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Investigation of Two Wnt Signalling Pathway Single Nucleotide Polymorphisms in a Breast Cancer-Affected Australian Population

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In the mammary gland, Wnt signals are strongly implicated in initial development of the mammary rudiments and in the ductal branching and alveolar morphogenesis that occurs during pregnancy. Previously, we identified two Wnt signaling pathway-implicated genes, *PPP3CA* and *MARK4*, as having a role in more aggressive and potentially metastatic breast tumors. In this study, we examined two SNPs within *PPP3CA* and *MARK4* in an Australian case-control study population for a potential role in human breast cancers. 182 cases and 180 controls were successfully genotyped for the *PPP3CA* SNP (rs2850328) and 182 cases and 177 controls were successfully genotyped for the *MARK4* SNP (rs2395) using High Resolution Melt (HRM) analysis. Genotypes of randomly selected samples for both SNPs were validated by dye terminator sequencing. Chi-square tests were performed to determine any significant differences in the genotype and allele frequencies between the cases and controls. Chi-square analysis showed no statistically significant difference ($p > .05$) for genotype frequencies between cases and controls for rs2850328 ($\chi^2 = 1.2$, $p = .5476$) or rs2395 ($\chi^2 = .3$, $p = .8608$). Similarly, no statistical difference was observed for allele frequencies for rs2850328 ($\chi^2 = .68$, $p = .4108$) or rs2395 ($\chi^2 = .02$, $p = .893$). Even though an association of the polymorphisms rs2850328 and rs2395 and breast cancer was not detected in our case-control study population, other variants within the *PPP3CA* and *MARK4* genes may still be associated with breast cancer, as both genes are implicated with processes involved in the disease as well as their mutual partaking in the Wnt signaling pathway.

■ **Keywords:** PPP3CA, MARK4, SNPs, association studies, genotyping, breast cancer risk, high resolution melt analysis, Dye terminator DNA sequencing

It has been well documented that Wnt signaling has a role in the regulation of cell fate in development and cell proliferation, morphology, polarity, migration, apoptosis and differentiation in a variety of tissue settings (Cadigan & Nusse, 1997). Using microarray analysis, we have previously (Gabrovska et al., 2011) identified two Wnt signaling pathway-implicated genes, *MARK4* and *PPP3CA*, as having a role in more aggressive and potentially metastatic human breast tumors. Gene expression profiles obtained via microarray analysis were replicated in Q-PCR analyses of the original tumor samples and continued to demonstrate a similar significant trend of expression in an extended sample population. We next decided to investigate functional SNPs within both of these genes for association in an Australian Caucasian case control breast cancer population.

In the mammary gland, Wnt signals are strongly implicated in initial development of the mammary rudiments, and in the ductal branching and alveolar morphogenesis that occurs during pregnancy (Brennan & Brown, 2004). In human breast cancer, evidence of β-catenin accumulation implies that the canonical Wnt signaling pathway is active in over 50% of carcinomas; however, specific mutations that might account for this activation have not yet

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been identified (Brennan & Brown, 2004). Although Wnt genes were initially discovered as oncogenes that contribute to mammary tumorigenesis in naturally occurring mouse models of breast cancer, definitive evidence linking Wnt signaling to human breast cancer has been slow to emerge (Howe & Brown, 2004). The pathway itself offers ample targets for cancer drug target development.

