Genetic association analysis identifies a role for ANO5 in prostate cancer progression

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Abstract

Anoctamins were originally identified as a family of calcium-activated chloride channels, but recently their roles in the development of different types of malignancies were suggested. Here, we evaluated the associations between 211 common single-nucleotide polymorphisms in 10 anoctamin genes with biochemical recurrence (BCR) after radical prostatectomy (RP) for localized prostate cancer. Four SNPs (ANO4 rs585335, ANO5 rs4622263, ANO7 rs62187431, and ANO10 rs118005571) remained significantly associated with BCR after multiple test correction (P < .05 and q = 0.232) and adjustment for known prognostic factors. Expression quantitative trait loci analysis found that ANO5 rs4622263 C and ANO10 rs118005571 C alleles were associated with decreased mRNA expression levels. Moreover, lower expression of ANO5 was correlated with more advanced tumors and poorer outcomes in two independent prostate cancer cohorts. Taken together, ANO5 rs4622263 was associated with BCR, and ANO5 gene expression was correlated with patient prognosis, suggesting a pivotal role for ANO5 in prostate cancer progression.

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1 | INTRODUCTION

Prostate cancer is one of the most common cancers affecting men worldwide. Most patients have clinically localized prostate cancer at the time of diagnosis but some develop aggressive prostate cancer, eventually leading to death. Radical prostatectomy (RP) is a widely used treatment for localized prostate cancer with excellent control; however, 20%-40% of patients still experience biochemical recurrence (BCR) within 10 years of RP.\(^1,2\) Although we currently have several predictors (prostate-specific antigen [PSA], Gleason score, and cancer stage) to risk stratify patients with prostate cancer, their clinical outcomes can vary widely. Identifying additional prognostic factors could further improve the management of personalized treatment for these patients.

Anoctamins, also known as transmembrane 16 proteins, are a family of calcium-activated chloride channels that comprise 10 members (ANO1-10). Anoctamins play important roles in regulating membrane excitability, ion homeostasis, and cell volume during endo/exocytosis, as well as in cell proliferation.\(^3\) Recent studies suggest that some anoctamins may be relevant to the proliferation and progression of a number of cancers, including gastrointestinal stromal tumors, breast cancer, and prostate cancer.\(^4-6\) Although the detailed mechanisms by which anoctamins regulate tumorigenesis are still unclear, it has been speculated that they can transiently increase intracellular calcium levels, which could activate the Ras/Raf/MEK/ERK signaling pathway to affect cell proliferation.\(^7\) Considering this, it is important to comprehensively investigate the relationship between anoctamins and clinical prostate cancer outcomes.

In a recent Nordic Twin Study of Cancer, genetic factors were estimated to account for 57% of the variation in prostate cancer risk,\(^8\) suggesting the existence of hereditary factors that influence prostate cancer initiation. However, only a few studies explored their impacts on the progression of prostate cancer.\(^9-11\) Therefore, the present study aims to investigate whether common genetic variants in anoctamin genes are associated with BCR-free survival, which may help identify candidate genes and provide insight into the etiology of prostate cancer progression.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment and data collection

In total, 641 patients were enrolled at three medical centers in Taiwan: Kaohsiung Medical University Hospital, Kaohsiung Veterans General Hospital, and National Taiwan University Hospital, as described previously.\(^12\) Biochemical recurrence was defined as two consecutive PSA measurements of 0.2 ng/mL or more after RP.\(^9,13-15\) The institutional review board of Kaohsiung Medical University Hospital approved this study, and all participants provided written informed consent in accordance with the institutional guidelines.

2.2 | Single-nucleotide polymorphism selection and genotyping

We selected 213 single-nucleotide polymorphisms (SNPs) from 10 anoctamin genes with a threshold of minor allele frequency (MAF) of >0.05 based on the 1000 Genomes data for Han Chinese in Beijing, China, and Southern Han Chinese.\(^16\) Single-nucleotide polymorphism genotyping was conducted using Affymetrix Axiom Genotyping Arrays at the National Centre for Genome Medicine, Taiwan, as described previously.\(^17\) SNPs that deviated from Hardy-Weinberg equilibrium (\(P < .005; N = 2\)) were removed, leaving a total of 211 SNPs for analyses.

