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Associating GWAS Information with the Notch Signaling Pathway Using Transcription Profiling

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Abstract: Genome-wide association studies (GWAS) have identified SNPs associated with breast cancer. However, they offer limited insights about the biological mechanisms by which SNPs confer risk. We investigated the association of GWAS information with a major oncogenic pathway in breast cancer, the Notch signaling pathway. We first identified 385 SNPs and 150 genes associated with risk for breast cancer by mining data from 41 GWAS. We then investigated their expression, along with 32 genes involved in the Notch signaling pathway using two publicly available gene expression data sets from the Caucasian (42 cases and 143 controls) and Asian (43 cases and 43 controls) populations. Pathway prediction and network modeling confirmed that Notch receptors and genes involved in the Notch signaling pathway interact with genes containing SNPs associated with risk for breast cancer. Additionally, we identified other SNP-associated biological pathways relevant to breast cancer, including the P53, apoptosis and MAP kinase pathways.

Keywords: GWAS, gene expression, Notch signaling pathway

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Introduction

The lifetime risk of developing breast cancer is about one in eight for women, with around 192,370 new invasive cases, 62,280 new in situ cases being diagnosed and 40,170 deaths in the United States each year.¹ Breast cancer is the second leading cause of cancer death among women in the US. While major inroads have been made in reducing mortality rates due to increased screening, digital mammography, specialized care, and the widespread use of therapeutic agents such as aromatase inhibitors, trastuzumab and others, defining the genetic architecture of breast cancer remains an important long-term goal for the development of more effective therapeutic strategies and early interventions. Recent advances in microarray technology and reduction in genotyping costs have made possible genome-wide association studies to identify genetic variants associated with risk for breast cancer.²⁻⁶ Although these studies are providing valuable clues about the broad patterns of genetic susceptibility to breast cancer, the ultimate goal of SNP and gene discovery is to identify and characterize the biological pathways and the molecular mechanisms underlying the disease. This is especially important in breast cancer, a group of biologically and genetically heterogeneous diseases with distinct oncogenic pathways and therapeutic targets. To date, there is little information associating GWAS data to known oncogenic pathways involved in breast cancer. This knowledge gap is hindering translation of discoveries from GWAS into clinical practice to develop clinically useful genetic tests as well as early therapeutic interventions and new targeted drugs.

The objective of this study was to investigate the association of GWAS information with the Notch signaling pathway. The rationale for choosing the Notch signaling pathway in this study was that apart from its involvement in breast cancer, the Notch signaling pathway is involved in many types of cancer including breast cancer, lung cancer, neuroblastomas, skin cancer, cervical cancer, and prostate cancer.⁷ However, it is less well characterized compared to other biological pathways involved in breast cancer such as the estrogen, kinase, apoptosis and P53 pathways which are enriched with SNPs associated with risk for breast cancer. Consequently, association of GWAS information with the Notch signaling pathway may provide proof of concept that this

approach could work and provide insights about the putative functional bridges between GWAS information and biological pathways that are less characterized and may not contain genes harboring mutations or SNPs associated with cancer. The Notch signaling pathway is extremely contextual-dependent, meaning that crosstalk and interaction with other pathways including those enriched by SNPs associated with risk for breast cancer would be very important in determining outcomes. With the exception of T-ALL (T-cell acute lymphoblastic leukemia),⁷ there are very few instances where mutations have been detected in solid tumors in Notch pathway genes, despite solid evidence that the pathway itself is very important to the biology of tumors.⁸⁻¹³ Thus, it is conceivable that the genes in the Notch signaling pathway may be regulated *in trans* by genetic variants located in genes in other biological pathways that crosstalk with the Notch signaling pathway. Therefore, modeling gene regulatory networks using GWAS information, gene expression data and genes involved in the Notch signaling pathway provides the best mechanism for understanding the potential molecular mechanisms underlying Notch dysregulation in breast cancer.

Our group⁸ among others^{9,10} have shown that the Notch signaling pathway plays a critical role in the development of breast cancer. Numerous cellular functions and microenvironment cues associated with tumorigenesis are modulated by Notch signaling, including cell fate, proliferation, apoptosis, adhesion, and angiogenesis.^{11,12} Additionally, Notch signaling plays an important role in the maintenance of breast tumor-initiating cells.¹³ Of the four known Notch receptors, three have been implicated in breast oncogenesis (Notch-1, -3, and -4) while one (Notch-2) has been suggested to have opposite roles and have a positive prognostic significance.¹⁴ Notch-2 has recently been associated with ER-positive breast cancer tumors.¹⁵ Both pan-Notch inhibitors and specific monoclonal antibodies (mAb) to individual Notch receptors are being developed for breast cancer. However, the molecular mechanisms underlying dysregulation and aberrant expression of Notch receptors and other genes involved in the Notch signaling pathway leading to breast cancer remain poorly understood. The association between the Notch signaling pathway and genes containing SNPs associated with risk for breast cancer could



provide putative functional bridges between GWAS information with an oncogenic pathway that does not harbor mutations, but is involved in cancer development and progression.^{8–13} Therefore, elucidating the association of GWAS information with the Notch signaling pathway may help to determine which patients may benefit from Notch inhibitors and to explore the role of Notch transmembrane receptors as potential drug targets and predictive markers.

We hypothesized that genes containing SNPs with large ($P \leq 10^{-5}$) and small to moderate ($P \sim 10^{-2} - 10^{-4}$) effects associated with risk for breast cancer directly or indirectly interact with the 4 Notch family members, other genes in the Notch signaling pathway, and potentially other biological pathways relevant to breast cancer. To formally test this hypothesis, we mined data from 41GWAS for SNPs and genes associated with risk for breast cancer, and two publicly available gene expression data sets derived from the Caucasian (42 cases and 143 controls)¹⁶ and Asian (43 cases and 43 controls)¹⁷ populations. Throughout this study, we have defined genes containing SNPs associated with risk for breast cancer as candidate genes, and assumed the gene and pathway as the units of association. We have assumed the P -value and correlation as measures of effect size for both GWAS and gene expression analysis.

Methods

Data sources

We mined the literature through PubMed searches and websites containing supplementary data on 41GWAS to identify SNPs and genes associated with risk for breast cancer. The search included terms (GWAS, GWA, WGAS, WGA, genome-wide, genomewide, whole genome, all terms + association, or + scan) in combination with breast cancer from the primary published reports through July 2010. All the reports were read and information was manually extracted and entered into the database. The inclusion criterion was that the study must include a sample size of ≥ 500 cases and ≥ 500 controls. We catalogued SNPs with large ($P \leq 10^{-5}$) and small to moderate ($P \sim 10^{-2} - 10^{-4}$) effect sizes. We chose this liberal statistical threshold to allow examination of genes containing borderline SNPs with small effect sizes and to include GWAS of various sizes accommodating publication bias while maintaining a consistent approach. SNPs mapping to intergenic regions were not used in this study. SNP

locations and gene names were verified using the dbSNP database and the chromosome report build 3.71. The Human Genome Nomenclature (HUGO) database was used to further check the authenticity of gene names and their aliases. The list of genes (gene symbols, full names), number of SNPs per gene, along with references of primary reports from which the GWAS information was derived are summarized in Table A in the Appendix, provided as supplementary data to this manuscript.

