

Biomarkers Associated with Tumor Heterogeneity in Prostate Cancer^{1,2}



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Abstract

BACKGROUND: Prostate cancers exhibit intratumor heterogeneity (ITH), like other cancer types. The ITH may affect diverse phenotypes such as treatment response, drug resistance, and clinical outcomes. It is crucial to consider ITH to understand tumorigenesis. **METHODS:** Genomic and transcriptomic profiles of prostate cancer patients were investigated to determine which markers are correlated with the degree of tumor heterogeneity. In addition, the correlation between the immune activity and clonality of tumors was examined. **RESULTS:** Tumor heterogeneity across all prostate cancer samples was variable. However, ITH events were dependent on genomic and clinical features. Interestingly, prostate-specific antigen score increased in tumors with multiple subclones, indicating high-grade tumor heterogeneity. On the other hand, CD8-positive T-cell activation decreased in highly heterogeneous tumors. Intriguingly, *PTEN* deletion was prominently enriched in high heterogeneity groups, with a strong association with heterozygous loss. Expression of major genes including *PTEN*, *CDC42EP5*, *RNLS*, *GP2*, *NETO2*, and *AMPD3* was closely related to tumor heterogeneity in association with *PTEN* deletion. **CONCLUSIONS:** In prostate cancer, ITH, a potential factor affecting tumor progression, is associated with *PTEN* deletion and cytotoxic T cell inactivation.

Translational Oncology (2019) 12, 43–48

Introduction

In many industrialized nations, prostate adenocarcinoma is one of the most common malignant diseases in men [1]. Prostate cancer (PC) is considered clinically heterogeneous. Some prostate cancers are indolent and localized, while others are aggressive and easily spread to other parts of the body. Therefore, it is necessary to understand key features related to tumor progression and invasiveness. Many cases of prostate cancer are multifocal; most radical prostatectomy specimens harbor morphologically and clonally distinct tumor foci [2–4]. Studies of metastatic tumors from primary PC have suggested that all of those tumors evolved from one clone, as they share a significant portion of genetic alterations [5,6]. The characteristics and diversity of a clone may explain the aggressiveness of prostate cancer.

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¹ Grant support: The study was supported in part by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B03034779).

² Disclosure of potential conflicts of interest: There are no potential conflicts of interest to disclose.

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Received 31 July 2018; Revised 9 September 2018; Accepted 9 September 2018

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1936-5233/19

<https://doi.org/10.1016/j.tranon.2018.09.003>

Therefore, it is important to perform a comprehensive genomic and transcriptomic characterization of the primary cancer lesion to understand the biology of the tumor and the factors associated with tumor progression. However, major factors that lead to tumor heterogeneity during prostate cancer progression are still not clear. Recently, next-generation sequencing provided a molecular portrait of genomic alterations. Furthermore, intratumor heterogeneity (ITH) has been inferred by clonality analysis [7]. In various types of cancer, the characterization of clonal heterogeneity may provide useful information for predicting patient prognosis and treatment response.

Herein, we investigated factors that are associated with ITH of prostate cancer through comprehensive genomic and transcriptomic analysis. We also investigated genomic alterations, altered pathways, and clinical features as the indicators of high ITH.

Materials and Methods

Dataset Collection

Genomic and transcriptomic alterations including somatic mutations, copy number alterations, and gene fusions were collected from The Cancer Genome Atlas Research Network (The Cancer Genome Atlas Research Network 2015) [8]. Gene expression data from RNA-seq were obtained from GDAC Firehose (<http://gdac.broadinstitute.org>). A total of 85 samples with clonality information were used to determine the association between genomic alteration and expression profile.

Gene Expression Analysis

Differentially expressed genes (DEGs) were identified using DESeq R package (www.huber.embl.de/users/anders/DESeq/). Significant DEGs by both ≥ 2 -fold change and adjusted P value $< .05$ were chosen. In order to identify overrepresented functions of an interesting group, gene set enrichment test was performed using Gene Set Enrichment Analysis (software [broadinstitute.org/gsea/](http://software.broadinstitute.org/gsea/)), based on REACTOME pathway database in the Molecular Signatures Database (MSigDB). To estimate the fractions of immune-associated cell types including CD8-positive T cells, CIBERSORT was applied using RNA-seq expression profiles [9]. It can infer relative proportions of each immune cell types using gene expression profiles.

