on known genes to further understand breast cancer development, there has also been emerging interest in researching epigenetics and gene regulation. One of the most surprising recent progresses in understanding the mechanisms of gene regulation has been the discovery of microRNAs (miRNAs) (Wightman et al., 1993). miRNAs are short, single-stranded RNAs of ~22 nucleotides that do not code for proteins themselves. miRNAs alter gene expression at a post-transcriptional level, regulating the amount of protein expressed from coding RNAs. Their regulatory effect is based on base pairing with mRNAs at the 3'-untranslated region, resulting in translational repression, or at the open reading frame, resulting in cleavage and degradation of the mRNA (Verghese et al., 2008). It has been reported that a single miRNA potentially binds to hundreds of mRNA targets and thus might down-regulate genes with different functions (Lim et al., 2005). It is estimated that one third of human genes are potential targets of miRNAs (Esquela-Kerscher & Slack, 2006). miRNAs are believed to play an important role in numerous biological processes, including development, metabolism, cell proliferation, differentiation and apoptosis (Davalos & Esteller).

Recent evidence indicates that miRNAs may also act as tumor suppressors and oncogenes, depending on which gene(s) they modulate (Zhang et al., 2007). miRNAs that negatively regulate tumor suppressor genes, or genes that control cell differentiation and apoptosis, may function as oncogenes if over-expressed in cancers. On the other

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hand, miRNAs that act as tumor suppressor genes by down-regulating oncogenes may lead to loss of oncogenic regulation if under-expressed in cancers (Zhang et al., 2007). Altered miRNAs, either in terms of structural changes or in the quantity of mature miRNAs, have been linked to cancer risk (Esquela-Kerscher & Slack, 2006). A recent paper identified a general decrease of mature miRNA levels in different kinds of human cancers compared with normal tissues (Lu et al., 2005). In breast cancer several miRNAs, including miR-196a, have been observed to be aberrantly expressed in breast cancer tissue compared with normal breast tissue (Iorio et al., 2005). miR-196a was highly expressed in breast cancer tissue (Iorio et al., 2005). It is plausible that variations such as mutations or single nucleotide polymorphisms (SNPs) in miRNAs which affect expression levels may play a role in breast cancer development.

SNPs in miRNA genes may alter miRNA expression and/or function, because miRNA binding to its mRNA target is dependent on sequence complementarity with the target. The SNP rs11614913 C>T in *hsa-miR-196a-2* has been implicated in lung cancer susceptibility (Tian et al., 2009). Two studies have also recently reported that this variant might be linked to breast cancer susceptibility (Hoffman et al., 2009; Hu et al., 2009). These two case-control studies demonstrated that the C-allele was significantly associated with increased breast cancer risk in both a Chinese population (Hu et al., 2009) and a US population (Hoffman et al., 2009). To build upon these findings, this study investigated whether there is an association between rs11614913 C>T and breast cancer susceptibility in a case-control cohort from Queensland, Australia.

Materials and Methods

Study Cohort

The study utilized 193 females diagnosed with breast cancer and 190 controls. The affected and control populations were matched for age and all were of Caucasian ethnicity as previously described (Curran et al., 2002). Briefly, this consisted of participant reporting of parental and grandparental ethnicity. The exclusion criteria for both included previous cancer or a family history of cancer, as determined by participant reporting. No other risk factors were controlled for. Samples were recruited through collaboration with the Gold Coast Hospital. Additional affected samples, as well as the entire control population, were obtained through the Genomics Research Centre (GRC) at Griffith University through volunteers responding to advertisements. Samples consisted of peripheral blood taken by GRC of hospital staff. All patients gave informed consent. The study was conducted under the approval of the Gold Coast Hospital and Griffith University Ethics Committees.

Genotyping

The SNP in this study was indentified in a previous study by Hu et al. (2009) and genotyped using a Polymerase chain reaction (PCR)-restriction length polymorphism assay. SNP analysis was carried out using GoTaq DNA Polymerase (Promega, USA) and *Hpy188I* (New England Biolabs, USA). The assays were carried out following the instructions from the company. The sequences of the forward primer and the reverse primer were 5'-GGGCTGAATTTCTTCCTTCC-3' and 5'-CTCGACGAAAACCGACTGAT-3', respectively. PCR amplification was performed in a 24-µl reaction mixture containing 60ng genomic DNA, 0.2µM each primer, 0.33mM dNTPs, 1X GoTaq buffer, 5.42 mM $MgCl_2$ and GoTaq polymerase. The reaction conditions were as follows; 95°C for 2 minutes, and 35 cycles of 95°C for 45 seconds, 59°C for 1 minute, and 72°C for 45 seconds, followed by 7 min at 72°C. Following amplification, 10µl of product was digested with the restriction enzyme *Hpy188I* at 37°C for 4 hours. Samples were then loaded into an ethidium bromide-stained 3% agarose gel for genotype determination.