We used two publicly available gene expression data sets based on the case control design as in GWAS design to evaluate and establish the expression levels of candidate genes and genes involved in the notch signaling pathway. The first data set involved the Caucasian population, and consisted of 143 histologically normal breast tissues derived from patients harboring breast cancer who underwent curative mastectomy and 42 invasive ductal carcinomas of various histological grades obtained from breast cancer patients. The data set has been fully described by the originators.¹⁶ Briefly, this data set consisted of histological data. Histologically-normal breast has the potential to harbor pre-malignant changes at the molecular level and thus provides a boon for identifying risk markers. We postulated that a histologically-normal tissue with tumor-like gene expression patterns might harbor substantial risk for future cancer development. Thus genes associated with these high-risk tissues would be considered to be malignancy-risk genes. From this assumption, it follows that these genes could serve as potential molecular predictors of breast cancer. Normal breast cancer tissue included histologically normal and benign. All samples were assessed for global gene expression profiles using the Affymetrix platform on U133 Plus 2.0 Array. The tumors were not associated with any known genetic risk factors such as BRCA1 or BRCA2 mutations. The microarray data from these samples including the raw probe-level hybridization intensities were downloaded from the NCBI's Gene Expression Omnibus (GEO) database under accession number GSE10780.

Most GWAS have been performed on Caucasian populations. It remains unclear to what extent findings from these studies can be extrapolated to non-Caucasians. To determine whether results found using data from Caucasian population could be



replicated in the Asian population, we used a second gene expression data set. The second data set involved a multi-ethnic Asian population, consisting of Malaysian breast cancer patients (Malays, Chinese and Indian). The data set has been described by.¹⁷ Briefly, the data set consisted of a total of 43 breast carcinomas and 43 patient-matched normal tissues collected from Kuala Lumpur, UKM and Putrajaya Hospitals in Malasia. The data set was generated using the Affymetrix platform's U133A Chip and was downloaded from GEO accession number GSE15852. The two data sets contained similar information, both involved ductal carcinomas with same tumor grades. The clinical and histological characteristics of the two gene expression data sets used are summarized in Table 1.

In each of the two microarray data sets described above, entries in the data matrix were expression values generated by Affymetrix's Microarray Analysis Suite 5.0 (MAS5) statistical algorithm.¹⁸ Following normalization and scaling, MAS5 signal values were summarized by Turkey's biweight estimation of the probe level intensities within each probe set. This was followed by a global normalization (linear scaling) to give all chips the same average intensity. These procedures yield robust weighted means called average-scaled differences that are proportional to the amount of a particular RNA transcript present in the sample after background correction, which we used as the input in this analysis, after filtering out spiked control genes.

Data analysis

As a first step, we mapped SNPs to the genes by matching gene names, SNP IDs and positions using the

information in the database (dbSNP). We then sorted and ranked the genes on the basis of P -values derived from GWAS, number of times the SNP in a particular gene has been replicated in multiple independent studies, and number of SNPs within each candidate gene. Genes containing SNPs with P -values $P \leq 10^{-5}$ were considered to have large effect size, whereas genes containing SNPs with P -values $P < 10^{-2} - 10^{-4}$ were considered to have small to moderate effect size. Relatively few SNPs mapped to candidate genes had P -values sufficiently small ($P \leq 10^{-5}$) or replicated in multiple independent studies to give conclusive evidence of association. Conversely, there were many genes containing several hundred SNPs with small to moderately significant P -values $P \sim 10^{-2} - 10^{-4}$. These would likely contain several false positives, but may also contain genuine effects of small magnitude. Consequently, in our data analysis, we considered all the 150 candidate genes containing SNPs identified by GWAS. Our rationale was that, the presence of greater than expected number of associated SNPs in genes of similar biological functions interacting with each other and their downstream targets in biological pathways gives a degree of confidence that the associations are genuine, even if none is individually highly significant. The overall P -value for SNPs replicated in multiple independent studies was estimated using Fisher's method.¹⁹

Briefly, we assumed that the P -values (P_i) are independent and uniformly distributed under their null hypotheses. Let P_i be the P -value for the corresponding statistic P_i where $[P_i = P_1, P_2, \dots, P_n]^T$ is a vector of P -values obtained by performing independent

Table 1. Clinical and histological characteristics of Caucasian and Asian patients used in this study to generate gene expression data.

ER, PR, Her2 and grade	Caucasian population			Asian population		
	ER	PR	HER2/neu	ER	PR	HER2/neu
Negative	25	38	43	18	17	22
Positive	55	42	12	25	26	21
Other	10	10	35	—	—	—
Total cases	90	90	90	43	43	43
Grade	Frequency			Frequency		
Well differentiated or 1	6			8		
Moderately differentiated or 2	27			24		
Poorly differentiated or 3	57			11		
Total cases	90			43		

test statistic $[T_i = T_1, T_2, \dots, T_n]^T$ on individual SNPs $[rs_i = rs_{s_1}, rs_{s_2}, \dots, rs_{s_n}]^T$. Assuming H as a continuous monotonic function, a transformation of the P -value can be defined as $Z_i = H^{-1}(1 - P_i)$.²⁰ The statistics for combining K independent P -values or for combining information from K SNPs is given by the following equation.²⁰

$$Z = \sum_{i=1}^K Z_i$$

where, Z denotes the sum of Z_i (Z-scores) of the transformed P -values for the K SNPs. The Z-scores were back transformed into P -values using Fisher's method.¹⁹

The challenge was how to represent a gene containing multiple SNPs within the gene and how to account for correlations among those SNPs. Correlations among P -values of SNPs within a gene exist because of linkage disequilibrium among SNPs. Correlations among SNPs will invalidate the existing methods for combining independent P -values. Furthermore, the SNPs within a gene may have antagonistic functions which could not be captured by combining P -values. Therefore, the method for combining P -values in independent SNPs described above cannot be directly applied to combining P -values of SNPs within a gene. Wang et al,²¹ suggested choosing the most significant SNP from each gene as a representative. The limitation of that approach is that genes that contain a number of SNPs jointly having significant risk effects, but individually making only a small contribution, will be missed in such a representation. Therefore, in this study, we considered the gene and pathway containing SNPs as the units of association. This allowed us to holistically unravel the genetic susceptibility architecture of breast cancer by jointly considering all common variation within the gene and all the genes in the pathway through pathway prediction and modeling gene networks using candidate genes (ie, genes containing SNPs associate with risk for breast cancer) and genes involved in the Notch signaling pathway.