Clonality and Tumor Purity Information

Clonality information was obtained from a pan-cancer analysis of the ITH, measured using PyClone and EXPANDS tools [7]. The number of subclones ranged from one to eight and represented the ITH level. In order to categorize high and low ITH, we defined a tumor as an oligoclone when there were one or two subclones; otherwise, the tumor was defined as a multiclonal [10]. Tumor purity information was collected from pan-cancer analysis of the tumor purity [11]. In brief, the purity levels were arbitrarily chosen from multiple estimators. The consensus purity estimation method is the median value for estimators after normalization.

Statistical Analysis

The significance of clinical outcomes of the selected genes was plotted using Kaplan-Meier survival analysis using the survival package in R (<http://CRAN.R-project.org/package=survival>). Log-rank test was used for survival analysis. Fisher's exact test was used for the statistical analysis of ITH and genomic mutations. $P < .05$ was considered statistically significant. Information gain (IG) was used to

select the informative features for discriminating cancers with high clonality. IG for tumor samples D and a feature a is defined as [12]:

$$IG(D, a) = Entropy(D) - \sum_{v \in \text{value}(a)} \frac{D_v}{D} Entropy(D_v)$$

where $\text{value}(a)$ is the set of all possible values for feature a and D_v is the subset of D which the feature a has value v .

Results

Genomic Profiles According to Degree of ITH

A total of 85 patients having clonality information with prostate cancer were evaluated. Patient characteristics according to clonality are shown in Table 1. Here, an oligoclone had one or two subclones, and a multiclonal had more than two subclones. Prostate-specific antigen (PSA) is one of the major markers used to diagnose prostate cancer. In the prostate cancer cohort, PSA level ($n = 472$) was significantly correlated with clinical outcome (Supplementary Figure S1). Although the association between tumor heterogeneity and level of PSA is not prominent, high level of PSA (>1.5) was more frequently observed in the group with multiclonal, indicating high ITH (Figures 1 and 2A). In the oligoclone group, only two patients had a high level of PSA, while there were seven such patients in the multiclonal group. Furthermore, the average level of PSA was 0.28 in the oligoclone group and 1.67 in the multiclonal group (Table 1). The analysis suggested that the level of tumor progression or invasiveness is substantially associated with ITH and PSA levels.

Several factors such as average PSA level, tumor mutation burden (TMB), and CD8 scores are associated with the number of clones (Figure 2). The data demonstrated that the level of PSA increased as the number of clones increased. TMB also increased slightly when the number of clones increased. On the other hand, the activation score of CD8 generally declined with the accumulation of clones. These results were not likely affected by tumor purity estimated by four different kinds of measurements including immunohistochemistry, as tumor purities of samples in PC were not different according to number of subclones (Supplementary Table S1).

Moreover, we measured the activation degree of immune cells adjacent to cancer cells using decomposition of RNA-sequencing data. When comparing the immune profiling based on tumor heterogeneity, the activation score of T cell (CD8+) showed slight differences ($P = .05$) between tumors with oligoclones and those with multiclonal (Supplementary Figure S2). On average, patients who had high heterogeneity showed a lower immune activation score of T cell compared to those with low heterogeneity. Generally, cancer cells are known to develop immunosuppression or avoidance [13]. Our analysis indicated that the immune avoidance mechanism works better by reducing the activation of T cells in multiclonal than oligoclone prostate cancer.

Association of PTEN Deletion and ITH

We also analyzed the association between ITH and mutational profiles, including somatic mutations and copy number alterations (CNAs), from whole-exome sequencing data. While most alterations did not show any difference in degree of heterogeneity, there were substantial deviations in *PTEN* CNA (Figure 3A). To observe the genomic factors that can lead to a separation of clonality, information gain (IG) was adopted, and *PTEN* CNA among many factors showed