MARK4 or MAP/microtubule affinity-regulating kinase 4, located on chromosome 19, was first isolated in 2001 by Kato et al. (2001) (calling it *MARKL1*) when it was found to be involved in human hepatocellular carcinogenesis with its expression correlating to the activity of the β-catenin/Tcf complex in HepG2 cells, therefore associating it with the Wnt signaling pathway. The MARK protein kinases were originally identified by their ability to phosphorylate a serine motif in the microtubule-binding domain of *tau* (proteins that stabilize microtubules) that is critical for microtubule binding (Trinczek et al., 2004). *MARK4* is predominantly expressed in the brain and readily phosphorylates *tau* and the related *microtubule associated protein 2 (MAP2)* and *MAP4* (Trinczek et al., 2004). Over expression of *MARK4* results in a thinning of the microtubule network, concomitant with a reorganisation of microtubules into bundles (Ebneth et al., 1999). *MARK4* is distinct from the other members of the MARK kinase family, in that it associates with microtubules, centrosomes, and neurite-like processes of neuroblastoma cells (Trinczek et al., 2004). In 2001 it was reported by Sun et al. (2001) that Drosophila PAR-1 also functions as a molecular switch by phosphorylating disheveled, thus enabling it to transduce the Wnt signal to β-catenin, linking polarity regulation by MARK kinases to molecular pathways that involve β-catenin in cancer (Kinzler & Vogelstein), neurodegeneration, and apoptosis (Zhang et al., 1998). *MARK4* has been shown to be down-regulated in response to decreased TCF/LEF1 activity (Kato et al., 2001). This feature suggests that it may play a role as a messenger in the Wnt-signaling pathway in humans.

PPP3CA has many alternative names but it is best known as *CCN1* (calcineurin 1). The Ca^{2+}/calmodulin-regulated protein phosphatise located on chromosome 4, was first detected in skeletal muscle and brain (Guerini & Klee, 1989). Enhanced expression of the Calcineurin family of secretory integrin-binding proteins has been correlated with multiple cancer-associated events, including tumor cell adhesion, proliferation, invasion and migration. Consequently, *PPP3CA* expression has been demonstrated to be elevated in various cancers, including breast cancer, with its expression directly correlated to patient prognosis (Dash et al., 2010). *PPP3CA* is also thought to play diverse roles in survival and angiogenesis (Dash et al., 2010). In breast cancers, over expression of *PPP3CA* in MCF-7 human breast cancer cells has been shown to u*p*-regulate MAPK and NFkB signaling in an integrin αvβ3-dependent manner, promoting cell survival and chemoresistance (Vellon et al., 2005) as well as to promote cell growth, migration and angiogenesis in vivo (Tsai et al., 2002). Additionally, elevated levels of *PPP3CA* gene expression has been demonstrated both in vitro and in vivo in highly aggressive breast cancer cell lines and in samples from patients with more advanced stages of malignancy (Xie et al., 2001). Furthermore, *Cyr61*, another member of the CCN family, is thought to be an essential mediator of estrogen and progesterone-stimulated growth of breast cancer cells and that the diverse functions of the gene indicated it may play a role at multiple stages of breast cancer development (Sampath et al., 2001). It is possible that members of the same family may have similar functions, thus implicating *PPP3CA* in mediation of estrogen and progesterone-stimulated growth of breast cancer cells.

For *MARK4*, the potentially functional rs2395 SNP located in the UTR-3' region was chosen, while for *PPP3CA* the potentially functional rs2850328 SNP located in the near -gene-5' region, specifying that the variation is within 2000 bases of the gene, but not in the transcript, was selected. Both SNPs are potentially functional as they are both in regions where microRNAs, transcription factors and chaperone proteins are often bound. While there is no published data about their functionality, the locations of both SNPs are in regions that are commonly bound and therefore worth further investigation. These particular SNPs were selected based on their high minor allele frequencies (MAF) from NCBI which were high enough so that linked effects from nearby SNPs could be detected and so appropriate statistical analysis could be performed. Here, we used HRM analysis and dye terminator sequencing (for validation) to examine these two SNPs in an Australian case-control study population for a potential role in human breast cancers.