Next, we matched the 150 candidate genes containing SNPs along with genes involved in the Notch signaling pathway to probes on the U133 Plus 2.0 Chips and U133A Human Chips, representing gene expression from the Caucasian and Asian populations,

respectively. The probes were extracted from the NetAffx Database using the batch query (Affymetrix Inc). We then used probes to extract the gene expression values for the candidate genes and genes involved in the Notch signaling pathway from gene expression data sets on Caucasian and the Asian populations, respectively.

On each data set containing candidate genes and genes involved in the Notch signaling pathway, we performed supervised analysis comparing mean gene expression profiles in cancer patients to mean gene expression profiles in cancer-free controls to identify significantly differentially expressed genes, which distinguished the two groups and were predictors of cancer, as demonstrated in Figures 1 and 2 for the Caucasian and Asian populations, respectively. We used the Benjamin and Hochberg²² procedure to correct for multiple testing. Genes were then ranked on estimated P -values and false discovery rate as shown in the Appendix Tables B and C for the Caucasians and Asians, respectively, and those passing a threshold ($P \leq 0.05$) were selected. We performed correlation analysis using Pearson correlation (r) coefficient procedure to identify genes with similar expression profiles, and to assess the association between candidate genes and genes involved in the Notch signaling pathway. In correlation analysis, genes were treated as the variables and their expression values as the measurements. The correlation coefficient between the candidate gene X and Notchgene Y [and between candidate genes] was computed using the following equation.

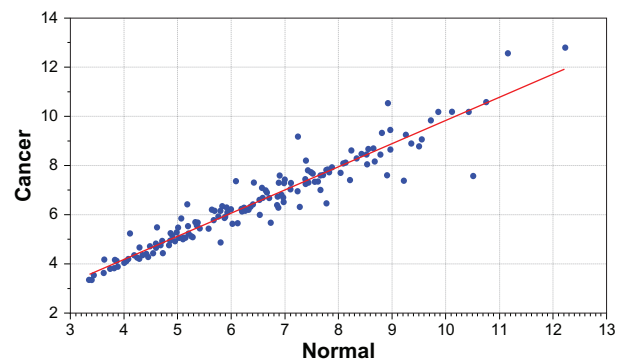


Figure 1. Distribution of mean expression values for candidate genes between breast cancer patients (y-axis) and normal subjects (x-axis) in the Caucasian population. Blue dots significantly deviating from the red line indicate differential expression. The genes, estimates of P -values and false discovery rates for each gene are presented in Table B in the Appendix provided as supplementary data.

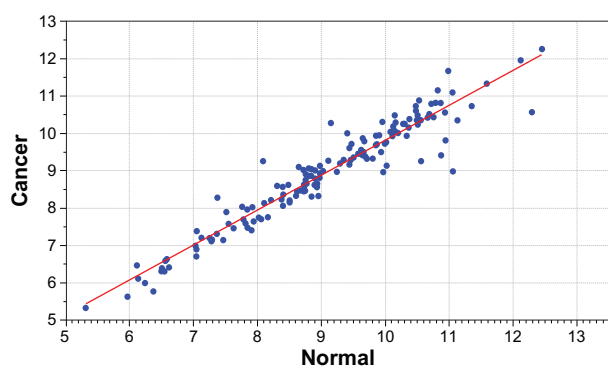


Figure 2. Distribution of mean expression values for candidate genes between breast cancer patients (y-axis) and normal subjects (x-axis) in the Asian population. Blue dots significantly deviating from the red line indicate differential expression. The genes, estimates of P -values and false discovery rates for each gene are presented in Table C in the Appendix provided as supplementary data.

$$r = \frac{1}{n-1} \sum_{i=1}^n \left[\left(\frac{X_i - \bar{X}}{S_X} \right) \left(\frac{Y_j - \bar{Y}}{S_Y} \right) \right]$$

where n is the sample size, X_i and Y_j [\bar{X} and \bar{Y}] are the expression [mean] expression values for the candidate gene and the gene involved in the Notch signaling pathway, respectively. S_X and S_Y are the standard deviations of the expression values for the candidate gene and the gene involved in the Notch signaling pathway, respectively. Both supervised and correlation analyses were performed using GenePattern.²³

Finally, we performed pathway prediction and network modeling using the Osprey System²⁴ to identify candidate genes which interact with genes involved in the Notch signaling pathway and other biological pathways relevant to breast cancer. The Osprey network modeling and visualization system is a very dynamic software platform which integrates experimental information from the literature with gene ontology information from the GO database about all the genes. Therefore, it allowed capturing all the genes that interact with the input genes (ie, candidate genes and genes in the Notch signaling pathway) that have been experimentally confirmed and are involved in the same biological process, which may have been missed during differential expression and co-expression analysis. Thus, is an optimal tool for pathway prediction, network modeling and in silico validation of predicted pathways and gene networks.

In pathway prediction and network visualization, we first performed pathway prediction using a set of candidate genes containing SNPs associated with risk for breast cancer and members of the Notch signaling pathway, which were differentially expressed between cases and controls in the Caucasian population. We repeated the same analysis for the Asian population. To determine whether genes containing SNPs with larger effects and SNPs replicated in multiple independent studies interact with genes in the Notch signaling pathway, we performed separate analysis for each set of genes. In pathway prediction, genes were represented by nodes and the interactions by vertices. Two genes were considered to share a genetic susceptibility architecture and network properties if they were interconnected as represented by the vertices and were correlated as determined by the correlation coefficient. To determine the functional relationships and biological properties of genes in the networks, we used the biological process category of the Gene Ontology classification built in the Osprey System to color-code the nodes (genes). We imposed level 3 filtering criteria to remove genes with spurious interactions, which could be less informative or could distort the reliability of network modeling. This approach also served as a validation step in that we randomly removed genes with fewer interactions and repeated the analysis.