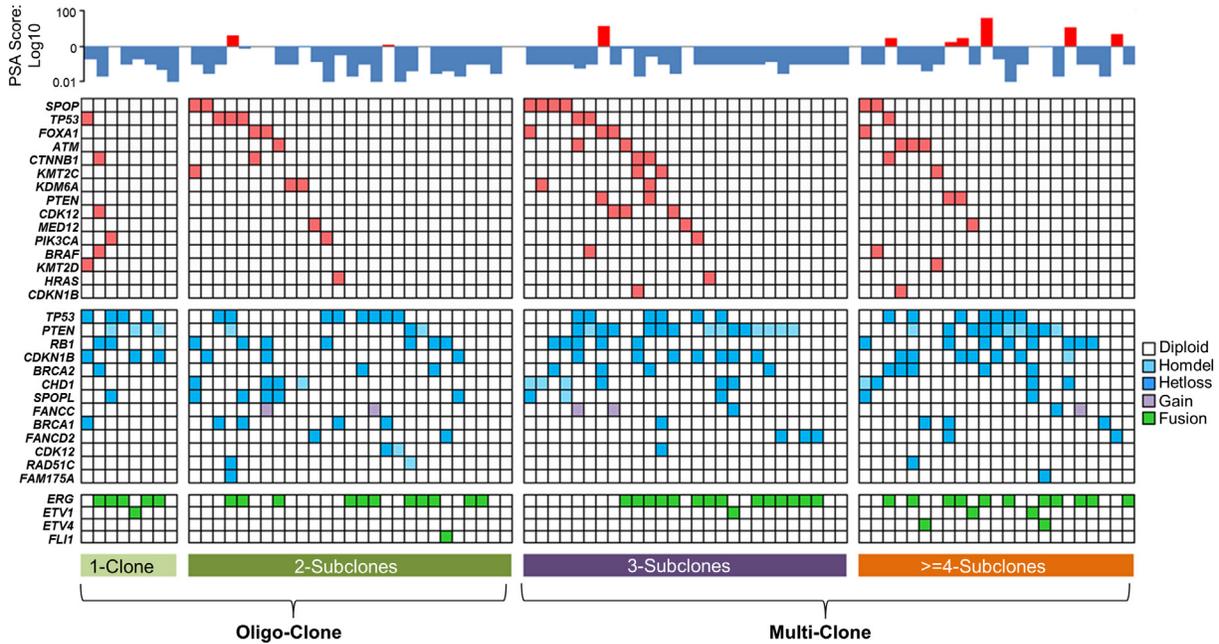


Figure 1. Heatmap of genomic alterations according to clonality. Landscape of somatic mutations with high frequency and copy number alterations from 85 prostate tumor samples (8 in 1 clone, 27 in 2 clones, 27 in 3 clones, and 23 in over 4 clones). When a tumor sample had one or two subclones, it was defined as an oligoclone; otherwise, the tumor was defined as a multiclonal. Bar plot at the top represents PSA values (log10). Patient samples with a level greater than zero are highlighted in red.

the highest IG score. Other subsets such as *PTEN* mutation, *ETV4* fusion, and *SPOPL* CNA also ranked high with regard to IG score but were not considered as important as *PTEN* CNA.

Tumors with wild type and those with *PTEN* deletion showed considerable differences between high- and low-heterogeneity groups ($P = .0027$; Fisher's exact test) (Figure 3B). Interestingly, PCs with *PTEN* deletion possessed an overwhelmingly large proportion of multiclonal tumors. Based on this result, we think that the *PTEN* deletion is one of the key markers associated with ITH.

We also analyzed the clonality among diploid, homozygous, and heterozygous *PTEN* deletion (Figure 3C). Our analysis revealed that multiclonal PCs are notably enriched in heterozygous *PTEN* deletion compared to diploid or homozygous deletion ($P = .001$). In some

types of PCs, *PTEN* heterozygous and homozygous deletions have different characteristics such as Gleason score [14], and our study suggested different ITH patterns in the two studied groups. However, further clinical and biological investigation is warranted.