Materials and Methods

Study Cohort

The population was recruited from the Gold Coast Hospital, Southport, and all samples were obtained from patients residing in the South East Queensland Region. The matched control samples were obtained via the Genomics Research Centre Clinic, Southport, with the research approved by Griffith University's Human Ethics Committee (Approval: MSC/07/08/HREC). The population comprised of healthy age/sex/ethnicity (Caucasian) matched controls certified as having no history of personal or familial cancer. The case-control population consisted of 188 cases and 182 healthy matched controls of which 182 cases and 180 controls were successfully genotyped for the *PPP3CA* SNP and 182 cases and 177 controls were successfully genotyped for the *MARK4* SNP. The remaining samples proved difficult to amplify efficiently or were not able to be definitively categorized during analysis. The mean age and median age of both

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populations were 57.52 years (*SD* 11.6) and 57 years (*SD* 11.4), respectively. Genomic DNA was extracted from peripheral blood using a standard salting out procedure as previously described (Lea et al., 1998). The DNA was then diluted down to a working concentration of 20ng/μL and genotyped using High Resolution Melt (HRM) analysis. A proportion of randomly selected samples of each genotype were then validated by Sanger sequencing using the Big Dye terminator sequencing reaction (Applied Biosystems) as described by the manufacturer.

Genotyping

All samples were genotyped using HRM with pre-amplification. The primers used for the *PPP3CA* SNP (rs2850328) were taken from the Chiocco et al. (2010) study with the following sequences: forward–CTCGCGTTCATTGGCTAGAG and reverse–GCAAAAGTGACACCTGAGCA. The primers used for the *MARK4* SNP (rs2395) were designed using Primer Express 3.0 (Applied Biosystems) and had the following sequences: forward- GCAGCCTGTTGCCCAATAA and reverse- TGGATTCCCACGCCTCTTC. For both variants, HRM was performed in a 25ul reaction containing: 1x buffer (Promega), 750nM of each primer, 75mM $MgCl_2$ (Promega), 5U HotStart Taq (Promega), 2.5mM final dNTPs, 40uM SYTO-9 (Invitrogen) and 40ng of DNA template. HRM analysis was then performed on a Rotor-Gene 6000 thermal cycler (Qiagen/Corbett Life Science, Australia) using the following thermal conditions: 1 cycle of 95°C for 3 min, 45 cycles of 95°C for 5 s, 60°C for 10 s (acquiring on Green). Following that, a HRM step was performed at (79–92°C for the PPP3CA SNP and 75–88°C for the MARK4 SNP) 2s/increment and one acquisition increment per 0.3°C. Data was resolved and analyzed on the Rotor-Gene 6000 Platform. Genotypes were determined by interpreting shifts in the amplification profiles and product melts of the samples using the Rotor-Gene software. The genotype results for both SNPs were validated using an ABI-3130 genetic analyzer (Applied Biosystems). The detailed genotyping method involving the sequencing for the samples used in this study has been previously published in Lea et al. (2004). Briefly, in a 10ul reaction 5ul of HRM product, 4 ul of RO water and 1ul of Exo-SAP-IT were mixed and incubated using the following cycling conditions: 37°C for 15 min and 80°C for 15 min. Subsequently, for each primer in a 20ul reaction the following were combined: 20ng/ul of the Exo-SAP-IT product, 650nM of each primer (one reaction per primer), 3.5ul of BDT v3.1 Ready Reaction Mix (100RR) and 1x Sequencing Buffer. The following cycling conditions were used: 1 cycle of 96°C for 1 min, 30 cycles of 96°C for 10 s, 50°C for 5 s. and 60°C for 4 min, 1 cycle of 4°C for 5 min and 1 cycle of 10°C for 5 min. A standard ethanol precipitation was performed on the products before they were loaded onto the ABI-3130 Genetic Analyzer.

Statistical Analysis

SPSS version 15 and Microsoft Excel were used to analyze the collected data. Genotype and allele frequencies were collated and Hardy-Weinberg equilibrium tested to check for genotyping accuracy. Linkage disequilibrium analysis was performed using the Haploview 4.2 computer program (Barrett et al. 2005). The power of the study was defined by the G-Power statistical package (Faul et al., 2007). Chi-square tests were performed to determine if significant differences existed in the genotype and allele frequencies between the case and control samples examined with significance threshold set at $p = .05$.

