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Original Article

Influence of CHIEF pathway genes on gene expression: a pathway approach to functionality

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Abstract: Background: Candidate pathway approaches in disease association studies often utilize a tagSNP approach to capture genetic variation. In this paper we assess gene expression patterns with SNPs in genes in the CHIEF pathway to help determine their potential functionality. Methods: Quantitative real-time RT-PCR was run to determine gene expression of 13 genes in normal colon tissue samples from 82 individuals. TagSNP genotype data were obtained from a GoldenGate Illumina multiplex bead array platform. Age, sex, and genetic ancestry adjusted general linear models were used to estimate beta coefficients and *p* values. Results: Genetic variation in *mTOR* (1 SNP), *NFKB1* (4 SNPs), *PRKAG2* (3 SNPs), and *TSC2* (1 SNP) significantly influenced their expression. After adjustment for multiple comparisons several associations between pathway genes and expression of other genes were significant. These included *AKT1* rs1130214 associated with expression of PDK1; *NFκB1* rs13117745 and rs4648110 with STK11 expression; *PRKAG2* rs6965771 with expression of NFκB1, PIK3CA, and RPS6KB2; *RPS6KB1* rs80711475 with STK11 expression; *STK11* rs741765 with PIK3CA and PRKAG2 expression; and *TSC2* rs3087631 with AKT1, IkBκB, NFκB1, PDK1, PIK3CA, PRKAG2, and PTEN expression. The higher levels of differential expression were noted for *TSC2* rs3087631 (percent difference ranges from 108% to 198% across genes). Many of these SNPs and genes also were associated with colon and rectal cancer risk. Conclusions: Our results suggest that pathway genes may regulate expression of other genes in the pathway. The convergence of these genes in several biological pathways involved in cancer further supports their importance to the carcinogenic process.

Keywords: Gene expression, colorectal cancer, mTOR, AKT1, STK11, PRKAG2, TSC2, PTEN

Introduction

Candidate pathway approaches in disease association studies often utilize a tagSNP approach to capture genetic variation associated with disease. Associations between single-nucleotide polymorphisms (SNPs) and outcomes, such as cancer, are often observed, although the interpretation of these associations is less clear. Associations with tagSNPs can result from being in LD with other disease-causing SNPs. *In silico* programs are available to help predict functionality based on their involvement in splicing, transcription, translation, and post-translation [1, 2], although studies have found that the prediction made by these programs do not correspond with associations observed in analytical studies [3]. Unfortunately, a lack of information on functionality of disease-associated SNPs can hamper interpretation of findings. Findings not supported by analysis of functionality are often deemed the result of chance or subjected to rigors of multiple comparisons adjustment. However, there are many ways to determine functionality of a SNP. One method is to determine if gene expression is influenced by SNP genotype. Changes in gene expression associated with specific genotypes provides some indication of functionality of that specific SNP.

The Convergence of Hormones, Inflammation, and Energy-Related Factors (CHIEF) pathway is composed of genes associated with these elements [4]; genetic variants within this pathway have been examined with colorectal cancer, although little is known about the functionality of those SNPs. One arm of the pathway con-

tains a serine/threonine protein kinase 11 STK11 or LKB1 and is involved in the regulation of mTOR, or mammalian target of rapamycin. STK11 responds to changes in cellular energy balance (ATP levels) [5, 6] and governs whole body insulin sensitivity [7, 8]. In cells with excess adenosine monophosphate (AMP) due to altered energy homeostasis, STK11 phosphorylates the AMP-dependent kinase such as PRKAG2 [5, 9-11], which in turn phosphorylates proximal substrates like tuberous sclerosis complex (TSC1 and TSC2). *mTOR* represses anabolic processes (ATP utilization) and enhances catabolic processes (ATP generation), restoring the system toward normal energy homeostasis. A different portion of the pathway that responds to insulin, estrogen, and androgen, and certain proto-oncogene growth factors contain PTEN (phosphatase tensin homolog deleted on chromosome 10). PTEN, a tumor suppressor, regulates metabolic signaling and is a negative regulator of cell growth in the insulin/IGF signaling pathways. PTEN acts as a metabolic regulator by modulating signaling via the phosphatidylinositol 3-kinase (PI3K; oncogene formal name PIK3CA) and the v-akt murine thymoma viral oncogene homolog 1 (*Akt1* also known as protein kinase B or PKB) pathway. Akt1-dependent phosphorylation negatively regulates the functioning of TSC1 and TSC2 and links to inflammation via NFκB [12].

Also involved in the pathway are the ribosomal protein S6 kinase (RPS6K) family which are involved in cell growth and regulation of insulin [13]. RPS6KB proteins are members of the AGC protein kinase family and require3-phosphoinositide-dependent protein kinase-1 (PDK1) phosphorylation for activation [14, 15]. PDK1 mediates the cellular influence of growth factors and insulin by activating both RSK and S6K and is essential for activation of Akt [16] NFκB plays a critical role in the regulation of inflammation and data have shown that RPS6KB is involved in a signaling pathway that involves angiotensin II activation of NFκB [17]. NFκB is an important nuclear transcription factor that regulates cytokines and is critical for the regulation of tumorigenesis, cell proliferation, apoptosis, response to oxidative stress, and inflammation. The IKK complex is a key regulator of NFκB's transcriptional activity. Vascular endothelial growth factor (VEGF) regulates S6K and IRS-1, and plays an important role in regulation of cell growth signaling within this pathway [18]; it is a major mediator of tumor angiogenesis [19].

