Original Article

Coexpression and expression quantitative trait loci analyses of the angiogenesis gene-gene interaction network in prostate cancer

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Background: Prostate cancer (PCa) shows a substantial clinical heterogeneity. The existing risk classification for PCa prognosis based on clinical factors is not sufficient. Although some biomarkers for PCa aggressiveness have been identified, their underlying functional mechanisms are still unclear. We previously reported a gene-gene interaction network associated with PCa aggressiveness based on single nucleotide polymorphism (SNP)-SNP interactions in the angiogenesis pathway. The goal of this study is to investigate potential functional evidence of the involvement of the genes in this gene-gene interaction network.

Methods: A total of 11 angiogenesis genes were evaluated. The crosstalks among genes were examined through coexpression and expression quantitative trait loci (eQTL) analyses. The study population is 352 Caucasian PCa patients in the Cancer Genome Atlas (TCGA) study. The pairwise coexpressions among the genes of interest were evaluated using the Spearman coefficient. The eQTL analyses were tested using the Kruskal-Wallis test.

Results: Among all within gene and 55 possible pairwise gene evaluations, 12 gene pairs and one gene (MMP16) showed strong coexpression or significant eQTL evidence. There are nine gene pairs with a strong correlation (Spearman correlation ≥0.6, $P<1\times10^{-13}$). The top coexpressed gene pairs are *EGFR-SP1* (r=0.73), *ITGB3- HSPG2* (r=0.71), *ITGB3- CSF1* (r=0.70), *MMP16-FBLN5* (r=0.68), *ITGB3-MMP16* (r=0.65), *ITGB3-ROBO1* (r=0.62), *CSF1- HSPG2* (r=0.61), *CSF1-FBLN5* (r=0.6), and *CSF1-ROBO1* (r=0.60). One cis-eQTL in *MMP16* and five trans-eQTLs (*MMP16-ESR1*, *ESR1-ROBO1*, *CSF1-ROBO1*, *HSPG2-ROBO1*, and *FBLN5-CSF1*) are significant with a false discovery rate q value less than 0.2.

Conclusions: These findings provide potential biological evidence for the gene-gene interactions in this angiogenesis network. These identified interactions between the angiogenesis genes not only provide information for PCa etiology mechanism but also may serve as integrated biomarkers for building a risk prediction model for PCa aggressiveness.

Keywords: Gene; expression; prostate cancer; single nucleotide polymorphisms (SNPs); interaction

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Introduction

Prostate cancer (PCa) accounted for 26% of cancer incidence and 9% of cancer deaths in American men in 2015 (1). PCa is a clinically heterogeneous disease but its risk classification is insufficient. About 30% of men are classified as a low risk group, but these men developed to high-grade cancer (2). Due to this inadequate classification, it is difficult for physicians to select a suitable treatment plan for PCa patients. Thus, understanding PCa etiology mechanisms and identifying biomarkers for improving prediction accuracy of PCa aggressiveness is essential to treating patients.

Angiogenesis plays an important role in prostate tumor growth and development. Angiogenesis involves the division and migration of endothelial cells and leads to microvasculature formation (3,4). Based on the genome-wide association studies (GWAS), angiogenesis genetic variants or single nucleotide polymorphisms (SNPs) in or near *IL-16* (rs4072111) and *FGFR2* (rs11199874, rs10749408 and rs10788165) are significantly associated with PCa aggressiveness (5,6). In candidate gene studies, several angiogenesis SNPs are shown to be associated with PCa prognosis. The pro-angiogenic genes include *VEGFs*, *FGF*, *EGF*, *HIF*, *TGF-β* and *TNF-α*; the anti-angiogenic genes include endostatin, *IFN*, *MMPs*, and *ILs*; and pro-/anti-angiogenic genes include *MMPs* and *ILs* (7). The functional mechanism of the majority of these genetic variants remain unclear.

It has often been shown that single gene effects are not sufficient to explain the complicated relationships among genes (8-11). Our previous study suggested an angiogenesis gene-gene interaction network associated with PCa aggressiveness (12). This network was built upon the SNP-SNP interactions of five gene pairs (*MMP16-ROBO1*, *MMP16-CSF1*, *FBLN5-CSF1*, *CSF1-HSPG2*, and *MMP16-EGFR)* and published protein-protein interactions. There are 12 genes identified (*FBLN5*, *ROBO1*, *E2F1*, *STAT1*, *HSPG2*, *MMP16*, *ITGB3*, *ESR1*, *EGFR*, *CSF1*, *SP1*, and *AP1*) in this network.