Results

We investigated the association of GWAS information with the Notch signaling pathway. GWAS information included a total 497 SNPs associated with risk for breast cancer. The SNPs were derived from 41 GWAS, totaling more than 250,000 cases and 250,000 controls, mostly (99%) from the Caucasian populations. From the total, 112 SNPs were located in intergenic regions and were not used in the analysis. The remainder, 385 SNPs mapped to 150 genes, of which 130 candidate genes matched probes on the U133 Plus 2.0 Chip for data on Caucasian population and 111 candidate genes matched probes on the U133A Chip for data on Asian population, and were used in the analysis. The discrepancy in the number of genes in the two data sets is due to differences in Chip density (ie, difference in probes and unique number of genes represented on the U133 Plus 2.0

and U133A Human Chips). The list of gene symbols, SNP (rs_IDs), number of SNPs per gene along with the primary sources (ie, references) are provided as supplementary material in Table A in the appendix. Genes involved in the Notch signaling pathway included the 4 members of the Notch family of transmembrane receptors, *NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4* and other genes involved in Notch signaling pathway including, *PTEN*, *HES1*, *HES2*, *SKP2*, *DICER*, *XIAP*, *JAG1*, *JAG2*, *HES1*, *HES2*, *HEY1*, *HEY2*, *FBXW7*, *SKPIA*, *CCNA1*, *CCNA2*, *IAP*, *MYC*, *VEGFA*, *VEGFB*, *VEGFC* and *TP53*, *NUMB*, *DLL1*, *DLK2*, *DLL3* and *DLL4*. We hypothesized that genes containing SNPs associated with risk for breast cancer interact with the members of the Notch family of transmembrane receptors and other genes involved in the Notch signaling pathway and other pathways relevant to breast cancer.

As a first step, we evaluated the expression of candidate genes and genes involved in the Notch signaling pathway by comparing normal breast to breast tumors in Caucasian and Asian populations using publicly available gene expression data described in the methods section, as demonstrated in Figures 1 and 2 for the Caucasian and Asian populations, respectively. We sought to identify candidate genes and members of the Notch signaling pathway that were significantly differentially expressed between breast cancer and normal tissue. Such genes would serve as molecular predictors of breast cancer. We then used the identified differentially expressed genes as the input for pathway prediction and network modeling.

Using supervised analysis, we identified 71 candidate genes and 12 genes involved in the Notch signaling pathway, with significant differences in expression profiles between the cases and controls in the Caucasian population. The list of significantly differentially expressed genes involved in the Notch signaling pathways included *HEY1* ($P = 5.00E-06$), *HEY2* ($P = 5.00E-06$), *JAG2* ($P = 5.00E-06$), *MYC* ($P < 0.002$), *NOTCH1* ($P = 3.00E-05$), *NOTCH2* ($P = 2.00E-05$), *NOTCH4* ($P = 5.00E-06$), *SP2* ($P < 0.04$), *DICER1* ($P < 0.0004$) and *FBXW7* ($P = 5.00E-06$). Repeating the same analysis using gene expression data from the Asian population, we identified 31 candidate genes and several genes

involved in the Notch oncogenic pathway (*PTEN*, $P5e-06$; *HEY2*, $P = 0.04$; *IAPP*, $P = 0.05$) with significant differences in expression profiles between cases and controls. A full list of significantly differentially expressed (and non significantly differentially expressed) candidate genes between breast cancer patients and controls, their estimated P -values and false discovery rates for the Caucasian and Asian populations are provided in Tables B and C, respectively, provided as supplementary data. As expected, not all candidate genes exhibited differences in expression profiles between cases and controls. In addition, not all candidate genes differentially expressed in the Caucasian population were replicated in the Asian population.

This suggests that like GWAS results, gene expression can be heterogeneous among populations, making it difficult to replicate results. The observed differences in expression profiles between cases and controls in the two populations can be attributed to several factors; including the fact that gene expression varies among populations,²⁵ differences in tissue procurement timing and storage, use of chips with different probe densities, as well as the genetic and phenotypic heterogeneity inherent in the GWAS data used in this study. Co-expression analysis however revealed that candidate genes that were not differentially expressed exhibited co-expression patterns with sets of genes distinguishing cancer from normal controls.

To formally test the hypothesis that candidate genes containing SNPs associated with risk for breast cancer interact with genes involved in the Notch signaling pathway, we performed pathway prediction and network modeling. As a first step, we performed pathway prediction and network modeling using the 71 candidate genes confirmed in the Causation population and all genes involved in the Notch signaling pathway. In addition we modeled the biological relationships of the genes in the predicted pathways and networks using Gene Ontology information and experimental information derived from the literature by text mining using the module built in the Osprey System. The key for GO information characterizing genes in the predicted pathways and networks according to the biological process in which they are involved is presented in Figure 3. The results of



- Signal transduction
- Transcription
- Metabolism
- Stress response
- Cell cycle
- DNA metabolism
- DNA repair
- Protein amino acid phosphorylation
- Protein degradation
- RNA processing
- Unknown
- Transport
- Cell organization and biogenesis
- Protein biosynthesis
- DNA replication
- DNA damage response
- Protein transport
- DNA recombination
- Protein amino acid dephosphorylation
- Carbohydrate metabolism

Figure 3. Color codes indicating the biological process in which genes reported in figures 4–7 are involved.

pathway prediction and network modeling for the Caucasian population are presented in Figure 4. For easy interpretation throughout the figures, names of candidate genes (ie, genes containing SNPs associated with risk for breast cancer) are shown in red. Names of genes involved in the Notch signaling pathway are shown in blue, while names of the new set of genes not identified by GWAS are shown in black. Nodes represent the genes and the vertices represent the interactions.

Members of the Notch family of transmembrane receptors *NOTCH1*, *NOTCH2*, were found to interact with genes containing SNPs associated with risk for breast cancer (Fig. 4). Interactions were found between *NOTCH1* and several DNA repair genes,

including *XRCC6*, *XRCC5*, and *CHEK1*; and between *NOTCH2* and *GSK3B*, *FANCA*, *MSH2*. Also observed were interactions between other members of the Notch signaling pathway, for example, *MYC*, *CNNAI1*, *JAG1*, *FBXW7*, *CNNA2*, *XIAP*, with genes containing SNPs associated with risk for breast cancer.