To assure the quality of genotyping, the RFLP assay was performed in a 96-well PCR plate, each containing three positive controls of DNA samples with known genotypes (CC, CT, TT). The concordance rate was 100%. DNA quality or quantity of 6 breast cancer samples and 19 control samples was insufficient for genotyping. Therefore the final analysis included 187 cases and 171 controls.

Statistical Analysis

To determine whether any significant differences in polymorphism frequencies occurred between the case and control population, allele and genotype frequencies were compared using the chi-square method. Both cases and controls were also analyzed to determine if they were in Hardy-Weinberg Equilibrium. Furthermore, odds ratios (ORs) with their 95% Confidence Interval (CI) were calculated to evaluate the potential association between rs11614913 and breast cancer susceptibility.

Results

We genotyped rs11614913 in both the affected group and the control group. The frequencies of the genotypes and alleles are listed in Table 1. The CC genotype was slightly more common in the breast cancer population, but in general both populations showed a similar distribution of genotypes. Chi-Square analysis of this data showed that no significant difference was observed between the breast cancer group and the control group samples for genotypes ($\chi^2 = 0.24$, $P = 0.888$) or for alleles ($\chi^2 = 0.16$, $P = 0.691$). Hardy-Weinberg equilibrium analysis was performed to exclude the possibility of experimental artefacts. We found that both cases ($\chi^2 = 0.41$, $p = .52$) and controls ($\chi^2 = 0.05$, $P = 0.83$) were in HWE. ORs were calculated (Table 2) and showed no association of rs11614913 with breast cancer susceptibility.

TABLE 1

Analysis Results, Allele and Genotype Frequencies for Tested SNP

rs11614913		Allele frequency		Genotype frequency			
Group	Frequency	C	T	CC	CT	TT	Total
Breast Cancer	N	222	152	68	86	33	187
	%	59.36	40.64	36.36	45.99	17.65	
Control	N	198	144	58	82	31	171
	%	57.89	42.11	33.92	47.95	18.13	
		(χ^2 = 0.16, P = .691)		(χ^2 = 0.24, P = .888)			

Since our cohort was comprised of Caucasians, we compared our control genotype frequencies for rs11614913 with those in a European population (HapMap) and found that the two populations corresponded closely (Table 3).

Discussion

Hoffman et al. showed in a cell based assay, that the variant allele T had an effect on *miR-196a* levels in vitro (Hoffman *et al.*, 2009); they were decreased compared with *miR-196a-C*. Furthermore they demonstrated in a whole-genome expression microarray (using RNA isolated from cells transfected with a *pre-miR-196a-C, pre-miR-196a-T,* and an empty vector) that *miR-196a-C* had a significantly greater impact on gene expression than *mir-196a-T*, suggesting that the variant has a diminished regulatory capacity (Hoffman et al., 2009). Among the genes that were differently expressed after enforced expression of *pre-miR-196a-C* were several potential tumor suppressors and oncogenes (Hoffman et al., 2009). According to this data it is plausible that *miR-196a* may have oncogenic properties.

A recent meta-analysis evaluated the association between rs11614913 *hsa-miR-196a-2* and breast cancer risk and included four studies with four different cohorts (Gao et al., 2011). The meta-analysis showed that rs11614913 polymorphisms significantly increased breast cancer risk for the C allele in the homozygote comparison (CC versus TT, OR = 1.30; 95% CI, 1.01-1.68) and the dominant model (CC versus CT/TT, OR = 1.11; 95% CI, 1.01-1.23) (Table 2).

In this case-control study of breast cancer in Caucasian women, we found that the common SNP rs11614913 was not associated with increased risk of breast cancer. Although the CC genotype frequency was higher in the breast cancer population, the ORs were not significant (CC versus TT, OR = 1.10; 95%CI, 0.60–2.01) and (CC versus CT/TT, OR = 1.11; 95% CI, 0.72–1.72) (Table 2). No significant *P*-values were found.

Our data contradict the findings of Hu et al. (Hu et al., 2009) and Hoffman et al. (2009), who both found a significant association between rs11614913 and breast cancer risk. The discrepancy between our results and these might be explained by population-specific factors, such as different genetic background, lifestyle, or environmental factors. For instance, the allelic frequency of rs11614913 is different between Han Chinese and Caucasian populations (Table 3). Whereas in the Chinese controls studied by Hu et al. (2009) the minor allele frequency of rs11614913 was 0.44 (C allele), the C allele was the common one in our Caucasian controls with a frequency of 0.58. In the case of Hoffman's study the participants were not matched for ethnicity. Although the population was predominantly Caucasian, ethnical diversity may have been crucial for the outcome of the study and certainly there seem to be ethnic differences in the allele frequencies associated with this SNP (Table 3). The results of Hoffman et al. did hold when only Caucasians were considered, but we do not know whether minor ethnic differences between our and their study may still be influencing the outcome. An additional and perhaps more important factor is that most of Hoffman's controls were diagnosed with benign breast diseases, which might be providing a subpopulation of controls with higher resistance to malignant transformation as opposed to dysplasia, influencing the outcome,