Among these angiogenesis genes, a majority of them were shown to be associated with PCa risk or prognosis. A key biological role of *FBLN5* in human cells is communication among cells and between cells to matrix. *FBLN5* is consistently downregulated in prostate tumors in data from expression microarray and RT-PCR (13). A recent study suggested *ROBO1* as a tumor suppressor in PCa (14). Several groups consistently reported an association between an overexpression of *E2F1*, a cell cycle-specific transcription factor, with progression of PCa, especially PCa metastasis (15-18). Patterson *et al.* (19) suggested that STAT1 expression affects the chemoresistant phenotype especially to docetaxel treatment. Therefore, STAT1 plays a key role in docetaxel resistance in PCa treatment. The HSPG2 is a five-domain proteoglycan that interacts with extracellular matrix components and cell-surface molecules. Expression of HSPG2 is associated with high Gleason scores (20), prostate tumor growth, and enhanced angiogenesis (21). Overexpression of HSPG2 is required for invasion of prostate tumors (22). *MMP16* has been shown to be down-regulated in malignant prostate tissues (23). Xu *et al.* [2103] identified *ITGB3* as one of the genes related to tumor metastasis in various PCa cell lines (24). Estrogen receptor 1 (*ESR1*) is a ligand-activated transcription factor with domains for binding to hormones and DNA. *ESR1* is associated with PCa risk because it stimulates proliferation of prostate cells and deregulates apoptosis (25). The EGFR binds the epidermal growth factor (EGF) and has been shown to play an important role in regulating prostate cellular growth and function (26-28). Overexpression of SP1 was observed in several cancers, including PCa, and increased angiogenesis and decreased cancer cell death (29). *AP1* activation is shown to be essential for inducing proliferation and anchorage independence in PCa cells (30).

This angiogenesis gene-gene interaction network is novel, but its functional mechanism is unclear. SNPs may impact the process of angiogenesis through influencing gene expressions. Gene expressions are involved in many important biological processes. It has been shown that 83% of genes are differentially expressed among individuals; evaluating gene expression variations may provide useful information for disease or other phenotype development (31). For identifying functional roles of genetic variants, using expression quantitative trait loci (eQTL) analyses to evaluate gene expressions as intermediate phenotypes has been applied to identify downstream genes (32,33). In addition to evaluating each gene individually, it has been shown that coexpressions can be used to demonstrate that these genes have a functional relationship, such as physical interaction between the encoded proteins. Evaluating coexpressions can also be used to identify a group of interactive genes (34). Therefore, the goal of this study is to evaluate functional mechanisms of this reported angiogenesis gene-gene interaction network through performing coexpression and eQTL analyses.

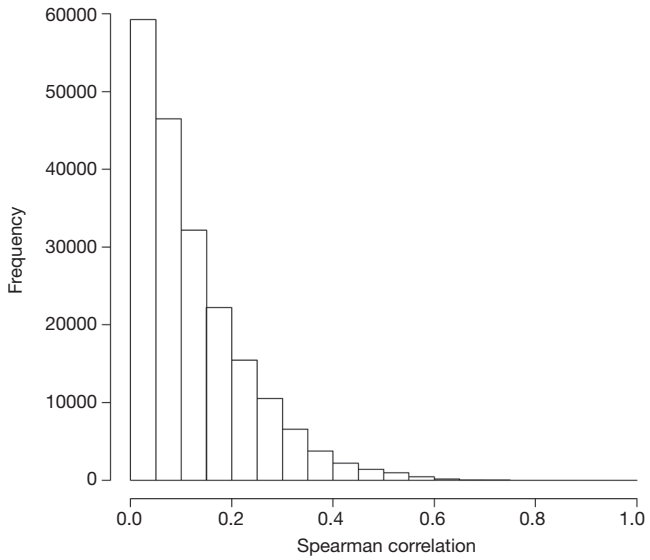

Figure 1 Distribution of coexpression strength of noise gene pairs. Gene coexpressions measured by the Spearman correlations for 10 random genes with all genes in the array (20,503 genes) from the 350 prostate tumor samples in the TCGA study. Only 0.12% coexpressions with a r≥0.6. TCGA, the Cancer Genome Atlas.

Methods

Gene and SNP selection

The 11 candidate genes (*FBLN5*, *ROBO1*, *E2F1*, *STAT1*, *HSPG2*, *MMP16*, *ITGB3*, *ESR1*, *EGFR*, *CSF1*, and *SP1*) were selected based on the reported angiogenesis gene-gene interaction network associated with PCa aggressiveness (12). The *AP1* was not included in the analysis due to lack of availability in the TCGA data. A total of 434 SNPs in these 11 angiogenesis genes was evaluated.

The Cancer Genome Atlas (TCGA) study population and genetic data

The TCGA SNP and gene expression data were used in this study. TCGA is a large-scale study led by the National Institutes of Health (NIH) to map the genomic changes that occur in more than 30 human cancer types. Its goal is to support new discoveries and accelerate research aimed at improving the prevention, diagnosis, and treatment of cancer (35). The eQTL and gene coexpression analyses were performed for Caucasians in this study due to small sample sizes of other race groups. Among 438 PCa patients recruited between 2009 and 2013 in the TCGA study, we applied the program LAMP-LD (36) to estimate ancestry based on the HapMap (37) data for the two major populations (Caucasian and African American). The ancestry test was performed using 347,481 SNP data from the blood samples. Caucasians were defined as European and those with greater than 80 percent European ancestry (38,39). The genotype call rate for our candidate SNPs was >95%. Among 411 PCa patients with both valid genotype and gene expression data, 350 patients were defined as Caucasian. RNA profiling was performed using Illumina HiSeq 2000 RNA Sequencing. The normalized RNAseq level 3 expression data were used in our analyses. The details are listed on the TCGA website (http://cancergenome.nih.gov).

Statistical analyses

All possible pairwise coexpressions among the candidate genes were evaluated using the Spearman coefficient (r). To our knowledge, there is no well-defined cutoff to define a strong coexpression. To identify a meaningful coexpression cutoff, we generated a null distribution of coexpressions from a set of 10 randomly selected genes with all 20,503 genes in the testing array. In this null distribution for coexpression (*Figure 1*), there were only 0.12% gene pairs with r≥0.6. Thus, we used a conservative cutoff of r=0.6 to define strong coexpressions as those with a r≥0.6.