In this study we test two hypotheses. First, we evaluate if variation in genes in the CHIEF pathway associated with colorectal cancer influence expression of those genes in normal colon tissue. Second, using a pathway approach, we assess if genetic variation in genes in this candidate pathway influence expression of other genes in that pathway. We hypothesize that genes in the same pathway could be influenced by variants in other pathway genes.

Materials and methods

Tissue samples

Eighty-two de-identified normal frozen colon tissues were obtained from the Cooperative Human Tissue Network (CHTN), and stored at -80°C. The age range of the sample donors was 17 to 92 (mean 60.48); 54% were male and 46% female, and from individuals with Caucasian (n=51), African American (n=23), Asian (n=1), and unknown (n=7) ethnicity.

Reverse transcription and quantitative real-time PCR

To maximize yield, total RNA was isolated utilizing Trizol (Invitrogen, Grand Island, NY) for homogenization, and the RNEasy Mini kit (Qiagen, Valencia, CA) for isolation using a protocol developed by Mauricio Rodriquez-Lanetty (unpublished) with minor alterations. Briefly, tissues (~25 mg) were homogenized in 150 μL Trizol using the Bullet Blender and stainless steel beads. Homogenate was placed in a new vial with 450 μL Trizol. After adding 100 μL chloroform, vials were shaken well, incubated for 2 minutes at room temperature, centrifuged, and the supernatant was placed in a new vial. An equal part of 100% ethanol was added, and the mixture placed in an RNEasy spin column. RNA was washed and eluted according to the RNEasy protocol. Total DNA and RNA were isolated from normal colon samples using the AllPrep DNA/RNA/Protein Mini Kit (Qiagen).

First strand cDNA synthesis was performed using the High Capacity RNA-to-cDNA kit (ABI, Carlsbad, CA) on 500 ng total RNA as measured by RNA 6000 Nano kit (Agilent, Santa Clara, CA). Quantitative real-time RT-PCR reactions

were performed on the ABI 7900HT Fast Real Time PCR System using Taqman primer/probe sets and Taqman Fast Universal PCR Master Mix no AmpErase® UNG (ABI). Experiments were run per manufacturer's protocol in triplicate on cDNA diluted 1:10 for 50 PCR cycles, retaining those with standard deviations (SD) <1. One observation was removed from the RPS6KB2 and STK11 analyses because the SD among the triplicates was >1. Samples were normalized to β-actin to generate gene expression levels using 2^[Ct(β-actin)-Ct(Marker)], where Ct represents the cycle threshold. Samples with β-actin Ct >30 were discarded (n=1 for AKT1, RPS6KB1, RPS6KB2, and VEGFA analyses; n=4 for all other genes). Gene of interest Ct ≥40 or undetermined were set to 40 (n=5 for mTOR; n=1 for RPS6KB1 and RPS6KB2; n=0 for all other genes). β-actin was chosen as the housekeeping gene because it has been shown that structural housekeeping genes such as β-actin have less variation in normal colon tissues than metabolic housekeeping genes such as GAPDH [20].

Case/control study

Associations between genotypes associated with expression and cancer risk come from data from two population-based case-control studies of colon cancer (cases n=1,555; controls n=1,956) and rectal cancer (cases n=754; controls n=959). Colon cancer cases were identified between October 1, 1991 and September 30, 1994 and included people living in the Twin Cities Metropolitan Area, Kaiser Permanente Medical Care Program of Northern California (KPMCP) and a seven-county area of Utah [19]. The rectal cancer study used identical data collection methods as the colon study, except cases came from the entire state of Utah and included incident cases of the rectosigmoid junction or rectum who were diagnosed between May 1997 and May 2001 in Utah and KPMCP [20]. Controls were matched to cases by sex and by 5-year age groups. At KPMCP, controls were randomly selected from membership lists; in Utah, controls 65 years and older were randomly selected from the Health Care Financing Administration lists and younger controls were randomly selected from driver's license lists. Controls were selected from driver's license and state-identification lists in Minnesota. Details of the study have been previously reported [19, 20].

Genotyping

TagSNPs were selected using the following parameters: LD blocks were defined using a Caucasian LD map and an r^2=0.8; minor allele frequency (MAF) >0.1; range=-1500 bps from the initiation codon to +1500 bps from the termination codon; and 1 SNP/LD bin. All markers were genotyped using a multiplexed bead-array assay format based on GoldenGate chemistry (Illumina, San Diego, California).

Statistical analysis for individual SNP effects on expression

Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC). Tests for Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium (LD) measures were calculated and stratified by race using the ALLELE procedure. The program STRUCTURE was used to compute individual ancestry for each sample assuming two founding populations [21, 22]. Higher order population model was assessed, but did not fit the population structure with the same level of repeatability and correlation among runs as the two-founding population model. Participants were classified by level of percent African ancestry. Genetic ancestry was used as a continuous variable when included in the models to adjust for possible confounding.