2.3 | Bioinformatics analysis

Bioinformatics and functional analyses were performed with multiple software tools and data sources: HaploReg\(^18\) for SNP functional prediction; lymphoblastoid cell data from the 1000 Genomes Project\(^16\) for expression quantitative trait loci (eQTL) analysis; and tumor gene expression data from the Memorial Sloan-Kettering Cancer Center (MSKCC) Prostate Oncogenome\(^19\) and The Cancer Genome Atlas (TCGA)\(^20\) projects for gene expression survival analysis.
| Characteristics | No BCR\(^a\) | BCR\(^a\) | \(P\) |
|-----------------|-------------|------------|-------|
| No. of patients, N (%) | 415 (64.7) | 226 (35.3) | |
| Age at diagnosis | | | |
| Median, y (IQR) | 66.0 (62.0-70.0) | 66.5 (61.0-71.0) | .102 |
| PSA at diagnosis, N (%) | | | |
| Median, ng/mL (IQR) | 9.3 (6.2-15.0) | 14.8 (8.4-26.3) | <.001 |
| Pathologic Gleason score, N (%) | | | |
| 2-6 | 117 (77.0) | 35 (23.0) | <.001 |
| 7-10 | 298 (60.9) | 191 (39.1) | |
| Pathologic stage, N (%) | | | |
| T1/T2 | 275 (76.2) | 86 (23.8) | <.001 |
| T3/T4/N1 | 139 (50.5) | 136 (49.5) | |
| Surgical margin, N (%) | | | |
| Negative | 320 (70.0) | 137 (30.0) | <.001 |
| Positive | 95 (51.6) | 89 (48.4) | |

Note: Subtotals do not sum to 641 due to missing data.
Abbreviations: BCR, biochemical recurrence; IQR, interquartile range; PSA, prostate-specific antigen.

\(^a\)With a median follow-up of 51 mo.

**TABLE 2** SNPs associated with BCR in prostate cancer patients receiving RP

| Gene SNP | Genotype | N | BCR | 5-y BFS | HR (95% CI) | \(P\) | HR (95% CI)\(^a\) | \(P^a\) |
|----------|----------|---|-----|---------|-------------|-------|----------------|-------|
| ANO3     | rs74754887 | AA | 509 | 170 | 63.5 | 1.00 | 1.00 |
|          |          | AG | 122 | 52  | 50.0 | 1.45 (1.11-1.90) | .007 | 1.33 (1.00-1.76) | .050 |
|          |          | GG | 10  | 4   | 33.8 | | |
| ANO4     | rs585335  | CC | 526 | 199 | 57.5 | 1.00 | 1.00 |
|          |          | CT | 112 | 26  | 74.6 | 0.58 (0.39-0.85) | .006 | 0.63 (0.42-0.95) | .025 |
|          |          | TT | 3   | 1   | 66.7 | | |
| ANO5     | rs4622263 | TT | 393 | 126 | 64.0 | 1.00 | 1.00 |
|          |          | TC | 215 | 82  | 55.7 | 1.35 (1.09-1.67) | .006 | 1.44 (1.16-1.79) | .001 |
|          |          | CC | 30  | 17  | 46.6 | | |
| ANO7     | rs62187431| CC | 490 | 187 | 56.4 | 1.00 | 1.00 |
|          |          | CG | 142 | 38  | 73.4 | 0.64 (0.45-0.89) | .008 | 0.67 (0.48-0.94) | .022 |
|          |          | GG | 6   | 1   | 83.3 | | |
| ANO10    | rs11800571| TT | 563 | 211 | 58.4 | 1.00 | 1.00 |
|          |          | TC | 78  | 15  | 76.9 | 0.45 (0.26-0.75) | .003 | 0.44 (0.26-0.75) | .002 |

Note: Subtotals do not sum to 641 due to missing data.
\(P < .05\) are in boldface.
Abbreviations: BCR, biochemical recurrence; BFS, BCR-free survival; CI, confidence interval; HR, hazard ratio; RP, radical prostatectomy; SNP, single-nucleotide polymorphism.