The eQTL analyses were applied to evaluate associations between one SNP and one gene expression. There are two types of eQTLs. Cis-eQTLs are eQTLs that map the approximate location of their gene-of-origin gene. In contrast, trans-eQTLs are those that map far from the location of their gene-of-origin gene. We performed a total of 4,774 (=11 gene expression × 434 SNPs) pairwise eQTL analyses, including both cis- (locally) and trans- (at a distance) eQTL analyses. The difference of gene expressions among the three genotypes (homozygous wild, heterozygous and homozygous variant types) was evaluated using the Kruskal-Wallis test. Adjusting for multiple comparisons, the false discovery rate (FDR) q value (40) was applied. We declared significance by using the FDR q value less than 20% as the cutoff. Linkage disequilibrium (LD), correlations between SNPs, was evaluated using r^2. The strong LD was defined as $r^2>0.8$.

Results

The participants' demographic and clinical characteristics are summarized in *Table 1*. All 55 possible pairwise

Table 1 Participant's demographic and clinical characteristics

Characteristics	N (%)
Age at pathology diagnosis (mean ± SD) (years)	61.5±6.6
Self-report race	
Whites	133 (38.0)
Not available	217 (62.0)
Pathology T stage	
T1	0
T2	134 (39.1)
T3	199 (58.0)
T4	10 (2.9)
Gleason score	
6	29 (8.3)
7	173 (49.6)
8	56 (16.1)
9	88 (25.2)
10	3 (0.9)
Vital status	
Alive	344 (98.6)
Dead	5 (1.4)

coexpressions among the 11 genes were evaluated using the Spearman correlations (*Table 2*). The *ITGB3* and *CSF1* are co-expressed with several genes in this angiogenesis network. Among them, 19 pairs (34.5%) had a correlation ≥0.5 and nine gene pairs (16.4%) had a strong coexpression (r≥0.6). These top co-expressed gene pairs are *EGFR-SP1* (r=0.73), *ITGB3- HSPG2* (r=0.71), *ITGB3- CSF1* (r=0.70), *MMP16-FBLN5* (r=0.68), *ITGB3-MMP16* (r=0.65), *ITGB3-ROBO1* (r=0.62), *CSF1-HSPG2* (r=0.61), *CSF1-FBLN5* (r=0.6), and *CSF1-ROBO1* (r=0.60). All these strong coexpressions have a P value $<1\times10^{-13}$.

Among the 4,774 eQTL tests, there were 21 significant tests with a FDR q value<0.2 (*Table 3*). These associations were found in a total of five gene pairs (*MMP16-ESR1*, *ESR1-ROBO1*, *CSF1-ROBO1*, *HSPG2-ROBO1*, and *FBLN5-CSF1*) and one SNP-expression pair within the same gene (*MMP16*, *cis-eQTL*). The most significant test is the association between rs2982705 in *ESR1* and *MMP16* expression (raw $P=7.5\times10^{-5}$, FDR q=0.12). rs162268 in *ROBO1* is significantly associated with ESR1 expression (raw $P=1.1\times10^{-4}$, FDR q=0.12). rs6788511 in *ROBO1* is associated with *CSF1* gene expression (raw $P=1.3\times10^{-4}$, FDR q=0.12). The box plots of the top six eQTL tests are shown in *Figure S1*. In the top eQTL list, several SNPs in the same gene were shown. We further evaluated LD among these SNPs in the same gene. The LD plots are

Table 2 Coexpressions among the 11 angiogenesis genes

Gene [chr]†	*FBLN5*	*ROBO1*	*E2F1*	*STAT1*	*HSPG2*	*MMP16*	*ITGB3*	*ESR1*	*EGFR*	*CSF1*	*SP1*
FBLN5 [14]	1										
ROBO1 [3]	0.34	1									
E2F1 [20]	−0.05	−0.24	1								
STAT1 [2]	0.07	0.38	−0.20	1							
HSPG2 [1]	0.55	0.46	−0.12	0.14	1						
MMP16 [8]	0.68*	0.50	−0.23	0.30	0.57	1					
ITGB3 [17]	0.47	0.62*	−0.36	0.29	0.71*	0.65*	1				
ESR1 [6]	0.52	0.50	−0.24	0.26	0.46	0.44	0.46	1			
EGFR [7]	0.09	0.57	−0.33	0.51	0.43	0.43	0.57	0.37	1		
CSF1 [1]	0.60*	0.60*	−0.21	0.38	0.61*	0.57	0.70*	0.59	0.45	1	
SP1 [12]	0.10	0.46	−0.34	0.47	0.33	0.44	0.46	0.29	0.73*	0.35	1

†, coexpression were measured using the Spearman correlations (r); *, strong coexpressions (r≥0.6 and $P<1\times10^{-13}$). chr, chromosome.