Results

Genotype and allele frequencies are summarized in Table 1. Hardy-Weinberg equilibrium (HWE) held for both SNP variants ($p > .05$). Linkage disequilibrium analysis was carried out between rs2850328 and rs2395 as outlined in the methods section. The two tested markers were found to not be in linkage disequilibrium and since both SNPs are on different chromosomes, no D' value could be produced. The observed allele frequencies were checked against the National Centre for Biotechnology Information (NCBI) SNP online database and found to be consistent with previously published findings.

Standard chi-square analysis was used to determine *p* values for genotype and allele frequencies which returned no statistically significant difference ($p > .05$) between cases and controls for genotype frequencies for rs2850328 ($\chi^2 = 1.2$, $p = .5476$) or rs2395 ($\chi^2 = .3$, $p = .8608$). Similarly, no statistical difference was observed for allele frequencies for rs2850328 ($\chi^2 = .68$, $p = .4108$) or rs2395 ($\chi^2 = .02$, $p = .893$). The power of the population used in this study was calculated to be 70% at $p = .05$, for a medium allele effect.

Three random samples of each genotype of rs2850328 and rs2395 were selected to be sequenced and all were in accord with the HRM genotyping, all showing the correct base change in the 174bp and 114bp amplicons respectively.

Discussion

To investigate the influence of two Wnt signaling pathway coding SNPs on breast cancer risk, we performed a case-control study using an Australian population. *PPP3CA* and *MARK4* were selected as suitable candidate genes from a previous study carried out in our laboratory (Gabrovska et al., 2011). For *MARK4*, the potentially functional rs2395 SNP located in the UTR-3 region, while for *PPP3CA* the potentially functional rs2850328 SNP located near the 5' gene region were chosen. We used HRM analysis and Dye terminator DNA Sequencing to examine these two SNPs in an Australian case-control study population for a potential role in human breast cancers.

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While the *MARK4* rs2395 SNP to our knowledge has not been previously investigated in an association study, the *PPP3CA* rs2850328 SNP was previously investigated in an association study on addiction vulnerability and differential isoform expression in Alzheimer's disease (Chiocco et al., 2010). In the study, the investigators looked at several *PPP3CA* SNPs including rs2850328 associated with addiction for an association with Alzheimer's disease. For rs2850328, they failed to find a significant association with the disease (p = .968) in a Caucasian population (n = 427). The genotypic and allelic frequency percentages of the Chiocco et al. study were in line with ours (Chiocco et al., 2010).

The observed allele frequencies were found to be consistent with the NCBI SNP online database information on both SNPs. The findings of this study did not find a positive association between these markers and breast cancer susceptibility in our population as no significant difference could be detected between the cases and healthy matched controls by either the genotype or allele frequencies for the investigated SNPs (all $p > .05$).

For rs2850328, even though the frequency of the CC homozygotes or the C allele varied, no significant association between the PPP3CA polymorphism and risk of breast cancer was found. The Chi-square analysis used to determine p values for genotype and allele frequencies returned no statistically significant difference ($p > .05$) between cases and controls for rs2850328 genotype frequencies ($\chi^2 = 1.2$, $p = .5476$) and allele frequencies ($\chi^2 = 0.68$, $p = .4108$) which was a similar finding as Chiocco et al. (2010) who also found no significance in the genotype frequencies ($p = .968$) or the allele frequencies ($p = .962$) for the same SNP in a population of addiction abusers and healthy controls.

Similarly for rs2395, even though the frequency of the AA homozygotes or the A allele was lower in the breast cancer cases compared to the healthy controls (6% versus 7.3% and 25.5% versus 26% respectively) no significant association between the *MARK4* polymorphism and risk of breast cancer was found. The Chi-square analysis returned no statistically significant difference ($p > .05$) between cases and controls for rs2395 genotype frequencies ($\chi^2 = 0.3$, $p = .8608$) and allele frequencies ($\chi^2 = .02$, $p = .893$).