To evaluate the strength of association between candidate genes and the Notch signaling pathway in the Caucasian population we estimated correlations between pairs of genes. Focusing on candidate genes with SNPs replicated in multiple GWAS studies. Significant correlations ($P < 0.05$) between Notch receptors and *FGFR2* (*NOTCH1*, $r = 0.20$; *NOTCH2*, $r = 0.37$; *NOTCH3*, $r = -0.23$) were observed. Significant correlations between other genes involved in the Notch signaling pathway and *FGFR2* were also observed, including with (*MYC*, $r = 0.27$; *DICER1*, $r = 0.36$; *HEY1*, $r = 0.44$; *HEY2*, $r = 0.57$; *JAG2*, $r = 0.47$). Other candidate genes which exhibited significant correlations with genes involved in the Notch signaling pathway included (*NOTCH1* versus *PGR*, $r = -0.24$; *NOTCH4* versus *PGR*, $r = -0.26$; *ESR1* versus *SKP2*, $r = -0.46$; *FANCA* versus *FBXW7*, $r = -0.44$; *ERBB2* versus *NOTCH3*, $r = 0.49$; *CDKN2A* versus *SKP2*, $r = 0.70$; *CDKN2A* versus *CHEK1*, $r = 0.68$; *CDKN2A* versus *CHEK2*, $r = 0.55$; *DICER1* versus *BLM*, $r = -0.56$; *HES1* versus *BLM*, $r = 0.47$; *HES2* versus *BLM*, $r = 0.52$; *FBXW7* versus *CHEK2*, $r = -0.49$; *FBXW7* versus *ERBB2*, $r = -0.41$ and *NOTCH3* versus *ERBB2*, $r = 0.49$). This confirms our hypothesis that candidate genes are associated with Notch receptors and other genes involved in the Notch signaling pathway. Supporting the validity of our observations, in addition to the Notch signaling pathway, we identified other biological pathways relevant to breast cancer. Additional biological pathways enriched by SNPs and relevant to breast cancer identified in this analysis included the P53 pathway, the apoptosis control pathways, MAP kinase pathways, the estrogen receptor pathway and the insulin growth factor pathway.

In general, the interactions between candidate genes and Notch signaling appears to be complex involving multiple pathways, suggesting that multiple interacting pathways are likely involved in the development and progression of breast cancer. The involvement of multiple pathways also indicates that interactions between the Notch signaling pathway and candidate

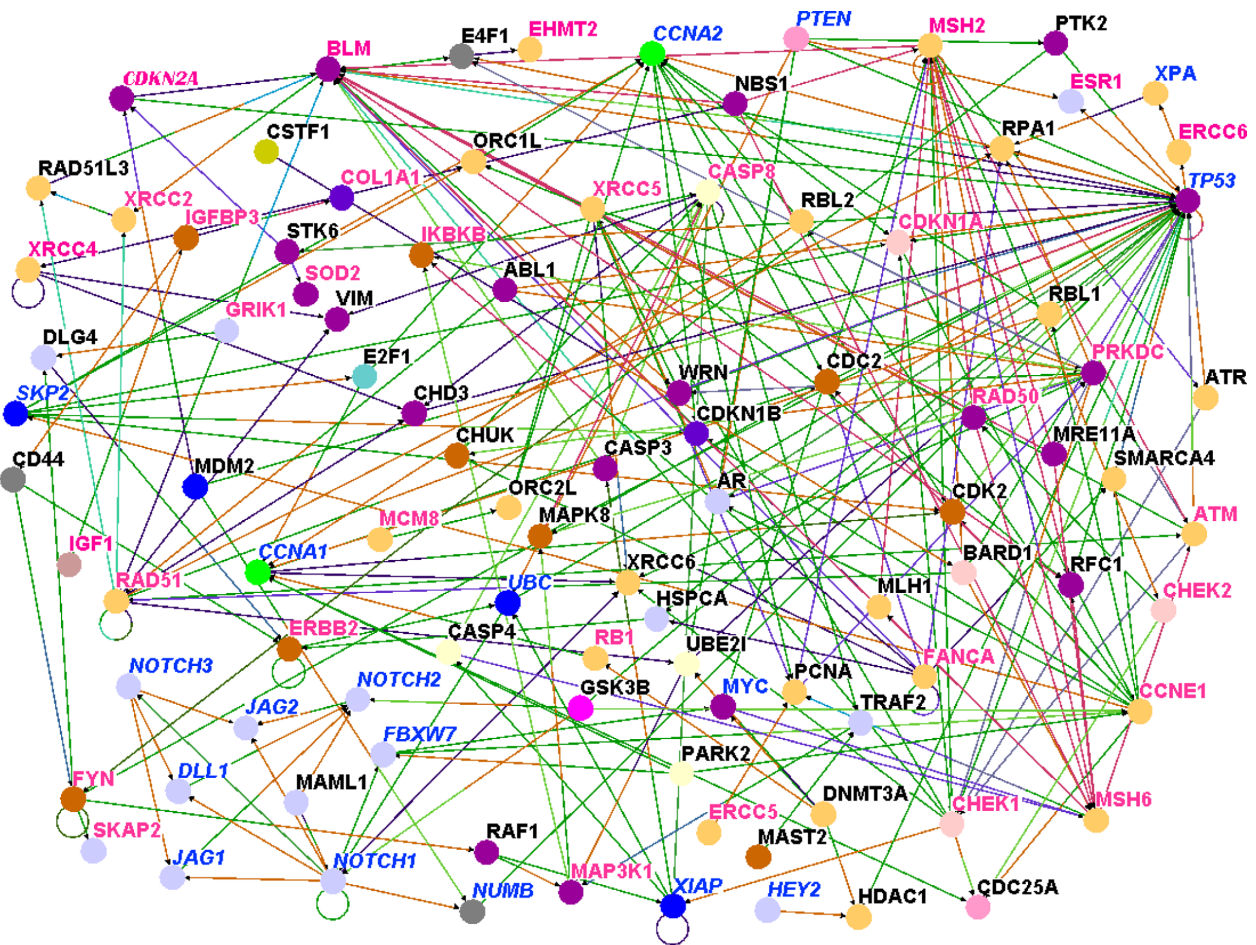


Figure 4. Results of pathway prediction and network modeling showing interactions between genes containing SNPs associated with risk for breast cancer and genes involved in the Notch signaling and other biological pathways based on the Caucasian population. Nodes represent genes and vertices represent interactions. The color code denotes the biological process in which the genes are involved as defined in Figure 3. The color codes in the vertices indicate the experimental techniques or a combination thereof used to confirm the relationship between the genes as determined by the experiments reported in the literature. Candidate genes containing SNPs associated with risk for breast cancer are shown in red, genes involved in the Notch signaling pathway are shown in blue and new genes not reported in GWAS studies are shown in black. For the full names of genes and number of SNPs per gene including GWAS references, please refer Table A in supplementary data.

genes containing SNPs associated with risk for breast cancer may involve multi-pathway crosstalk. A clear example was the involvement of NUMB, which is involved in the Notch signaling pathway but also controls P53 tumor suppressor activity in breast cancer.²⁶ NUMB is a cell fate determinant, which by asymmetrically partitioning at mitosis, controls cell fate choices by antagonizing the activity of the plasma membrane receptor of the Notch family.²⁷

Of particular interest were the three-way interactions among genes containing SNPs with large ($P \leq 10^{-5}$) and small to moderate ($P \sim 10^{-2} - 10^{-4}$) effects and genes involved in the Notch signaling pathway; and the interactions among candidate genes. This indicates that the genetic susceptibility architecture of breast cancer is complex and that even genes contain-

ing SNPs with small effects often considered as noise in GWAS analysis could potentially have significant effects on development and progression of cancer. These results demonstrate that pathway prediction and network modeling could potentially increase the power of GWAS analysis by taking into account complex interactions which could not be realized using GWAS alone.