Table 3 The significant expression quantitative trait loci (eQTL) results†

Gene_Exp	Gene_SNP	SNP	Min/Maj	MAF	P value	FDR q value
MMP16	*ESR1*	rs2982705	G/C	0.39	7.5×10^{-5}	0.12
ESR1	*ROBO1*	rs162268	C/T	0.35	1.1×10^{-4}	0.12
		rs162263	C/A	0.32	2.0×10^{-4}	0.12
		rs328047	C/T	0.36	2.2×10^{-4}	0.12
		rs162429	G/C	0.34	3.7×10^{-4}	0.15
CSF1	*ROBO1*	rs6788511	A/C	0.24	1.3×10^{-4}	0.12
		rs3821603	T/G	0.21	1.5×10^{-4}	0.12
		rs1457659	G/A	0.25	1.5×10^{-4}	0.12
		rs6787349	T/C	0.25	2.8×10^{-4}	0.12
		rs7610686	A/C	0.23	4.4×10^{-4}	0.16
		rs17375110	A/G	0.23	6.2×10^{-4}	0.16
		rs2271151	T/C	0.22	6.5×10^{-4}	0.16
		rs6788434	T/C	0.22	6.8×10^{-4}	0.16
		rs17016466	C/T	0.22	6.9×10^{-4}	0.16
		rs17375496	T/C	0.23	9.2×10^{-4}	0.19
MMP16	*MMP16*	rs10100297	C/T	0.37	2.0×10^{-4}	0.12
		rs6994019	A/C	0.31	7.7×10^{-4}	0.17
		rs10955542	A/C	0.29	7.9×10^{-4}	0.17
HSPG2	*ROBO1*	rs1457659	G/A	0.25	2.7×10^{-4}	0.12
		rs6787349	T/C	0.25	6.2×10^{-4}	0.16
FBLN5	*CSF1*	rs3093045	C/G	0.02	6.0×10^{-4}	0.16

†, significance is defined as a false discovery rate (FDR) q value<0.2. Gene Exp, gene for a gene expression; Gene_SNP, gene for a SNP; Min/Maj, minor and major allele; MAF, minor allele frequency.

listed in *Figure S2*. The majority of SNPs in the same gene have strong LD (r^2>0.8).

The results of coexpression and eQTL analyses are summarized in *Figure 2* and *Table 4*. Our findings demonstrate 11 gene-gene interactions in the angiogenesis network. Among the previous SNP-SNP interactions in the five gene pairs (*MMP16-ROBO1*, *MMP16-CSF1*, *FBLN5-CSF1*, *CSF1-HSPG2*, and *MMP16-EGFR*), two of them (*FBLN5-CSF1* and *CSF1-HSPG2)* are supported by direct coexpression and/or eQTL results. The interaction between *FBLN5* and *CSF1* was observed in both coexpression (r=0.6) and eQTL analyses (rs3093045 in *CSF1* associated with the *FBLN5* expressions, $P=6.0\times10^{-4}$). The interaction between *CSF1* and *HSPG2* was supported by the strong coexpression (r=0.61). For three other gene pairs (*MMP16-ROBO1*, *MMP16-CSF1*, and *MMP16-EGFR*), no direct gene interactions were observed but they may have interacted through another gene. As shown in *Table 4*, our findings suggest that the interaction of *MMP16* and *ROBO1* may be through *ESR1* or *ITGB3*. For *MMP16* and *CSF1*, they may be interacted through *FBLN5* or *ITGB3*, while the crosstalk of *MMP16* and *EGFR* may be through *ITGB3*.

Conclusions

Our findings reveal potential functional mechanisms for the angiogenesis gene-gene interaction network, which has a reported association with PCa aggressiveness (12). This study successfully provides direct and indirect functional evidence of these gene-gene interactions. Among the five

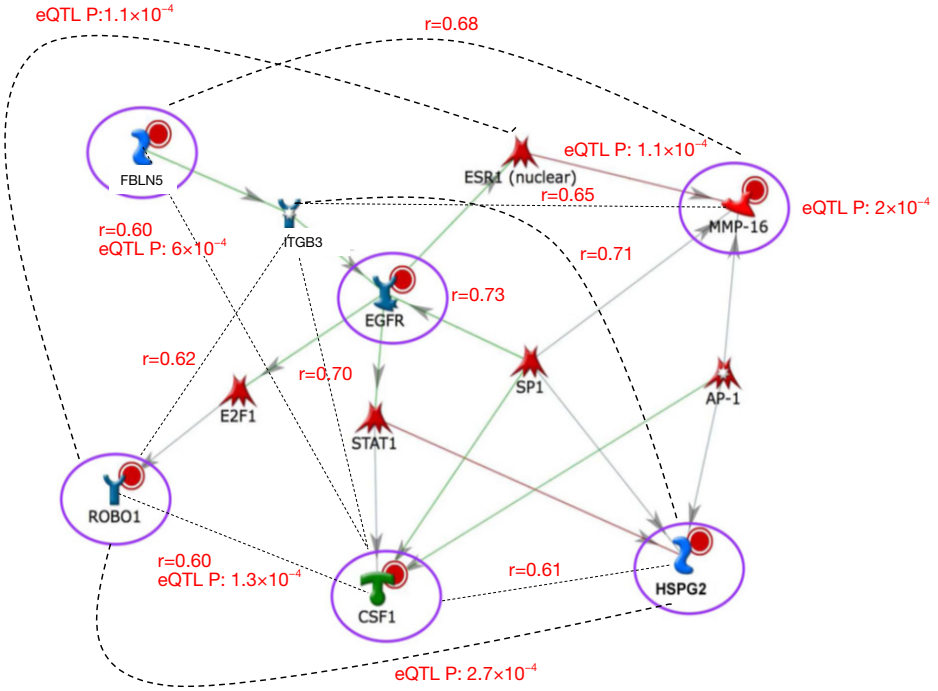

Figure 2 Coexpressions and eQTL results in the angiogenesis network of prostate cancer aggressiveness. r, the Spearman coefficient; eQTL P, P value of expression quantitative trait loci analysis using the Kruskal-Wallis test. The genes and solid lines are from on the published network. The dashed lines are based on the coexpression and eQTL results. The circled genes are based on the SNP-SNP interactions in (12).

gene pairs (*MMP16-ROBO1*, *MMP16-CSF1*, *FBLN5-CSF1*, *CSF1-HSPG2*, and *MMP16-EGFR)* with SNP-SNP interactions (12), two of them (*FBLN5-CSF1*, *CSF1-HSPG2)* are supported by direct coexpression and/or eQTL results; the other three gene pairs may interact indirectly through another gene in this network.