\(^a\)Adjustment for age, PSA at diagnosis, pathologic Gleason score, stage, and surgical margin.
2.4 | Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software version 19.0.0 (IBM). A two-sided $P < .05$ was considered to represent statistical significance; $q$ values were calculated to reduce the probability of false positive findings.21

3 | RESULTS

The basic characteristics of the 641 patients who underwent RP for localized prostate cancer are presented in Table 1. The median age of all patients was 66 years (interquartile range [IQR] 61.5-70.0), and median PSA was 11.0 ng/mL (IQR 7.0-18.4). Most patients had a Gleason score of 7-10 (489, 76.3%), stage T1/T2 (361, 56.8%), and a negative surgical margin (457, 71.3%). Biochemical recurrence was observed in 226 (35.3%) patients during a median follow-up of 51 months. Univariate Cox regression indicated that PSA, Gleason score, stage, and surgical margin were significantly associated with BCR ($P < .001$).

Cox regression analysis was performed to assess associations between a total of 211 common SNPs of 10 anoctamin genes and BCR (Table S1). Ten SNPs showed evidence of association at $P < .05$ and $q = 0.232$, indicating that 23.2% of the 10 SNPs were likely to be false positives. Therefore, seven SNPs with the lowest $P$ (ANO3 rs74754887, ANO4 rs585335, ANO4 rs1354228, ANO5 rs4622263, ANO7 rs62187431, ANO7 rs76832527, and ANO10 rs118005571) were considered noteworthy after multiple test correction. Since rs585335 and rs1354228 in ANO4 and rs62187431 and rs76832527 in ANO7 were in strong linkage disequilibrium ($r^2 > .80$), ANO4 rs1354228 and ANO7 rs76832527 were also excluded due to their lower significance, leaving five SNPs for further analyses (Table 2; Figure 1).

Multivariate Cox regression analysis was then performed to evaluate the robustness of these five SNPs on BCR with adjustment for known risk factors (Table 2). The risk of BCR significantly decreased with the number of ANO4 rs585335 minor allele T, ANO7 rs62187431 G, and ANO10 rs118005571 C ($P = .025$, .022, and .002, respectively), but significantly increased with the number of ANO5 rs4622263 C ($P = .001$).

We performed eQTL analysis using HapMap lymphoblastoid cell line data to test if these SNPs could influence gene expression. The minor allele C at rs4622263 was correlated with lower ANO5 expression ($P = .013$; Figure 2A), and rs118005571 C was correlated with lower ANO10 expression ($P = .038$). According to these results, we hypothesized that lower ANO5 and higher ANO10 expression would correlate with worse outcomes in prostate cancer. The associations between gene expression and prostate cancer outcomes were investigated in MSKCC and TCGA cohorts. Lower levels of ANO5 expression were associated with more aggressive forms of prostate cancer, higher Gleason score, pathologic stage, and shorter time to BCR/disease-free survival in both cohorts (Figure 2B,D). However, no significant associations were found between ANO10 expression levels and prostate cancer (Figure 2C).

4 | DISCUSSION

We conducted a comprehensive analysis of anoctamin gene SNPs with prostate cancer recurrence and found that ANO5 rs4622263 was associated with unfavorable prognosis. This genotype-outcome association was pronounced, even in the presence of known predictors of prostate cancer outcome. Moreover, rs4622263 influenced ANO5 expression. These findings suggest that ANO5 may have biological roles in prostate cancer and provide new insights into the mechanism of prostate cancer progression.