A major concern in genome-wide association studies is that majority of the GWAS studies ~99% (based on this study) have been conducted on Caucasian populations. To determine whether results of pathway prediction and network modeling observed in the Caucasian population could be replicated in the Asian population, we performed pathway prediction using the 31 differentially expressed candidate genes

yet another confirmation of association between the Notch signaling pathway and candidate genes. *PTEN* is a major regulator of the Notch signaling pathway. In general, the biological pathways identified in the Caucasian population were replicated in the Asian populations. This is an interesting result in that it demonstrates that although gene expression may vary across populations presumably due to environmental factors, the biological pathways underlying breast cancer development and progression could be the same in different populations. Environmental factors were not considered in this study.

Both the validity and reproducibility of results from GWAS studies particularly the ones with small to moderate effect sizes ($P \sim 10^{-2}$ – 10^{-4}) have been challenged.²⁸ Therefore, to determine whether genes involved in the Notch signaling pathway interact with genes that contain SNPs with good evidence of association (ie, “winner” SNPs) ($P \leq 10^{-5}$), we removed genes containing SNPs with small effects.

We then repeated pathway prediction combining genes involved in the Notch signaling pathways and genes containing SNPs with large effects. Genes containing SNPs with larger effects ($P \leq 10^{-5}$) included *ABCC4*, *BTNL8*, *CASP8*, *COL1A1*, *ECHDC1*, *ESR1*, *FBN1*, *FGFR2*, *GRIK1*, *MLK4*, *LOC643714*, *LSP1*, *NEK10*, *PPP2R2B*, *RAD51L1*, *RFN146*, *SLC4A7*, *TOX3*, *STXBP4*, *TGFB1*, *MAP3K1*, *H19*. The results of genes containing SNPs with large effects are presented in Figure 6. We found that genes containing SNPs with large effects interact with each other and with genes involved in the Notch signaling pathway and other biological pathways involved in breast cancer. In addition, we also identified genes with small effects, for example, the *IKBKB* ($P = 0.002$).

To further address the problem of reliability of GWAS data, we performed additional analyses combining genes containing SNPs replicated in multiple independent studies with genes involved in the Notch Signaling pathway. Genes containing SNPs reported

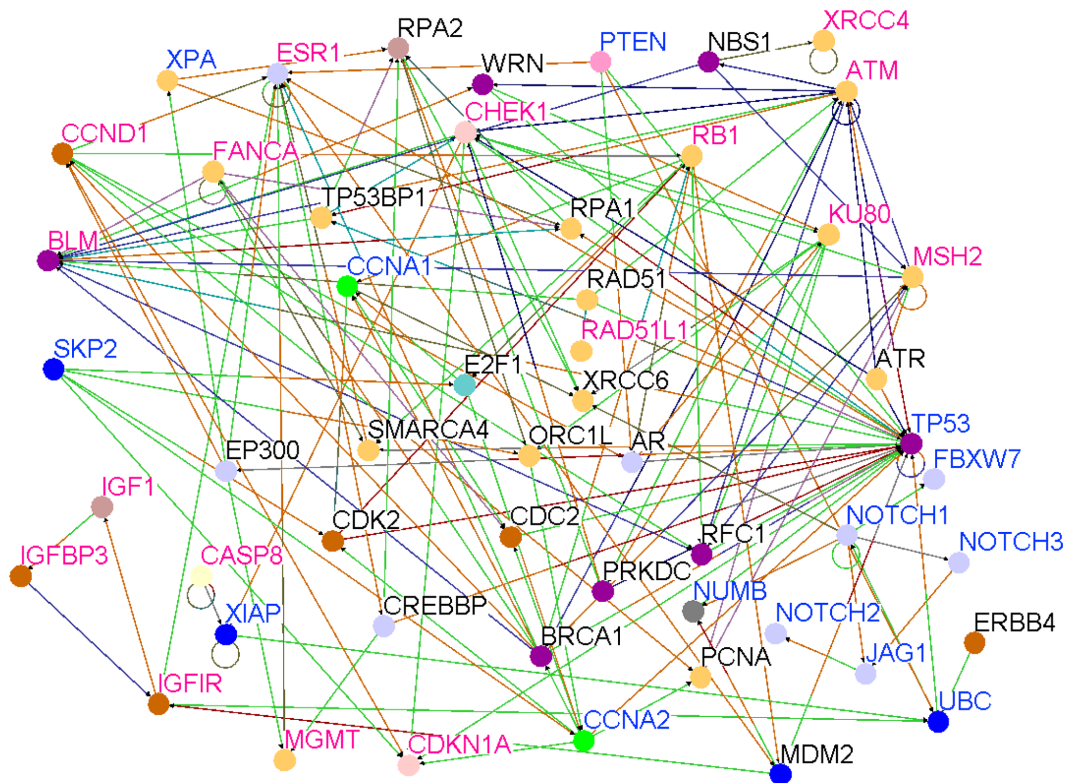


Figure 6. Results of pathway prediction and network modeling showing interactions between genes containing SNPs associated with risk for breast cancer and genes involved in the Notch signaling and other biological pathways. The results are based on genes containing SNPs replicated in multiple independent GWAS. Nodes represent genes and vertices represent interactions. The color code denotes the biological process in which the genes are involved as defined in Figure 3. The color codes in the vertices indicate the experimental techniques or a combination thereof used to confirm the relationship between the genes as determined by the experiments reported in the literature. Candidate genes containing SNPs associated with risk for breast cancer are shown in red, genes involved in the Notch signaling pathway are shown in blue and novel genes are shown in black.



in multiple independent studies included *A2BP1*, *ADH1B*, *ALPL*, *ATM*, *BLM*, *CASP8*, *CCND1*, *CDKN1A*, *CHEK1*, *CSB*, *PMBT1*, *ECHDC1*, *EHMT*, *ERBB4*, *ESR1*, *FANCA*, *FGFR2*, *HCN1*, *ICAM5*, *IGF1*, *IGFIR*, *IGFBP3*, *KU80*, *LSP1*, *MGMT*, *MSH2*, *NEIL2*, *POLB*, *POLK*, *RAD51L1*, *RB1*, *RNF146*, *RPA1*, *RPA2*, *SKAP2*, *SOD2*, *MAP3K1*, *H19*, *TOX3*, *TGFB1*, *TNPI1*, *XPA*, *XPC*, *XRCC3*, *XRCC4*. The results are shown in Figure 7. We found that genes containing SNPs replicated in multiple independent studies interact with each other and with genes involved in the Notch signaling pathway, and genes involved in other breast cancer-relevant biological pathways. In addition, we identified novel genes not yet identified by GWAS (genes shown in black).