General linear models adjusted for age, admixture, and sex were used to estimate SNP beta coefficients and their corresponding *p* values, having applied a log10(2^[ΔCt])+10 transformation to the gene expression values to achieve approximate normality. Co-dominant models were initially used; the best fitting inheritance model is presented. Adjusted medians of the gene expression values were calculated in a manner similar to McGreevy et al using the parameter estimates from the QUANTREG procedure, including the intercept and beta coefficients for genotype and covariates age, admixture, and sex [23]. Percent difference in expression by genotype was calculated as 100 times the absolute difference of the adjusted medians across genotypes divided by the maximum of the absolute value of the adjusted medians. For simplicity the number of genotype categories was restricted to two by substituting the dominant model in place of the additive or co-dominant model. Adjustments for multiple comparisons used the step-down Bonferroni

Table 1. Gene expression influenced by SNPs in genes

Gene	SNP (Model)	Median $2^{\Delta Ct}$			Percent Difference[c]	β[d]	*p*
		Genotype 1	Genotype 2	Genotype 3			
MTOR	**rs2024627 (R)**	**0.0337**	**0.0146**		**56.68%**	**-0.548**	**0.011**
NFKB1	rs1801[a] (R)	0.0655	0.119		44.96%	0.323	0.019
	rs3821958[a,b] (R)	0.0688	0.1001		31.27%	0.222	0.032
	rs3774964[a,b] (R)	0.0661	0.1034		36.07%	0.243	0.022
	rs3755867[a,b] (R)	0.0701	0.1005		30.25%	0.276	0.037
PRKAG2	rs6965771	0.0921	0.0511	0.0289	47.76%	-0.173	0.013
	rs7784818	**0.1121**	**0.0681**	**0.0476**	**46.38%**	**-0.172**	**0.003**
	rs9648724	**0.0592**	**0.0993**	**0.1224**	**46.03%**	**0.212**	**0.004**
TSC2	**rs3087631 (R)**	**0.0796**	**-0.047**		**159.05%**	**-0.767**	**0.003**

[a]Pairwise r^2 values ranging from 0.61 to 0.88 among white population. [b]Pairwise r^2 values ranging from 0.59 to 0.84 among black population. [c]Percent difference=100*(absolute difference of adjusted medians)/maximum of absolute value of adjusted medians). A dominant model was used in place of additive model in order to limit tables to one value per expression/genotype pair. Significant associations after adjustment for multiple testing are in bold. [d]Median $2^{\Delta Ct}$ values, β coefficients and corresponding *p* values from models adjusted for age, genetic admixture, and sex.

correction, taking into account the degree of correlation of the SNPs within genes using the SNP spectral decomposition method proposed by Nyholt [24] and modified by Li and Ji [25].

The Database for Annotation, Visualization and Integrated Discovery (DAVID), which provides functional annotation tools to assist in the understanding of biological meaning of genes when analyzing larger gene lists [26, 27] was used to further examine the candidate genes. The thirteen genes were uploaded to DAVID using their official gene symbol for Homo sapiens. The tools utilized in this study were principally the Pathways and the Functional Annotation Clustering tools. The Pathways tool provides access to six pathway databases; in this analysis we compared the pathways from the PANTHER Classification System and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, as both databases included all thirteen genes from our list in their pathway analyses. The databases identified a list of pathways that were associated with genes from our gene list, the group of specific genes within each pathway, and provided the *P*-values (crude and Bonferroni-adjusted for number of genes in the pathway assessed), and the fold enrichment scores for each gene. Only pathways that had a *P*-value of <0.05 were considered in our analysis. The fold enrichment score measured the amount of enrichment (over-expression) of the pathway in the gene list as compared to the background population. The Functional Annotation Clustering tool groups together heterogeneous but highly similar annotations of the genes by measuring the "degree of co-association" and using this as the basis of the relationship between the annotation terms of the genes in the list [26, 27]. The clusters were also assigned an enrichment score, which ranks the groups according to relative importance; the score is the geometric mean of the individual annotation term enrichment *P*-values. An enrichment score of 1.3 was seen as equivalent to a *P*-value of 0.05, however a higher score indicated more enriched terms within the cluster; therefore any score ≥1.3 is considered significant.

Results

Genetic variation in *mTOR*, *NFκB1*, *PRKAG2*, and *TSC2* influenced the expression of their respective genes (**Table 1**). The percent difference in gene expression by genotype ranged from 30% for *NFκB1* rs3755867 to 159% for *TSC2* rs3087631. For *mTOR* rs2024627, *PRKAG2* rs6965771 and rs7784818, and *TSC2* rs3087631, gene expression decreased for the rare homozygote variant, while for the other SNPs, expression significantly increased in the presence of the rare homozygote variant.

Many more associations between pathway genes and expression of other genes were observed than for within gene SNPs and expression (**Table 2**). AKT1 expression was influenced by *PRKAG2*, *STK11*, and *TSC2* genotypes; IKBκB expression was altered by *mTOR*, *PIK3CA*, *PRKAG2*, *TSC2*, and *VEGFA*; mTOR expression