Among 55 possible gene pairs in the angiogenesis network, 12 gene pairs (21.8%) have strong coexpressions or significant eQTL (or SNP-expression) results. Among them, the interactions of two gene pairs (*CSF1-ROBO1* and *FBLN5-CSF1*) are supported by both coexpressions and eQTL results in this study. The relationships between these genes are understudied, although these genes individually are shown to associate with PCa risk and progression. Among the coexpression gene pairs identified, *CSF1* and *ITGB3* were involved in multiple coexpression pairs, thus a network correlation was implied. To assess a potential biological relevance of these genes and explore the underlying functional mechanism, we updated our genetic regulatory network, which were reported previously (41) (*Figure 2*). The interconnectedness of biochemical process, coexpression and eQTL networks of the identified genes showed that the six proteins were involved directly or indirectly in the EGFR signaling pathway. This network suggested that these genes are regulated by multiple proteins like receptors and transcription factors. The most prominent protein in the network is CSF1 which was directly co-expressed or interacted with the four angiogenesis proteins (*Table 4*, FBLN5, ROBO1, HSPG2, and ITGB3). Indeed the CSF1-ITGB3 pair showed a strong coexpression (r=0.7). Although studies of crosstalk between *ITGB3* and *CSF1* in PCa are limited, both *CSF1* and *ITGB3* were included in a gene signature of vitamin D exposure in breast cancer (42).

Potential functional role, relation to angiogenesis, and relation to prostate etiology of the 11 candidate genes are briefly summarized in *Table 5*. The exact role of colony stimulating factor-1 (CSF1) in PCa is not fully established yet. However, previous studies on other cancers, such as that of breast, ovary and endometrial tissues, reported that an overexpression of CSF1 was observed in cancer patients (69,70), increased tumor angiogenesis (69), promoted metastatic potential in breast cancer (64) and was associated with poor outcome in ovarian cancer (65). These biological roles of CSF1 were also confirmed in a murine pancreatic cancer study (41). Recently in their preclinical study, Garcia

Table 4 Summary table of results of co-expressions, eQTL analyses, and SNP-SNP interactions in the angiogenesis network

Gene 1	Gene 2	Co-ex r[†]	eQTL P value[‡]	SNP-SNP int[§]	Possible mechanism
CSF1	*ROBO1*	0.60	1.3×10^{-4}		
FBLN5	*CSF1*	0.60	6.0×10^{-4}	Yes	
EGFR	*SP1*	0.73			
ITGB3	*HSPG2*	0.71			
ITGB3	*CSF1*	0.70			
MMP16	*FBLN5*	0.68			
ITGB3	*MMP16*	0.65			
ITGB3	*ROBO1*	0.62			
CSF1	*HSPG2*	0.61		Yes	
MMP16	*ESR1*		7.5×10^{-5}		
ESR1	*ROBO1*		1.1×10^{-4}		
MMP16	*MMP16*		2.0×10^{-4}		
HSPG2	*ROBO1*		2.7×10^{-4}		
MMP16	*ROBO1*			Yes	*MMP16-ESR1-ROBO1* (eQTL P: 7.5×10^{-5} and 1.1×10^{-4}); *MMP16-ITGB3-ROBO1* (r=0.65 and 0.62)
MMP16	*CSF1*			Yes	*MMP16-FBLN5-CSF1* (*MMP16-FBLN5*: r=0.68; *FBLN5-CSF1*: r=0.60, eQTL P: 6×10^{-4}); *MMP16-ITGB3-CSF1* (r=0.65 and 0.70)
MMP16	*EGFR*			Yes	*MMP16-ITGB3-EGFR* (r=0.65 and 0.57)

[†], expression measured using the Spearman correlation; [‡], the Kruskal-Wallis test P value; [§], significant SNP-SNP interactions associated with prostate cancer aggressiveness (12). eQTL, expression quantitative trait loci.

et al. [2014] demonstrated that CSF1 can be a potential target for PCa treatment (66).

Integrins play important roles in signal transduction and are known to be involved in carcinogenesis, including colorectal (54), lung (71), and prostate cancers (59). The ITGB3 is a key player in tumor growth and metastasis and a key regulator in reactive oxygen species-induced migration and invasion of cancer cells (54). Ni *et al*. reported that downregulated expression of ITGB3 reduced cell proliferation, migration, and invasion in cancer cells (55). For PCa, ITGB3 is a part of a gene expression signature for detecting the presence of prostate tumors in stroma with 97% accuracy (59). ITGB3 expression was significantly lower in tumor-adjacent stroma compared to normal stroma (60). These results were consistent in the immunohistochemistry assays: ITGB3 protein showed lower protein expression in tumor-adjacent stroma compared to the normal stroma.