Functional Enrichment of Tumors with *PTEN* Deletion and ITH

PTEN CNA was positively correlated with mRNA expression (Figure 4A). As expected, the gene expression of genes with homozygous and heterozygous *PTEN* deletion was lower than that with diploid and gain. Moreover, we studied the kinds of genes differentially affecting clonality according to *PTEN* deletion. In order to identify genes associated with both *PTEN* deletion and clonality, we extracted DEGs for six possible combinations for *PTEN* deletion

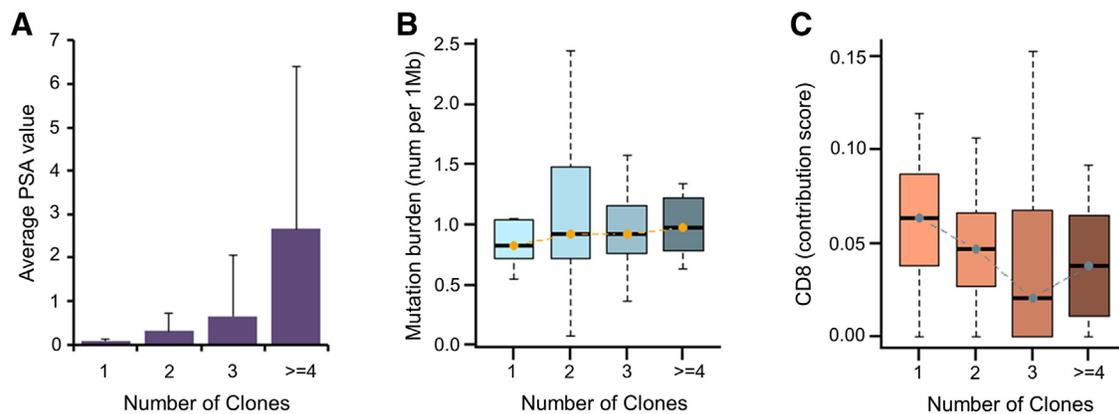


Figure 2. Correlations between ITH and several factors in prostate cancer samples. (A) Bar plot of average PSA values according to number of clones. (B) Box plot of TMB according to number of clones. (C) Box plot of contribution scores of CD8 T-cell signature according to number of clones.

Table 1. Demographic and Clinical Characteristics of Prostate Cancer Patients

Characteristic	Oligoclone (n = 35)	Multiclone (n = 50)	P Value
Age, median (years)	62 (44-71)	61.5 (47-73)	.904
PSA value, average	0.28 (0-4.09)	1.67 (0-37.4)	.108
Preoperative PSA, average	11.15 (2.2-87)	10.54 (1.6-37.4)	.831
Gleason Score			.407
3 + 3	5 (14.3%)	5 (10.0%)	
3 + 4	10 (28.6%)	19 (38.0%)	
4 + 3	11 (31.4%)	9 (18.0%)	
≥ 8	9 (25.7%)	17 (34.0%)	
Tumor cellularity (pathology)			.374
<20%	2 (5.7%)	1 (2.0%)	
21%-40%	3 (8.6%)	6 (12.0%)	
41%-60%	7 (20.0%)	8 (16.0%)	
61%-80%	12 (34.3%)	26 (52.0%)	
81%-100%	11 (31.4%)	9 (18.0%)	
Pathologic stage			.454
pT2a/b	1 (2.9%)	2 (4.0%)	
pT2c	14 (40.0%)	19 (38.0%)	
pT3a	8 (22.9%)	19 (38.0%)	
pT3b	8 (22.9%)	9 (18.0%)	
pT4	3 (8.6%)	1 (2.0%)	
Not available	1 (2.9%)	0 (0.0%)	
Ethnicity			.895
Caucasian	32 (91.4%)	45 (90.0%)	
African descent	2 (5.7%)	4 (8.0%)	
Asian	1 (2.9%)	1 (2.0%)	

(+ and -) and clonality (oligo and multi). Among them, only two combinations had significantly differentially expressed genes (Figure 4B). One was the test for PTEN Del (+) & multiclonal vs. PTEN Del (-) & oligoclone. The other was the test for PTEN Del (+) & multiclonal vs. PTEN Del (-) & multiclonal. Several differentially expressed genes such as *PTEN*, *CDC42EP5*, *RNLS*, *GP2*, *NETO2*, and *AMPD3* were correlated with tumor heterogeneity in the presence of *PTEN* deletion (≥2-fold

change, adjusted *P* value <.05). In other words, high heterogeneity of a tumor with *PTEN* deletion may be dominantly driven by the expression of those genes.