While it is probable that these two potentially functional SNPs have no significant association with the risk of

TABLE 1

Genotype and Allele Frequencies for PPP3CA (rs2850328) and MARK4 (rs2395) SNPs

PPP3CA (rs2850328)		Cases	Controls	Total
Genotypes				
	TT	89 (49%)	78 (43.3%)	167
	TC	78 (42.8%)	87 (48.3%)	165
	CC	15 (8.2%)	15 (8.3%)	30
	Total	182	180	362
	HWE	0.716	0.1727	
	χ^2	1.2		
	P VALUE	0.5476		
Alleles				
	T	256 (70.3%)	243 (67.5%)	499
	C	108 (29.7%)	117 (32.5%)	225
	Total	364	360	724
	HWE	0.716	0.1727	
	χ^2	0.68		
	P value	0.4108		
Mark4 (rs2395)	Cases	Controls	Total	
Genotypes				
	GG	100 (55%)	98 (55.4%)	198
	GA	71 (39%)	66 (37.3%)	137
	AA	11 (6%)	13 (7.3%)	24
	Total	182	177	359
	HWE	0.7315	0.6829	
	χ^2	0.3		
	P value	0.8608		
Alleles				
	G	271 (74.5%)	262 (74%)	533
	A	93 (25.5%)	92 (26%)	185
	Total	364	354	718
	HWE	0.7315	0.6829	
	χ^2	0.02		
	P value	0.893		

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breast cancer, it is possible that different polymorphisms within these genes contribute to susceptibility in both our and other populations. This may be especially the case for weak allele effects, or for other SNPs in the genes not in linkage disequilibrium, which our population would have poor power to detect. A larger population may therefore be required to detect modest differences in these markers if their relative strength is small. It would be useful to undertake studies to determine functional effects, if any, of these SNPs.

Other variants within the *PPP3CA* and *MARK4* genes may still be associated with breast cancer, as both genes are associated with processes involved in the disease as well as their mutual participation in the Wnt signaling pathway. The involvement of *MARK4* in human hepatocellular carcinogenesis through down-regulation in response to decreased TCF/LEF1 activity (Kato et al., 2001) suggests its role as a messenger in the Wnt-signaling pathway in humans. Over expression of PPP3CA in MCF-7 HBC cells has been shown to promote cell survival and chemoresistance (Vellon et al., 2005), cell growth, migration and in vivo angiogenesis (Tsai et al., 2002). In addition, up-regulation of PPP3CA has been demonstrated both in vitro and in vivo in highly aggressive breast cancer cell lines and in samples from patients with more advanced stages of malignancy (Xie et al., 2001), a finding that is consistent with our previous study (Gabrovska et al., submitted).

Conclusion

This was the first study to examine the tested SNPs in the context of breast cancer risk. Even though an association of the polymorphisms rs2850328 and rs2395 and breast cancer was not detected in our case-control study population, exploring these SNPs in other breast cancer populations may be worthwhile. Given what is currently known about *PPP3CA* and *MARK4* and their involvement in the Wnt signaling pathway and other biological processes involved in breast cancer, both genes are suitable candidates for further investigations. Investigations of *PPP3CA* and *MARK4* in a cancer context may enrich our understanding of the role of these genes in carcinogenesis and in particular making them suitable targets for breast cancer therapies.

List of Abbreviations

Bp: base pair
CI: confidence interval
DNA: deoxyribonucleic acid
DNTPs: deoxynucleosides
HRM: high resolution melt
MAF: Minor allele frequency
MARK4: MAP/microtubule Affinity-Regulating Kinase 4 Gene
NCBI: National Centre for Biotechnology Information
OR: odds ratio
PPP3CA: Protein Phosphatase 3, Catalytic subunit, Alpha isoform (calcineurin A alpha) gene
Q-PCR: quantitative real time polymerase chain reaction
SNP: short nucleotide polymorphism
WNT: wingless

Authors' Contributions

P.N.G. participated in the design of the study, carried out sample preparation, genotyping, statistical analysis and drafted the manuscript. R.A.S. participated in the design of the study and helped to draft the manuscript. L.M.H. participated in the design of the study and helped to draft the manuscript. L.R.G. conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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