Table 2. Gene expression influenced by SNPs in genes in the pathway

Genotype		Gene Expression																							
		AKT1		IKBKB		MTOR		NFKB1		PDK1		PIK3CA		PRKAG2		PTEN		RPS6KB1		RPS6KB2		STK11		TSC2		VEGFA	
Gene	SNP (Model)	β[h]	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
AKT1	rs1130214 (R)									**-0.45**	**0.04**																
MTOR	rs2295080 (D)			0.33	0.04																						
NFKB1	rs13117745[a]																					-0.25	<0.01				
	rs1801[b] (R)									0.44	0.03	0.28	0.02	0.29	0.02												
	rs3821958[b,c] (R)											0.19	0.04														
	rs3774964[b,c] (R)											0.22	0.02														
	rs12509517[b]																			-0.17	0.05					-0.27	0.02
	rs3755867[b,c] (R)									0.41	0.03																
	rs4648090																					-0.21	0.02				
	rs4648110[a]																					**-0.24**	**<.01**				
	rs4648127 (D)																	0.32	0.03	0.32	0.05						
PIK3CA	rs7644648 (D)			0.36	0.03					0.35	0.02																
	rs7651265 (D)			0.43	0.02					0.43	0.01											0.25	0.04				
	rs1607237 (R)					-0.48	0.04																				
PRKAG2	rs1362236					-0.32	0.03																			0.27	0.05
	rs1001117 (D)																	-0.23	0.01	-0.22	0.03						
	rs6965771 (D)			-0.38	0.02	-0.43	0.01	**-0.30**	**<0.01**	-0.39	0.01	**-0.29**	**<0.01**			-0.41	0.03	-0.29	0.01	**-0.36**	**<0.01**			-0.31	0.04	-0.31	0.05
	rs7784818 (R)	-0.34	0.02	-0.37	0.04					-0.34	0.04					-0.39	0.05	-0.25	0.03	-0.28	0.03					-0.46	0.01
	rs9648724															0.37	0.01	0.17	0.05								
PTEN	rs1903858[e] (D)											-0.16	0.05	-0.20	0.02												
	rs2735343[e] (D)													-0.18	0.03												
RPS6KB1	rs8071475 (R)																					**-0.54**	**<0.01**				
	rs1292034[f]											-0.16	0.04														
STK11	rs8111699 (R)															0.46	0.04										
	rs741765[g] (R)	0.86	0.03									**0.62**	**0.01**	**0.83**	**<0.01**	**1.02**	**0.05**	**0.57**	**0.05**								
TSC2	**rs3087631 (R)**	**-0.65**	**0.01**	**-0.74**	**0.01**			**-0.59**	**<.01**	**-0.78**	**0.00**	**-0.51**	**<0.01**	**-0.47**	**<0.01**	**-1.16**	**<.01**									-0.60	0.02
VEGFA	rs3025030					-0.33	0.02																				
	rs3025033			-0.26	0.04							**-0.18**	**0.01**														

[a]r^2=0.81 among white population, 0.66 among black population. [b]Pairwise r^2 values ranging from 0.56 to 0.88 among white population. [c]Pairwise r^2 values ranging from 0.59 to 0.84 among black population. No homozygote variants. [e]r^2=0.96 among white population, 1.00 among black population. [f]Out of HWE among white population (p=0.0004). [g]Only two homozygote rare variants. [h]β coefficients and corresponding *p* values for models adjusted for age, admixture, and sex; significant associations after adjustment for multiple comparisons in bold fond.

Table 3. Percent difference in expression by genotypes

Genotype		Gene Expression												
		AKT1	IKBKB	MTOR	NFKB1	PDK1	PIK3CA	PRKAG2	PTEN	RPS6KB1	RPS6KB2	STK11	TSC2	VEGFA
Gene	SNP (Model)	PD[h]	PD	PD	PD	PD	PD	PD	PD	PD	PD	PD	PD	PD
AKT1	rs1130214 (R)					77.35%								
MTOR	rs2295080 (D)		71.26%											
NFKB1	rs13117745[a]											45.36%		
	rs1801[b] (R)					59.61%	35.90%	57.88%						
	rs3821958[b,c] (R)						18.80%							
	rs3774964[b,c] (R)						34.23%							
	rs12509517[b]										36.21%			58.59%
	rs3755867[b,c] (R)					63.51%								
	rs4648090											36.07%		
	rs4648110[a]											49.21%		
	rs4648127[d] (D)									60.85%	55.91%			
PIK3CA	rs7644648 (D)		40.62%			45.05%								
	rs7651265[d] (D)		54.36%			44.67%						31.86%		
	rs1607237 (R)			58.26%										
PRKAG2	rs1362236			34.02%										54.70%
	rs1001117 (D)									48.64%	24.64%			
	rs6965771 (D)		71.68%	61.04%	46.37%	65.12%	46.53%		57.44%	45.52%	31.78%		62.17%	39.32%
	rs7784818 (R)	37.07%	69.22%			46.17%			54.29%	46.63%	42.34%			50.63%
	rs9648724								57.65%	47.56%				
PTEN	rs1903858[e] (D)						22.81%	36.63%						
	rs2735343[e] (D)							24.32%						
RPS6KB1	rs8071475 (R)											64.96%		
	rs1292034[f]						44.16%							
STK11	rs8111699 (R)								68.62%					
	rs741765[g] (R)	84.70%					81.37%	67.71%	96.12%	66.55%				
TSC2	rs3087631 (R)	198.28%	143.02%		166.18%	146.68%	142.45%	108.38%	150.09%					110.74%
VEGFA	rs3025030			49.59%										
	rs3025033		71.64%				18.87%							

[a]r^2=0.81 among white population, 0.66 among black population. [b]Pairwise r^2 values ranging from 0.56 to 0.88 among white population. [c]Pairwise r^2 values ranging from 0.59 to 0.84 among black population. [d]No homozygote rare variants. [e]r^2=0.96 among white population, 1.00 among black population. [f]Out of HWE among white population (p=0.0004). [g]Only two homozygote rare variants. [h]Percent difference (PD)=100*(absolute difference of adjusted medians)/(maximum of absolute value of adjusted medians); medians adjusted for age, admixture, and sex. Dominate model used in place of additive to limit tables to one value per expression/genotype pair.