TABLE 2

Results of ORs in the Meta-Analysis and our Caucasian Population

Polymorphism	Cases/controls	OR (95% Confidence Interval)			
		CC vs. CT	CC vs. TT	CC vs. CT/TT	CC/CT vs. TT
meta-analysis rs11614913 (Gao et al., 2011)	3287/4298	1.08 (0.97–1.19)	**1.30*** **(1.01–1.68)**	**1.11*** **(1.01–1.23)**	1.22 (1.00–1.50)
Queensland rs11614913	187/171	1.12 (0.70–1.78)	1.10 (0.60–2.01)	1.11 (0.72–1.72)	1.03 (0.60–1.78)

Note: *Bold-faced values indicate significant difference at α = 0.05 level.

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TABLE 3

Genotype and Allele Frequencies of SNP rs11614913

Population	Genotypes			Allele frequencies	
	CC	CT	TT	C	T
European*	0.310	0.500	0.190	0.56	0.44
Asian Han Chinese*	0.273	0.432	0.295	0.49	0.51
Asian Japanese*	0.156	0.422	0.422	0.37	0.63
Sub-Saharan African*	0.733	0.250	0.017	0.86	0.14
Han Chinese controls Hu et al. (Hu et al., 2009)	0.199	0.473	0.328	0.44	0.56
Hoffman et al. controls (Hoffman et al., 2009)	0.356	0.491	0.152	0.60	0.40
Italian controls Catucci et al. (Catucci et al., 2009)	0.428	0.443	0.130	0.65	0.35
German controls Catucci et al. (Catucci et al., 2009)	0.390	0.465	0.144	0.62	0.38
Caucasian controls Queensland	0.339	0.480	0.181	0.58	0.42

Note: * Control populations from HapMap, data from http://www.ncbi.nlm.nih.gov

particularly if rs11614913 can affect malignant transformation as their own results suggest.

On these lines, it is possible that genetic variants in the genes that *miR-196a* targets may contribute to its ability to influence breast cancer risk. Altered sequence in target mRNAs, including SNPs previously overlooked for being non-amino acid changing may enhance or reduce the ability of the *miR-196a* to block translation. Differences in SNP frequency between our population and those of Hu et al. and Hoffman et al. may explain why this study did not detect any association with breast cancer risk. Our population size is also smaller than both studies and it may also be that the effect of the rs11614913 SNP on breast cancer risk is relatively weak and thus a larger sample size may be needed to detect it.

However, our data do correspond well with the findings of Catucci et al. (2009), who genotyped an Italian and a German cohort, comprised of 2,003 and 2,651 participants, respectively. According to their data, there was a lack of association between rs11614913 and breast cancer risk. In terms of genotype frequencies both of these populations were similar to our Australian cohort, as the C-allele was the predominant allele. As both of the sub-populations in the Catuccu et al. study were larger than that of Hoffman et al., it is likely that the risk profile for rs11614913 is complex and that, despite the smaller size, this study reinforces that possibility.

Although our results did not suggest any association of rs11614913 in *miR-196a* with breast cancer risk, there has been evidence that *miR-196a* may play an important role in carcinogenesis. Potential targets of *miR-196a* have been identified, among them are genes that participate in diverse cancer-related pathways (Yekta et al., 2004); The cleavage of *HOX* gene products like *HOXB8* mRNA was shown to be partly the result of *miR-196a* (Yekta et al., 2004). *HOX* genes regulate several genes that in turn regulate large networks of other genes, thus are involved in several crucial biological processes. In addition, *LSP1* and *TOX3* are potential targets of *miR-196a* as determined by the mirBase database (http://www.mirBase.org). These two are novel breast cancer susceptibility genes that have been recently discovered in a whole-genome association study (Easton et al., 2007). The roles of these genes in breast cancer development remain to be investigated, but the rs11614913 SNP may modulate that role.

Conclusion

miRNAs play an important role in many biological processes. A better understanding of their pathways, their mechanisms and their targets could lead to progress in breast cancer prognosis, classification, and treatment. At present, little is known about whether SNPs in miRNAs can alter breast cancer risk. Therefore, future characterisation of SNPs of miRNAs may help to investigate their contribution to cancer development.

Our data suggested lack of association between SNP rs11614913 in *hsa-mir-196a-2* and breast cancer risk. As there is a discrepancy between our results and previous findings, it is important to assess the role of rs11614913 in breast cancer by further larger studies investigating different ethnic groups. Functional studies should also attempt to more closely probe the mechanisms behind *hsa-mir-196a-2* function to help inform good recruiting methodologies for future susceptibility studies.

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