Among 12 interacted gene pairs, only *EGFR-SP1* interaction was previously reported. The epidermal growth factor receptor (EGFR) is involved in the proliferation of epithelial cells and tumorigenesis. Our previous study and a meta-analysis study identified *EGFR* in both analyses of gene and pathway levels associated with PCa risk (12,63). Specificity protein 1 (Sp1) is a transcription factor that affects the expression of genes involved in various cellular processes and oncogenesis. Further, expression of Sp1 is associated with the prognosis of patients (67) and can contribute to predicted PCa recurrence (68). Among two SNPs in the promoter region of *EGFR*, -216G/T was located in a *Sp1* recognition site. Transient transfection assay showed significantly increased promoter activity in the -216G allele as compared with the -216T allele. These findings were confirmed in an additional transient transfection assay in the *Sp1*-deficient cell line and electrophoretic mobility shift assay, which confirmed a significantly higher binding efficiency of Sp1 protein to the T allele compared with the G allele (72). Liu *et al*.

Table 5 Summary of functional role, relation to angiogenesis and prostate cancer (PCa) etiology of the 11 candidate genes

Gene	Functional role	Relation to angiogenesis	Relation to prostate cancer etiology
FBLN5	*FBLN5* is one of the extracellular matrix (ECM) glycoproteins and interacts with other ECM components, such as laminin, elastin, endostatin and fibronectin (43). These FBLN proteins are involved in the formation and stabilization of basement membranes, fibers, and connective tissues (44). Fibulins are also involved in fibrogenesis, vasculogenesis and tumorigenesis (45)	*FBLN5* regulate angiogenesis, thus is an endogenous inhibitor of angiogenesis during development via the control of *VEGF* and angiopoietins (46)	The expression of Fibulin-5 is consistently down-regulated in prostate tumors and is impaired in various human cancer including PCa (13)
ROBO1	*ROBO1*, a roundabout (ROBO) immunoglobulin, is involved in cell motility and migration (47)	SLIT2-ROBO receptor signaling is involved in angiogenesis. ROBO1-dependent tumor angiogenesis is affected by SLIT2 released from tumor cells. Interaction with ROBO4 can stabilize the vasculature and inhibit VEGF-induced endothelial cell migration and permeability, and prevent pathologic angiogenesis in animal study (48)	*ROBO1* acts like a natural inhibitor of metastasis (14). Thus, *ROBO1* acts as a tumor suppressor gene and downregulation of *ROBO1* was significantly associated with invasive PCa (14)
E2F1	*E2F1* is a cell cycle-specific transcription factor and induces apoptosis as a protection from malignant transformation and suppression of tumorigenesis (49)	Expression of *E2F1* regulates VEGF-C and VEGFR-3 in cancer cells through transcriptional control. In addition, VEGFR-3 also positively regulates *E2F1* in a feedback circuit. This E2F1-VEGF-C/VEGFR-3 loop is a main mechanism for angiogenesis in tumor (49)	Overexpression of *E2F1* is associated with progression of PCa, especially PCa metastasis (15-18)
STAT1	STAT family members are phosphorylated by the receptor associated kinases, and translocate to the cell nucleus where they act as transcription activators. STAT1 mediates the expression of a variety of genes, which is thought to be important for cell viability in response to different cell stimuli	Activated *STAT1* signaling has tumor suppressive roles by promoting apoptosis and by inhibiting angiogenesis, tumor growth and metastasis (50,51)	*STAT1* expression affects the chemoresistant phenotype, especially to docetaxel treatment. Therefore, *STAT1* plays a key role in resistance in PCa treatment (19)
HSPG2	The *HSPG2* is a five-domain proteoglycan that interacts with extracellular matrix components and cell-surface molecules	*HSPG2* is required for activating *VEGFR* signaling of endothelial cells for vascular invasion. Thus, *HSPG2* plays a critical role in endochondral bone formation by promoting angiogenesis (52)	Expression of *HSPG2* is associated with high Gleason scores (20), prostate tumor growth, and enhancing angiogenesis (21). Overexpression of *HSPG2* is required for invasion of prostate tumors (22)
MMP16	MMP16 activates pro-MMP2 and can promote the migration and invasion of tumor cells by denaturing collagens and other ECM proteins in basement membrane (53)	MMPs, including *MMP16* have been implicated in angiogenesis, and tumor cell invasion (53)	*MMP16* has been shown to be down-regulated in malignant prostate tissues (23)