Table 4. Summary of SNPs associated with gene expression

Symbol	SNP	Chromosome Location	Function		Regulation Potential	Major/Minor Allele	MAF[a] NHW	MAF[a] Hispanic	MAF[a] AA	OR (95% CI)[b] Colon	OR (95% CI)[b] Rectal
AKT1	rs1130214	14q32.32	Intronic	TFBS[h]	0.15	G/T	0.29	0.26	0.41	1.25 (0.98, 1.59)	1.29 (0.93, 1.77)
mTOR	rs2024627	1p36.2	Intronic		NA	C/T	0.26	0.21	0.60	1.16 (1.01, 1.33)[e]	0.97 (0.80, 1.17)
mTOR	rs2295080	1p36.2	5'UTR	TFBS	0.28	T/G	0.29	0.27	0.77	**1.21 (1.06, 1.39)**	1.06 (0.88, 1.29)
NFKB1	rs13117745	4q24	Intronic		0	C/T	0.15	0.10	0.27	0.64 (0.39, 1.05)	1.55 (0.85, 2.79)
NFKB1	rs1801	4q24	Intronic		0.02	G/C	0.36	0.46	0.29	1.02 (0.84, 1.24)	1.20 (0.92, 1.57)
NFKB1	rs3821958	4q24	Intronic		0	A/G	0.41	0.47	0.41	1.02 (0.86, 1.22)	**1.28 (1.00, 1.64)**
NFKB1	rs3774964	4q24	Intronic		0.15	A/G	0.36	0.43	0.40	1.04 (0.86, 1.26)	**1.34 (1.03, 1.75)**
NFKB1	rs12509517	4q24	Intronic		0	G/C	0.29	0.40	0.08	1.04 (0.82, 1.33)	**1.44 (1.04, 1.99)**
NFKB1	rs3755867	4q24	Intronic		0	A/G	0.31	0.43	0.32	1.04 (0.84, 1.28)	1.23 (0.91, 1.64)
NFKB1	rs4648090	4q24	Intronic		0.09	G/A	0.14	0.11	0.15	0.64 (0.37, 1.13)	1.14 (0.57, 2.28)
NFKB1	rs4648110	4q24	Intronic		0.03	T/A	0.20	0.14	0.29	**0.66 (0.46, 0.97)**	1.30 (0.81, 2.09)
NFKB1	rs4648127	4q24	Intronic		0	C/T	0.06	0.03	0.00	1.07 (0.87, 1.31)	1.18 (0.86, 1.61)
PIK3CA[e]	rs7644648	3q26.3	Unknown		NA	A/G	0.18	0.23	0.39	1.11 (0.96, 1.27)	1.12 (0.91, 1.37)
PIK3CA	rs7651265	3q26.3	Unknown		NA	A/G	0.11	0.14	0.11	1.17 (0.61, 2.24)	2.32 (1.02, 5.30)
PIK3CA	rs1607237	3q26.3	Intronic		NA	T/C	0.41	0.38	0.13	1.02 (0.85, 1.23)	0.88 (0.68, 1.12)
PRKAG2	rs1362236	7q36.1	Intronic		0	C/T	0.17	0.19	0.05	0.97 (0.64, 1.47)	0.98 (0.55, 1.73)
PRKAG2	rs1001117	7q36.1	Intronic		0	C/T	0.33	0.26	0.35	0.91 (0.79, 1.04)	1.08 (0.89, 1.31)
PRKAG2	rs6965771	7q36.1	Intronic		0.09	C/T	0.28	0.31	0.12	**0.88 (0.77, 1.00)**	0.97 (0.80, 1.18)
PRKAG2	rs7784818	7q36.1	Intronic		0	A/G	0.49	0.43	0.35	1.01 (0.86, 1.18)	1.28(1.02, 1.61)
PRKAG2	rs9648724	7q36.1	Intronic	TFBS	0.17	G/A	0.25	0.19	0.17	**0.74 (0.56, 1.00)**	0.67 (0.43, 1.04)
PTEN	rs1903858	10q23.3	intronic		0	T/C	0.32	0.39	0.42	0.94 (0.82, 1.08)	1.20 (0.98, 1.46)
PTEN	rs2735343	10q23.3	intronic		0.20	G/C	0.34	0.40	0.42	0.92 (0.81, 1.06)	**1.23 (1.01, 1.50)**
RPS6KB1[c]	rs8071475	17q23.1	Intronic	TFBS	0	T/C	0.25	0.23	0.35	0.99 (0.87, 1.13)[e]	0.86 (0.71, 1.03)
RPS6KB1	rs1292034[d]	17q23.1	Intronic	TFBS	0	T/C	0.45	0.44	0.82	0.92 (0.76, 1.12)	0.85 (0.65, 1.11)
STK11	rs8111699	19p13.3	Intronic		NA	C/G	0.46	0.54	0.63	1.10 (0.94, 1.29)	1.03 (0.82, 1.29)
STK11	rs741765	19p13.3	Intronic		0.10	G/A	0.22	0.29	0.22	1.17 (0.87, 1.57)	**1.48 (1.01, 2.18)**
TSC2	rs3087631	16p13.3	3'UTR	miRNA[h]	0.08	A/T	0.18	0.17	0.62	**0.66 (0.47, 0.93)**	0.77 (0.48, 1.23)
VEGFA	rs3025030	6p12	Intronic		0.01	G/C	0.15	0.19	0.08	1.14 (0.73, 1.80)	0.53 (0.25, 1.11)
VEGFA	rs3025033	6p12	Intronic		0.31	A/G	0.16	0.23	0.19	1.46 (0.98, 2.18)	0.61 (0.32, 1.15)