To address the problem of publication bias and to determine whether members of the Notch signaling pathway interact with candidate genes with small

to moderate effects, we performed further analyses combining the genes involved in the Notch signaling pathway and candidate genes containing SNPs with small effects. We found that genes containing SNPs with small effects interact with genes involved in the Notch signaling pathway (results not presented because we captured the same results in the four figures reported above). In addition, we identified novel genes not reported in GWAS studies, including *MDM2*, *CHUK*, *AR*, *WRN*, *XRCC6*, *ABL1*, *HDAC1* and *RAF1*.

Overall, in all the analysis, we confirmed our hypothesis that genes containing SNPs associated with risk for breast cancer (regardless of effect size) interact with genes involved in the Notch signaling pathway as well as other biological pathways known to be relevant to breast cancer. Additionally, we identified novel genes not yet reported by GWAS. These

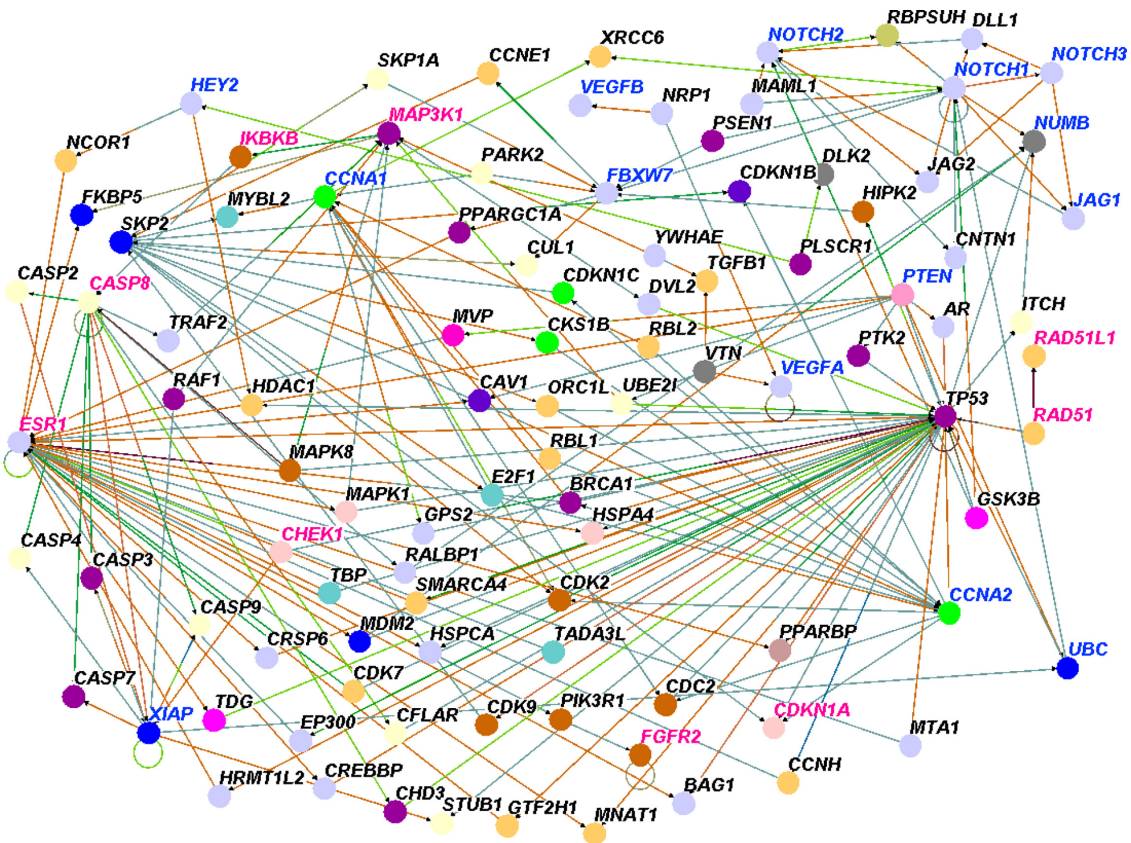


Figure 7. Results of pathway prediction and network modeling showing interactions between genes containing SNPs associated with risk for breast cancer and genes involved in the Notch signaling and other biological pathways. The results are based on genes containing SNPs with the largest effect size ($P < 10^{-5}$). Nodes represent genes and vertices represent interactions. The color code denotes the biological process in which the genes are involved as defined in Figure 3. The color codes in the vertices indicate the experimental techniques or a combination thereof used to confirm the relationship between the genes as determined by the experiments reported in the literature. Candidate genes containing SNPs associated with risk for breast cancer are shown in red, genes involved in the Notch signaling pathway are shown in blue and novel genes are shown in black.



results demonstrated that GWAS information can be leveraged with biological knowledge and gene expression data to infer the association between gene expression and breast cancer. The association of the Notch oncogenic pathway with genes containing SNPs associated with risk for breast cancer demonstrates that integrative analysis combining GWAS information, gene expression data and biological knowledge is a powerful approach to identifying molecular markers underlying GWAS findings.

Discussion

In this study, we provide evidence of association between breast cancer risk candidate genes and the Notch signaling pathway, an important oncogenic pathway involved in many aspects of tumor development, growth and progression, and a potential therapeutic target. Additionally, we identified other biological pathways including the ESR1 pathway, IGF pathway, the Map kinase pathway, the apoptosis pathway and the P53 pathway enriched by SNPs associated with risk for breast cancer. This suggests that regulation of the Notch pathway by candidate genes from GWAS is complicated and potentially involves multi-dimensional crosstalk between the Notch signaling pathway and other oncogenic pathways. Our results tend to agree with those in a recent association study, Fu et al,¹⁵ which showed the association between *NOTCH2* and the *P53* pathway in ER-positive breast tumors.