Table 5 *(continued)*

Table 5 *(continued)*

Gene	Functional role	Relation to angiogenesis	Relation to prostate cancer etiology
ITGB3	The ITGB3 is a key player in tumor growth and metastasis and a key regulator in reactive oxygen species-induced migration and invasion of cancer cells (54). Downregulated expression of ITGB3 reduced cell proliferation, migration, and invasion in cancer cells (55)	ITGB is required for angiogenesis (56). *ITGB3* antagonists promoted tumor regression by inducing apoptosis of angiogenesis (57). Enhanced angiogenesis and tumor growth were also observed in animal lacking *ITGB3* (58)	*ITGB3* was identified as one of the genes related to tumor metastasis (24). *ITGB3* is a part of a gene expression signature for detecting the presence of prostate tumors in stroma with 97% accuracy (59). *ITGB3* expression was significantly lower in tumor-adjacent stroma compared to normal stroma (60)
ESR1	Estrogen receptor 1 (*ESR1*) is one of ligand-activated transcription factors with domains for binding to hormones and DNA and is present in the vascular endothelium	*ESR1* enhanced ROCK-II signaling pathway promote angiogenesis in endothelial cells, and cell migration (61)	*ESR1* is associated with PCa risk by stimulating proliferation of prostate cells and deregulating apoptosis (25)
EGFR	EGFR is involved in the proliferation of epithelial cells and tumorigenesis	Constitutive activation of *EGFR* upregulates p38 MAPK, resulting in activation of HIF-1α and further upregulations of MMP-1 and VEGF, leading to increase in angiogenesis in transformed cells (62)	The *EGFR* binds the *EGF* and plays an important role in regulating prostate cellular growth and function (26-28). Multiple studies identified *EGFR* in both analyses of gene and pathway levels associated with PCa risk (12,63)
CSF1	CSF1 controls the production, differentiation, and function of macrophages	*CSF1* recruits tumor associate macrophages (TAMs), which stimulate angiogenesis in PCa. TAMs produce a variety of pro-angiogenic factors that include *VEGF*, basic fibroblast growth factor, tumor necrosis factor α (TNFα), and others	Overexpression of *CSF1* was observed in various cancer patients, increased tumor angiogenesis, promoted metastatic potential in breast cancer (64) and was associated with poor outcome in ovarian cancer (65). A recent animal study demonstrated that *CSF1* can be a potential target for PCa treatment (66)
SP1	*SP1* is a transcription factor that affects the expression of genes involved in various cellular processes and oncogenesis	Overexpression of *SP1* increased angiogenesis (29)	Over expression of *SP1* was observed in prostate cancer, and decreased cancer cell death (29). Expression of *SP1* is associated with PCa prognosis (67), and recurrence (68)

demonstrated that -216 SNP in the *EGFR* promoter was associated with altered promoter activity by Sp1 binding and gene expression both *in vitro* and *in vivo* (72). SP1 has been shown to directly or indirectly regulate some PCa related genes, of which *EGFR* is one (73).

ROBO1, a roundabout (ROBO) immunoglobulin, is involved in the cell motility and migration (47). Khusial *et al*. [2010] reported that expression of *ROBO1* affect process of motility in cancer cells (74). Parray *et al*. demonstrated that *ROBO1* acts as a tumor suppressor gene and downregulation of *ROBO1* was significantly associated with invasive PCa. Further, the study suggested that ROBO1 is a promising biomarker to differentiate metastatic cases from early stage cases. ROBO1 acts like a natural inhibitor of metastasis; therefore, this protein provides an opportunity to develop novel therapies targeting ROBO1 for treating metastatic PCa (14). Regarding Fibulin 5 (FBLN5), it is one of the extracellular matrix (ECM) glycoproteins and interacts with many ECM components, such as laminin, elastin, endostatin, and fibronectin (43). These FBLN proteins are involved in the formation and stabilization of basement membranes, fibers, and connective tissues (44). Furthermore, fibulins are involved in fibrogenesis, vasculogenesis, and tumorigenesis (45). The expression of Fibulin-5 is impaired in various human cancer tissues including PCa (13). These expression data were confirmed by immunohistochemistry results. The study also reported that Fibulin-5 was predominantly located in the stroma, (with a strong gradient from the periurethral to the peripheral zone) and lost in prostate tumors.

This study evaluated both cis- and trans-eQTLs in the angiogenesis network. Among the six primary gene pairs of eQTL results, only one set of *MMP16* contains cis-eQTLs. Five other sets are gene pairs that interacted at a distance. Our study findings demonstrate that the gene-gene interaction network, conducted based on the SNP-SNP interaction and bioinformatics approach, can be an effective tool for identifying potential trans-genes, which is beneficial for understanding the etiology of PCa prognosis. Another large-scale eQTL study also has demonstrated that some disease-associated SNPs affect multiple genes in *trans-eQTLs*, which are known to be changed for individuals with diseases, and these results have been successfully replicated (75).

The strength of this study is that it defines strong coexpressions ($r \geq 0.6$) based on the empirically null distribution of coexpressions in the same testing dataset. To our knowledge, there is no standard cutoff to define strong coexpressions. Using these conservative criteria, we still can identify nine strong coexpressions. The confirmed coexpressions, which are validated in several datasets, can be used to identify gene clusters with functional interactions. A study examined mRNA coexpressions in 60 human datasets and identified 8,805 gene coexpressions in at least three datasets (34). The clinical use of coexpressions is also promising. It has been shown that coexpression provides a better prediction and classification than single-gene expression in PCa progression even after controlling for clinical variables (76).

These findings provide functional evidence to support the association among genes in the angiogenesis network. The conventional prediction model only considers an additive effect of individual biomarkers. Several studies show that interactions of multiple biomarkers are more powerful than individual biomarkers (12,76-78). The integrated biomarkers (such as SNP-SNP interaction, coexpression, or eQTL results) of these genes may be useful for building a risk prediction model for PCa aggressiveness. Future large scale gene expression, eQTL, protein expression studies, or functional experiments are warranted to further validate the interactions of angiogenesis genes.