[a]Minor allele frequency (MAF) based on non-Hispanic white (NHW) controls from case/control study (n=2746 NHW; n=150 Hispanic; n=96 African-American (AA). [b]Odds ratio (OR) and 95% confidence interval (CI) from multiple logistic regression analysis models adjusted for age, center, race, and sex using inheritance models presented in **Tables 1** and **2**; homozygote rare compared to homozygote common for additive model, heterozygote/homozygote rare compared to homozygote common for dominant model, and homozygote rare compared to homozygote common/heterozygote for recessive model. [c]Included *RPS6KB1* tagSNPs in analysis because *RPS6KB1* rs180519 (in high LD with rs1292034) interacts with *PIK3CA* rs7640662 for rectal cancer. [d]TagSNP was not included on case/control study platform so the MAF and ORs refer to rs180519 with G/A major/minor alleles; rs1292034 and rs180519 are in high LD with r^2=0.965 according to 1000 Genomes. [e]Colorectal ORs and 95% CIs based on dominant model rather than recessive model, as presented in **Tables 1** and **2**. [h]Transcription factor binding site (TFBS). If a non-coding SNP is located at a TFBS of a gene, then it may affect the level, location or timing of gene expression. miRNA binding site prediction can inhibit protein translation through binding to the end of a mRNA. Regulatory Potential Score downloaded from the UCSC genome bioinformatics web site is used for SNPs outside of the coding region.

Table 5. A bioinformaticss assessment of convergence of genes in biological pathways

Gene Groupings	PANTHER pathways	*p* value	Bonferroni	Fold Enrichment	Gene Groupings	*KEGG Pathways*	*p* value	Bonferroni	Fold Enrichment	Cluster#[1]	SNP Influence on Gene Expression in Pathways
PIK3CA, NFKB1, IKBKB	B cell activation[2]	0.048	0.70	7.8	*AKT1, PIK3CA, NFKB1, IKBKB*	B cell receptor signaling	0.0006	0.03	20.9	***7**	NFKB1 -> PIK3CA; PIK3CA -> IKBKB[3]
AKT1, PIK3CA, NFKB1, IKBKB, PTEN	Inflammation mediated by chemokine and cytokine signaling	0.031	0.55	3.6	*AKT1, PIK3CA, NFKB1, IKBKB*	Chemokine signaling	0.008	0.32	8.4	**7**	NFKB1 -> PIK3CA; PIK3CA -> IKBKB
AKT1, VEGFA, PIK3CA	**VEGF signaling pathway**	0.041	0.65	8.5	*AKT1, VEGFA, PIK3CA*	**VEGF signaling**	0.013	0.45	15.6	**7**	VEGFA -I PIK3CA
AKT1, PIK3CA, PTEN	p53 feedback loops 2	0.027	0.49	10.6	*AKT1, PIK3CA, PTEN*	**Endometrial cancer**	0.006	0.25	22.6	**7**	PTEN -I PIK3CA
						Melanoma	0.012	0.42	16.5	**7**	
AKT1, PIK3CA, NFKB1, IKBKB	**Apoptosis signaling**	0.015	0.31	6.8	*AKT1, PIK3CA, NFKB1, IKBKB*	**Apoptosis**	0.001	0.04	18.0	**3, 7**	NFKB1 -> PIK3CA; PIK3CA -> IKBKB
AKT1, PIK3CA, NFKB1, IKBKB	**T cell activation**	0.012	0.26	7.3	*PDK1, AKT1, PIK3CA, NFKB1, IKBKB*	**T cell receptor signaling**	0.0008	<0.01	18.1	**7**	AKT1 -I PDK1; NFKB1 -> PIK3CA; NKFB1 -> PDK1; PIK3CA -> IKBKB; PIK3CA -> PDK1
AKT1, RPS6KB2, PIK3CA, RPS6KB1, MTOR	PDGF signaling	0.005	0.12	6.1	*AKT1, RPS6KB2, PIK3CA, RPS6KB1, MTOR*	**ErbB signaling**[4]	<0.0001	<0.01	22.5	**1, 7**	RPS6KB1 -I PIK3CA; PIK3CA -I MTOR
AKT1, TSC2, PIK3CA, PTEN	Insulin/IGF-protein kinase B signaling cascade	0.005	0.12	10.0	*AKT1, PRKAG2, TSC2, RPS6KB2, PIK3CA, RPS6KB1, MTOR, IKBKB*	**Insulin signaling**	<0.0001	<0.01	23.2	3	MTOR -> IKBKB; PIK3CA -> IKBKB; PIK3CA -I MTOR; PRKAG2 -I MTOR; PRKAG2 -I AKT1; PRKAG2 -I IKBKB; PRKAG2 -I PIK3CA; PRKAG2 -I/> RPS6KB1; PRKAG2 -I RPS6KB2; PRKAG2 -I/> PTEN; PRKAG2 -I TSC2; PTEN -I PRKAG2; PTEN -I PIK3CA; RPS6KB1 -I PIK3CA; TSC2 -I AKT1; TSC2 -I PIK3CA; TSC2 -I PTEN; TSC2 -I PRKAG2
AKT1, RPS6KB2, PIK3CA, RPS6KB1, PTEN	PI3 kinase	0.001	0.02	9.6							
AKT1, PIK3CA, MTOR, PTEN	Hypoxia response via HIF activation	0.0003	0.01	25.1	*AKT1, PIK3CA, MTOR, PTEN*	**Glioma**	0.0004	0.02	24.8	3	PIK3CA -I MTOR; PTEN -I PIK3CA
AKT1, STK11, PRKAG2, TSC2, RPS6KB2, RPS6KB1, MTOR	p53 by glucose deprivation	4.45E-10	1.11E-08	54.9	*AKT1, STK11, VEGFA, TSC2, RPS6KB2, PIK3CA, RPS6KB1, MTOR*	**mTOR signaling**	<0.0001	<0.01	60.2	**1, 3, 6**[5]	PIK3CA -I MTOR; PIK3CA -> SKT11; PRKAG2 -I AKT1; PRKAG2 -I MTOR; PRKAG2 -I PIK3CA; PRKAG2 -I RPS6KB1; PRKAG2 -I RPS6KB2; PRKAG2 -I TSC2; PRKAG2 -I VEGFA; TSC2 -I AKT1; TSC2 -I PIK3CA; TSC2 -I PRKAG2; TSC2 -I VEGFA; VEGFA -I MTOR; RPS6KB1 -I PIK3CA; RPS6KB1 -I SKT11; SKT11 -> AKT1; SKT11-> PIK3CA; SKT11 -> PRKAG2; SKT11 -> RPS6KB1