Several studies have now attempted pathway-based approaches to dissect the genetic susceptibility architecture of common diseases, for example, in inflammatory diseases,²⁹ in bipolar disorder,³⁰ in multiple sclerosis,³¹ in breast cancer,^{32,33} prostate cancer,³⁴ and in seven common diseases.³⁵ To our knowledge, this is the first study to associate GWAS information with the Notch signaling pathway. This is an important finding because although GWAS as demonstrated in this and other studies²⁻⁶ can effectively map loci contributing to phenotypes of interest in breast cancer, they offer limited insights about the biological mechanisms by which SNPs confer risk. Of particular interest is the association of Notch signaling with multiple DNA repair genes including the *XRCC5* and *XRCC6*, which participate in non-homologous end joining for chromosomal DNA double-strand break

repair, and they are essential for the efficient removal of apurinic/apyrimidinic sites near double-strand breaks.³⁶ *WRN*, a member of the RecQ family of DNA helicases, interacts with *XRCC5* and *XRCC6* heterodimers (also known as Ku70/80).³⁷ *WRN* and the serine/threonine protein kinase *ATM*, a master regulator of the cellular DNA damage response, cooperatively participate in an intra-S checkpoint in cells with collapsed replication forks.³⁸ Protein kinase *CHEK1* participates in all known cell cycle checkpoints.³⁹ Checkpoint kinase 2 (*CHEK2*) is an important signal transducer of cellular responses to DNA damage, and it is considered a tumor suppressor; germ line defects of *CHEK2* predispose to familial breast cancer and some other types of malignancies.⁴⁰ *FANCA* participates in the repair of DNA interstrand crosslinks⁴¹ and *MSH2*, a gene frequently mutated in familial non-polyposis colon cancer,⁴² is a homolog of *Drosophila* MutS, a mismatch repair component.⁴³

One possible explanation for these findings is that Notch signaling may be necessary for the survival of cells that are deficient in DNA repair. Also, DNA repair pathways are especially active in cells with stem-like phenotypes, potentially including tumor-initiating cells.^{44,45} Interestingly, an increase in Notch activity is part of the response to radiation in breast cancer-initiating cells,⁴⁶ endothelial cells⁴⁷ and glioma stem cells.⁴⁸

A number of genes involved in cell proliferation and survival are represented in our analysis. Among them, *GSK3B* is a protein kinase involved in glycolysis metabolism but also in proliferation, differentiation and survival that is downstream of the *Wnt* and *AKT* pathways⁴⁹. *GSK3B* itself, as well as the *WNT* and *AKT* pathways are considered potential therapeutic targets in breast cancer.⁵⁰ Interestingly, *Drosophila* Notch is known to interact genetically with the homolog of *GSK3B*.⁵¹ Additional interactions worth noting are those with *IKK α* (*CHUK*) and *IKK β* (*IKBKB*). We have reported that Notch-1 interacts and cooperates with *IKK α* in cervical⁵² and breast cancer cells,⁵³ and Vilimas et al have shown that it interacts with the *IKK* signalosome in T-ALL cells.⁵⁴ These findings emphasize the potential cancer relevance of the Notch-NF- κ B cross-talk. Similar considerations can be made for Raf-1, a key member of the Ras-Raf-MAP kinase pathway. We showed



in 2002 that Ras-mediated transformation requires Notch-1.⁵⁵ A number of cell cycle and proliferation-related genes containing breast cancer-related SNPs also show significant interaction with Notch pathway genes (eg, *CDK2*, *CDC2*, *PCNA*, *cyclin E1*), as does multi-functional oncogene c-Myc, which is known to be a direct Notch target and genetically interact with *Notch-1* in T-ALL.⁵⁶ Protein kinase c-Abl is another interesting Notch-interacting gene. In *Drosophila*, there is evidence of non-canonical interactions between Notch and Abl mediated by accessory protein Disabled.⁵⁷ The anti-apoptotic mediator *XIAP* has been reported to physically interact with Notch-1, resulting in a direct interference with *XIAP* ubiquitination and degradation.⁵⁸ Lipid phosphatase *PTEN*, which is frequently defective in breast cancer, has been shown to be regulated by Notch in other models.^{59,60} Histone deacetylase 1 (*HDAC1*) is displaced by Notch during the process of Notch-mediated transcriptional activation.⁶¹ We have recently revealed a two-way feedback between *Notch-1* and the estrogen receptor alpha (*ESR1*) in breast cancer cells,^{8,53} as well as between *Notch-1* and *ERBB2*.⁶²

The E3 ubiquitin ligase *MDM2*, which targets tumor protein *p53* for proteasomal degradation, was identified in our analysis as a novel candidate in both Caucasian and Asian populations. In a mouse model, it has been shown that Notch suppresses *p53* in lymphomagenesis through repression of the ARF-MDM2-*p53* tumor surveillance network.⁶³ Interestingly, the endocytic protein *NUMB*, which is a negative regulator of Notch activity, can be found in a complex with *p53* and *MDM2*, thereby preventing ubiquitination and degradation of *p53*.²⁶

Thus, an analysis of interactions of genes that contain breast cancer-related SNPs with Notch pathway genes reveals genes and gene products that have been suggested to cross-talk with Notch signaling in other models, including invertebrate models,⁶⁴ supporting the validity of our approach. Moreover, this analysis detected additional candidates for functional interactions with Notch, including numerous DNA repair and checkpoint genes, the androgen receptor (*AR*), free radical detoxifying enzyme superoxide dismutase 2 (*SOD2*) and other genes relevant to breast carcinogenesis and to responsiveness to therapeutic agents including chemotherapeutics

and radiation. The association between the Notch signaling pathway and GWAS information is important in that numerous cellular functions and microenvironment cues associated with tumorigenesis are modulated by Notch signaling, including proliferation, apoptosis, adhesion, epithelial-to-mesenchymal transition and angiogenesis.^{11,64} Given that Notch signaling is activated in a wide variety of human breast cancer cases, components of the Notch pathway have been evaluated as prognosis markers and drug targets.¹⁴ In breast cancer clinical specimens, mRNA expression of Notch-1 and Notch ligand Jagged-1 have been shown to correlate strongly with poor prognosis.⁶⁴⁻⁶⁶ Loss of Notch-negative regulator NUMB, has been described in approximately 50% of human breast cancers.⁶⁷ Notch-4 expression, as detected by immunohistochemistry, correlates with Ki67, a well-known proliferation marker in infiltrating breast carcinomas of ductal or lobular histologies.⁸ Conversely, and consistent with published in vitro data, expression of Notch-2 appears to have a positive prognostic significance.⁶⁸

Although the results of this study offer valuable clues about association of GWAS information with the Notch signaling pathway and other pathways relevant to breast cancer, limitations in interpreting these results must be acknowledged. We have used results of genome-wide association studies and publicly available gene expression data in this analysis. Therefore, interpretation of our results is inherently subject to the constraints of such data. Key limitations include but are not limited to the fact that GWAS information was derived from results obtained from different studies conducted using different platforms, sample sizes, cryptic population stratifications, different phenotypes, and potentially different analysis techniques all of which could potentially affect our results.

However, the results presented in this study demonstrate conclusively that genes containing SNPs associated with risk for breast cancer interact with genes involved in the Notch signaling pathway and other biological pathways relevant to breast cancer. Important work remains to be done to determine how the SNPs disrupt the genes and pathways, leading to cancer development and progression. Such work is beyond the scope of this paper, but the work reported here is the first step in that direction.



Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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