Anoctamins are a family of calcium-activated ion channels and phospholipid scramblases.22 They have been shown to control compartmentalized calcium signaling by tethering endoplasmic reticulum (ER) calcium storage to the plasma membrane and promoting the release of calcium from ER via inositol trisphosphate and ryanodine receptors to regulate cell proliferation.23 Anoctamins can also function as phospholipid scramblases when activated by increases in calcium levels, whereby they promote cell death by moving phosphatidylserine from the inner to the outer leaflet of the plasma membrane.24 Further, the expression of several members of...
FIGURE 2  Functional analyses of candidate single-nucleotide polymorphisms. A, Expression quantitative trait loci analyses identify two significant associations between rs4622263 and ANO5, and rs118005571 and ANO10 in 523 HapMap lymphoblastoid cell lines. B, Lower expression of ANO5 is correlated with prostate cancer, higher Gleason score, and stage, and shorter time to biochemical recurrence in Memorial Sloan-Kettering Cancer Center cohort. C, Expression of ANO10 is not altered over the course of prostate cancer progression. D, ANO5 expression is consistently correlated with prostate cancer progression in the The Cancer Genome Atlas cohort. Values between brackets denote the number of patients. Rho: Spearman’s rank correlation coefficient. RP, radical prostatectomy
the anoctamin family, such as ANO1, ANO5, ANO7, and ANO9, is correlated with the development of malignant tumors. Expression of ANO5 is downregulated in thyroid cancer, and knockdown of ANO5 promotes cell migration and invasion via regulation of the JAK/STAT3 signaling pathway. However, the exact role of ANO5 in prostate cancer has yet to be determined. The position of rs4622263 overlaps with a GATA binding protein 1-bound region and is predicted to alter the regulatory motif transcription factor CP2-like 1, which has been implicated in maintenance of pluripotency and self-renewal in embryonic stem cells and cancers, according to the experimental chromatin immunoprecipitation sequencing data in the HaploReg database. These data provide a possible explanation of the mechanisms underlying the association between ANO5 rs4622263 and prostate cancer recurrence we observed.

A previous genome-wide meta-analysis of more than 25,000 men revealed no significant association between common genetic variants and prostate cancer-specific survival. A possible reason might be the heterogeneous patient cohorts with different ethnic background and differences in patient care, which could dilute the effect of SNPs on prostate cancer-specific survival. In contrast, another genome-wide association study focused only on the northern European ancestry in Sweden and identified an association between the AOX1 locus and prostate cancer-specific survival. Therefore, the strengths of our study include a well-characterized Taiwanese patient cohort from a defined geographical region, with complete medical information and significant follow-up times, as well as a comprehensive coverage of common genetic variants across all genes of the anoctamin family.

There are several limitations to the present study. Firstly, our genetic association analyses were limited to a single multicenter study. As our cohort was based on a Taiwanese patient population, it is not clear whether these results would apply in other ethnic groups. Although we used the q-value for multiple test correction, it is still possible that some of our findings could be false discoveries. The limited sample size of the current study does not provide sufficient power to detect SNP associations with a low MAF; thus, additional cancer-related genes might have been overlooked. Also, we have not determined the precise mechanisms for the effect of ANO5 rs4622263 on prostate cancer progression, but functional annotation in HaploReg database provides clues to potential mechanisms, as described. Therefore, additional larger studies with multiethnic groups are needed to confirm our results, and further functional studies are also warranted to investigate the exact functions of rs4622263 or ANO5 on prostate cancer progression.

In conclusion, the present study identified that rs4622263 is associated with BCR and may regulate ANO5 gene expression. Lower expression of ANO5 is correlated with worse BCR/disease-free survival of prostate cancer. ANO5 may impede the progression of prostate cancer, and rs4622263 could be a promising prognostic biomarker for personalized therapies.

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CONFLICT OF INTEREST
The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES
1. Freedland SJ, Humphreys EB, Mangold LA, et al. Risk of prostate cancer-specific mortality following biochemical recurrence after radical prostatectomy. JAMA. 2005;294(4):433-439.
2. Roehl KA, Han M, Ramos CG, Antenor JA, Catalona WJ. Cancer progression and survival rates following anatomical radical retropubic prostatectomy in 3,478 consecutive patients: long-term results. J Urol. 2004;172(3):910-914.
3. Hartzell C, Putzier I, Arreola J. Calcium-activated chloride channels. Annu Rev Physiol. 2005;67:719-758.
4. Britschgi A, Bill A, Brinkhaus H, et al. Calcium-activated chloride channel ANO1 promotes breast cancer progression by activating EGFR and CAMK signaling. Proc Natl Acad Sci USA. 2013;110(11):E1026-E1034.
5. Liu W, Lu M, Liu B, Huang Y, Wang K. Inhibition of Ca(2+)-activated Cl(−) channel ANO1/TMEM16A expression suppresses tumor growth and invasiveness in human prostate carcinoma. Cancer Lett. 2012;326(1):41-51.
6. West RB, Corless CL, Chen X, et al. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. Am J Pathol. 2004;165(1):107-113.
7. Kunzelmann K. TMEM16, LRRC8A, bestrophin: chloride channels controlled by Ca(2+) and cell volume. Trends Biochem Sci. 2015;40(9):535-543.
8. Mucci LA, Hjelmborg JB, Harris JR, et al. Familial risk and heritability of cancer among twins in Nordic countries. JAMA. 2016;315(1):68-76.
9. Huang EY, Chang YJ, Huang SP, et al. A common regulatory variant in SLC35B4 influences the recurrence and survival of prostate cancer. J Cell Mol Med. 2018;22(7):3661-3670.
10. Li W, Middha M, Bicak M, et al. Genome-wide scan identifies role for AOX1 in prostate cancer survival. Eur Urol. 2018;74(6):710-719.
11. Szulkin R, Karlsson R, Whitington T, et al. Genome-wide association study of prostate cancer-specific survival. Cancer Epidemiol Biomarkers Prev. 2015;24(11):1796-1800.
12. Huang S-P, Huang L-C, Ting W-C, et al. Prognostic significance of prostate cancer susceptibility variants on prostate-specific antigen recurrence after radical prostatectomy. *Cancer Epidemiol Biomarkers Prev*. 2009;18(11):3068-3074.