[1]Enrichment Scores for Clusters: 1=4.692, 3=3.072, 6=2.651, 7=2.627. [2]Bolded terms are associated with the cluster reference. [3]-I indicates action blocked; -> indicates enhanced activity; in some instances different SNPs within gene were involved in blocking and enhancing activity. [4]PDGF is a growth factor (protein) regulating cell growth, particularly as it pertains to angiogenesis. ErbB belongs to the EGFR (epidermal growth factor receptor) family and as such ErbB signaling is involved with the development of tumors. [5]Regulation of glucose metabolic process is in cluster 2 and involves AKT1, MTOR, and PRKAG2. Glucose metabolic process is in cluster 4 and contains *PDK1, AKT1, PRKAG2* and *PIK3CA* (*AKT1* regulated expression of PDK1).

was influenced by *PIK3CA*, *PRKAG2* and *VEGFA*; NFκB1 expression was altered by *PRKAG2* and *TSC2*; PDK1 was influenced by *AKT1*, *NFκB1*, *PIK3CA*, *PRKAG2*, and *TSC2*; PIK3CA was altered by *NFκB1*, *PRKAG2, PTEN, RPS6KB1, STK11, TSC2,* and *VEGFA*; PRKAG2 expression was altered by *NFκB1*, *PTEN*, and *TSC2*; PTEN was influenced by *PRKAG2*, *STK11*, and *TSC2*; RPS6KB1and RPS6KB2 were influenced by *NFκB1* and *PRKAG2* with RPS6KB1 also influenced by *STK11*; STK11 expression was influenced by *NFκB1*, *PIK3CA* and *RPS6KB1*; TSC2 expression was influenced by *PRKAG2*; and VEGFA expression was influenced by *NFκB1, PRKAG2* and *TSC2*. The percent difference in gene expression across genotypes varied, with ranges from around 35% for many genes to much higher levels of expression as noted for *TSC2* rs3087631 which influenced expression of AKT1 (198%), IKBKB (143%), NFκB1 (166%), PDK1 (147%), PIK3CA (142%), PRKAG2 (108%), PTEN (150%), and VEGFA (111%) (**Table 3**).

Most of the tagSNPs associated with gene expression had limited prior information on potential functionality (**Table 4**). Five SNPs (*AKT1* rs1130214, *mTOR* rs2295080, *PRKAG2* rs9648724, and *RPS6KB1* rs8071475 and rs1292034) were identified as transcriptional factor binding sites and *TSC2* rs3087631 was identified as a miRNA site. Several of these SNPs (n=13) were predicted to have small regulation potential. Corresponding colon and rectal cancer risk associated with these tagSNPs for the most part was modest.

Discussion

Genes in this candidate pathway, which we labeled CHIEF, were selected because of their involvement in hormones, inflammation, and energetic factors. The genes and SNPs we examined were part of a candidate pathway and were evaluated here because of prior associations with colon and rectal cancer [28]. Our findings suggest that several SNPs we had previously identified as being associated with colon or rectal cancer influenced expression of genes in the pathway. While several SNPs located within genes regulated that gene's expression, gene expression was more frequently altered by SNPs in other genes in the pathway. To further evaluate and validate the inter-relatedness of genes and common functions and pathways, we utilized bioinformatics tools. As shown in **Table 5**, these genes are collectively involved in many pathways that could influence cancer risk.

Gene expression levels were altered for four genes by genetic variants in those genes. *mTOR* rs2024627, four SNPs in *NFκB1* (rs1801, rs3821958, rs3774964, rs3755867), three SNPs in *PRKAG2* (rs6965771, rs7784818, and rs9647824), and *TSC2* rs3087631 influenced their respective gene expression. All of these genes had been associated with colon or rectal cancer previously in our studies [28]. *TSC2* rs3087631 had the greatest impact on *TSC2* expression, with a 159% relative difference in expression between the common homozygote and heterozygote variants and the homozygote rare variant and also was associated with levels of expression of several other genes in the pathway. The homozygote rare genotypes of SNPs in *NFκB1* were associated with a slight increase in *NFκB1* expression, and with lower levels of pathway gene expression.