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Footnote

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aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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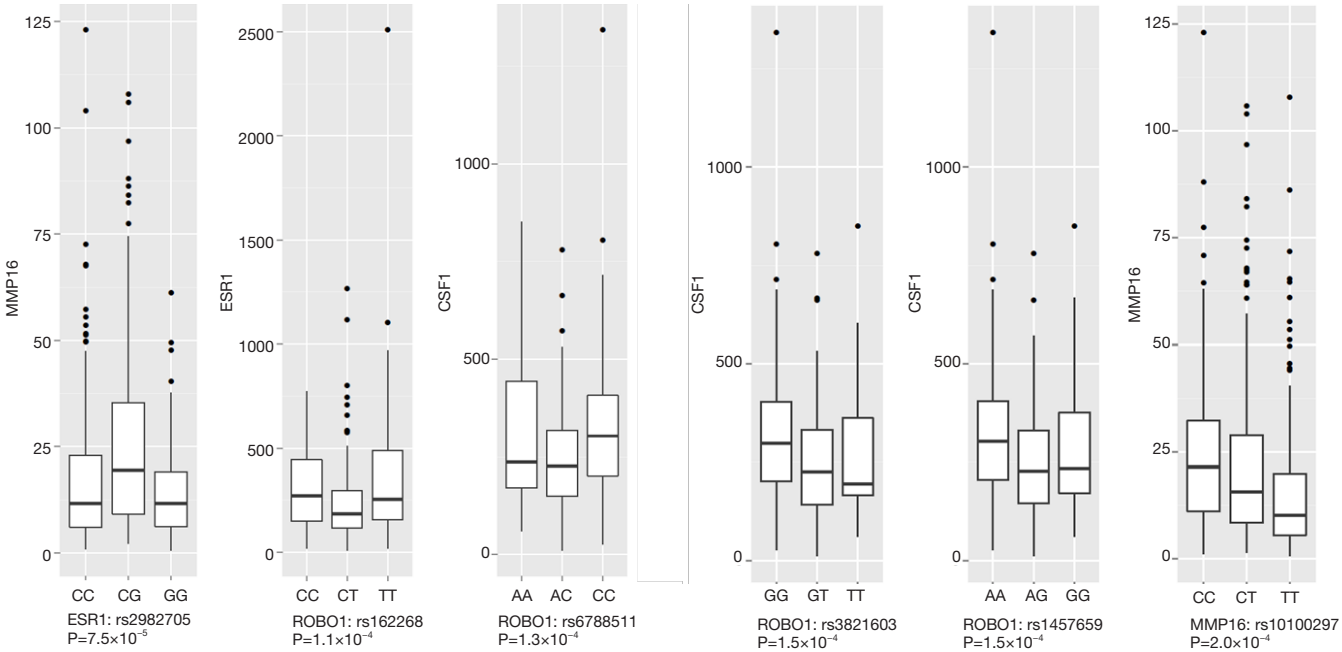

Figure S1 Top six eQTL resutls. eQTL, expression quantitative trait loci.

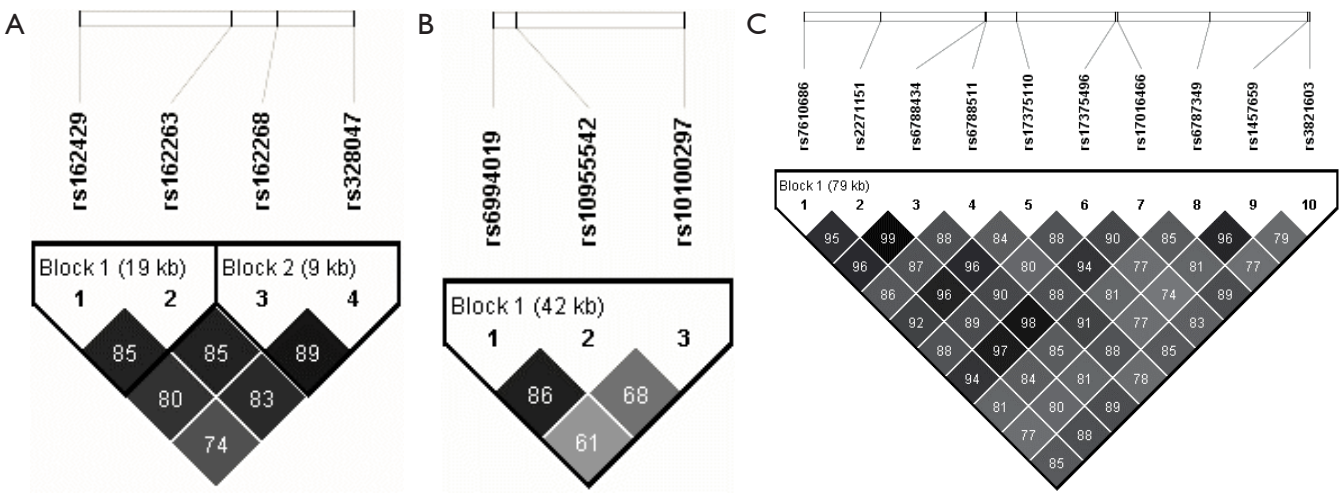

Figure S2 Linkage disequilibrium plots for the SNPs in the top eQTL list. (A) Four *ROBO1* SNPs with significant *ESR1-ROBO1* eQTL results; (B) three *MMP16* SNPs with the significant *MMP16-MMP16* eQTL results; (C) ten *ROBO1* SNPs with the significant *CSR1-ROBO1* eQTL results. The values in the triangles are r^2. The strong LD is defined based on r^2>0.8. SNP, single nucleotide polymorphism; eQTL, expression quantitative trait loci.