13. Freedland SJ, Sutter ME, Dorey F, Aronson WJ. Defining the ideal cutpoint for determining PSA recurrence after radical prostatectomy. *Prostate-specific antigen*. *Urology*. 2003;61(2):365-369.

14. Huang C-Y, Huang S-P, Lin VC, et al. Genetic variants in the Hippo pathway predict biochemical recurrence after radical prostatectomy for localized prostate cancer. *Sci Rep*. 2015;5:8556.

15. Huang S-P, Lévesque E, Guillemette C, et al. Genetic variants in microRNAs and microRNA target sites predict biochemical recurrence after radical prostatectomy in localized prostate cancer. *Int J Cancer*. 2014;135(11):2661-2667.

16. 1000 Genomes Project Consortium; Abecasis GR, Auton A, Brooks LD, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491(7422):56-65.

17. Huang C-N, Huang S-P, Pao J-B, et al. Genetic polymorphisms in oestrogen receptor-binding sites affect clinical outcomes in patients with prostate cancer receiving androgen-deprivation therapy. *J Intern Med*. 2012;271(5):499-509.

18. Ward LD, Kellis M. HaploReg v4: systematic mining of putative causal variants, cell types, regulators and target genes for human complex traits and disease. *Nucleic Acids Res*. 2016;44(D1):D877-D881.

19. Taylor BS, Schultz N, Hieronymus H, et al. Integrative genomic profiling of human prostate cancer. *Cancer Cell*. 2010;18(1):11-22.

20. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature*. 2008;455(7216):1061-1068.

21. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA*. 2003;100(16):9440-9445.

22. Kunzelmann K, Tian Y, Martins JR, et al. Anoctamins. *Pflugers Arch*. 2011;462(2):195-208.

23. Cabrita I, Benedetto R, Fonseca A, et al. Differential effects of anoctamins on intracellular calcium signals. *FASEB J*. 2017;31(5):2123-2134.

24. Brunner JD, Lim NK, Schenck S, Duerst A, Dutzler R. X-ray structure of a calcium-activated TMEM16 lipid scramblase. *Nature*. 2014;516(7530):207-212.

25. Chang Z, Cai C, Han D, et al. Anoctamin5 regulates cell migration and invasion in thyroid cancer. *Int J Oncol*. 2017;51(4):1311-1319.

26. Bera TK, Das S, Maeda H, et al. NGEP, a gene encoding a membrane protein detected only in prostate cancer and normal prostate. *Proc Natl Acad Sci USA*. 2004;101(9):3059-3064.

27. Li C, Cai S, Wang X, Jiang Z. Identification and characterization of ANO9 in stage II and III colorectal carcinoma. *Oncotarget*. 2015;6(30):29324-29334.

28. Pelton TA, Sharma S, Schulz TC, Rathjen J, Rathjen PD. Transient pluripotent cell populations during primitive ectoderm formation: correlation of in vivo and in vitro pluripotent cell development. *J Cell Sci.* 2002;115(Pt 2):329-339.

29. Tun HW, Marlow LA, von Roemeling CA, et al. Pathway signature and cellular differentiation in clear cell renal cell carcinoma. *PLoS ONE*. 2010;5(5):e10696.

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