As we hypothesized, the majority of variation in gene expression was the result of genetic variants in the pathway influencing expression of other pathway genes. Although a pathway approach to evaluating expression in genes within the pathway is not commonly done, it is reasonable given what we know about the genes and the pathway. We observed that genetic variants in *IkBκB*, *PDK1* and *RPS6KB2* did not alter expression of any genes within the pathway, although their expression was altered by several genes. AKT1 is phosphorylated by PDK1 [29], leading to partial activation of AKT. Full activation of AKT1 occurs upon phosphorylation by the TORC2 complex of the mTOR protein kinase. Our findings suggest that variation in *AKT1* can decrease expression of PDK1, which in turn may decrease phosphorylation of AKT1. Phosphorylation turns protein activity on and off, altering function at post-translational level. This may imply a feedback loop. Another example of a potential feedback loop in our data is between TSC2 and AKT1. TSC2 functions downstream of Akt and upstream of mTOR and is inactivated when phosphorylated by Akt. Depression of TSC2 in turn would increase activation of mTOR and S6K since TSC2 functions as a tumor suppressor to inhibit cell growth and functions as an inhibitor of mTOR and S6K (RPS6KB) [30]. However in our data *TSC2* altered expression of AKT1 rather than AKT1

altering expression of TSC2. IκB has been shown to block the TSC1/TSC2 complex [31], however in our data *TSC2* rs3087631 reduced expression of IκBκB.

Our data also show increased gene activation that is supported in the literature. PIK3CA has been shown to activate PDK1 [32] and we observed increased PDK1 expression with *PIK3CA* rs7644648 and rs7651265. NFκB family is involved in many biological pathways and is activated by many stimuli such as cytokines and oxidant-free radicals. PIK3CA is transcriptionally regulated through the NFκB pathway [33] and we observed increased PIK3CA expression with several NFκB SNPs.

AMP-activated protein kinase (AMPK) is a key regulator of energy homeostasis. The official gene name for AMPK is *PRKA* with subunits A1, A2, B1, B2, and G1, G2, and G3; we observed associations with colon cancer for the G2 subunit (PRKAG2). In our data *PRKAG2* influenced expression of AKT1, IKBκB, MTOR, NFκB1 (which also regulated its expression), PDK1, PIK3CA, PTEN (which also regulated its expression), RPS6KB1, RPS6KB2, TSC2 (which also regulated its expression) and VEGFA. AMPK is involved in numerous pathways. AMPK signaling has been shown to inhibit inflammatory response induced with the NFκB pathway through a non-phosphorylation pathway and has been shown to inhibit oxidative stress [34]. In our data PRKAG2 reduced expression of both IKBκB and NFκB1. In addition to the anti-inflammatory mechanisms associated with AMPK, it plays a key role in the LKB1 (STK11)/AMPK/TSC/mTOR pathway [35]. AMPK can inhibit TSC2 and mTOR along this pathway [36]; mTOR activates S6K. We observed that PRKAG2 was inversely related to expression of TSC2, mTOR, and RPS6KB2. PI3Ks and PTEN feed into this pathway and modulate AMPK expression; variants in PRKAG2 decreased expression of both PIK3CA and PTEN.

TSC2 rs3087631 was associated with reduced expression of several components of this pathway, including AKT1, IKBκB, NFκB1, PDK1, PIK3CA, PRKAG2, PTEN, and VEGFA. TSC2 is central to the mTOR pathway where PTEN suppressing PI3K, PI3K and PDK1 enhances AKT, and AKT inhibits TSC2. AMPK can phosphorylate TSC2 to activate it. The TSC1/TSC2 complex modulates NFκB activity by regulating AKT signaling that activates NFκB [37]. TSC2-deficient cells have been shown to have reduced NFκB activation [37]. *TSC2* rs3087631 was inversely related to TSC2 expression as well as expression of NFκB1 and IKBκB.

Several considerations should be made when evaluating these results. First, there were few samples and they included only normal colon tissue. Because of the small sample size it was difficult to evaluate recessive models that may have been associated with colorectal cancer risk. Since the samples were limited to normal colon tissue, we were unable to evaluate expression or changes in gene expression in tumors. Other genes and genetic variants in the pathway may have been important; this study included genes where we previously observed an association with CRC risk. We evaluated functionality by associations with gene expression, however, lack of association with gene expression does not rule out functionality of that gene or SNP. While gene expression studies mainly test the influence of that SNP to alter transcription, SNPs could have an impact through other mechanisms, such as altering protein expression and stability. Thus, while gene expression can provide an indication of functionality of a given SNP, SNPs can be functionally significant without altering gene expression. Finally, gene expression could be influenced by non-genetic factors, including diet and lifestyle factors, that could influence gene expression independently or in conjunction with genetic factors. We were able to only evaluate independent genetic factors in this study.

Conclusions

In conclusion, several genes in our candidate pathway influenced expression of other genes in that pathway in normal colon tissue. Our results suggest that genetic variation in these genes may importantly regulate expression of other genes in the pathway. The convergence of these genes in several biological pathways involved in cancer further supports their importance to the carcinogenic process.

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Disclosure of conflict of interest

No authors have any competing interest to report.

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