According to previous studies, several pathways such as WNT, PI3CA, and androgen receptor signaling are known to regulate the progression of prostate cancer [15]. Particularly, the PI3CA pathway was significantly affected by tumor heterogeneity (Figure 4, C and D). It is assumed that the altered PI3CA pathway is closely related to higher ITH with *PTEN* deletion.

Discussion

In this study, we presented genomic and transcriptomic factors associated with the degree of ITH. Deletion of *PTEN* tumor suppressor gene occurs at high frequency in prostate cancer and is associated with clinical outcome and aggressive metastatic potential [16,17]. Although many studies regarding *PTEN* deletion in prostate cancer have been reported, the association with ITH has not been clarified. We demonstrated that it was frequently observed in tumors with high heterogeneity. It is still questionable whether the alteration of *PTEN* directly or indirectly causes tumor heterogeneity during prostate cancer progression.

Previous studies have reported that most *PTEN* deletions of primary tumors were concordantly found in metastatic sites [5,6,18]. It is highly possible that *PTEN* deletions are early occurring mutations as they tend to be observed in multiple lesions. A study on ERG rearrangements and *PTEN* deletions in prostate cancer indicated that they are early events during tumor progression [14]. That prior study has reported that *PTEN* heterozygous deletions showed higher frequency than homozygous deletions in diverse tissues obtained from benign prostate tissue to high-grade prostatic intraepithelial neoplasia and prostate cancer. In our study, both types

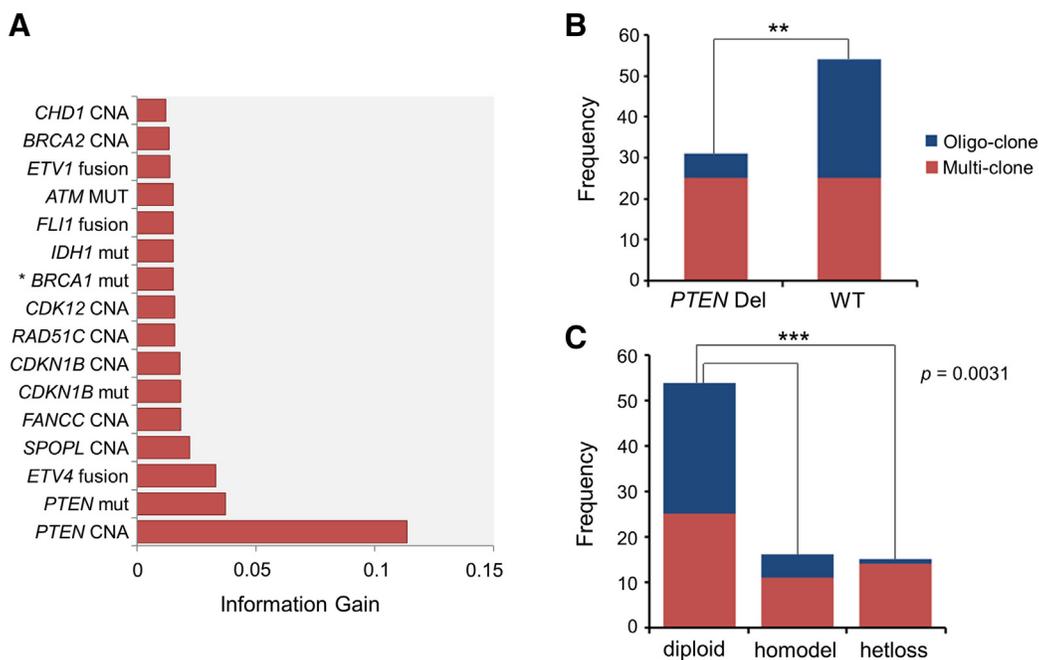


Figure 3. *PTEN* deletion as an indicator of tumor heterogeneity in prostate cancer. (A) Contribution score of genomic and transcriptomic features. Information gain for each feature was measured between oligo- and multiclonal samples. (B) Comparison of clonality between tumor samples with *PTEN* deletion and those with wild type. (C) Comparison of clonality among diploid, homozygous, and heterozygous *PTEN* deletion.

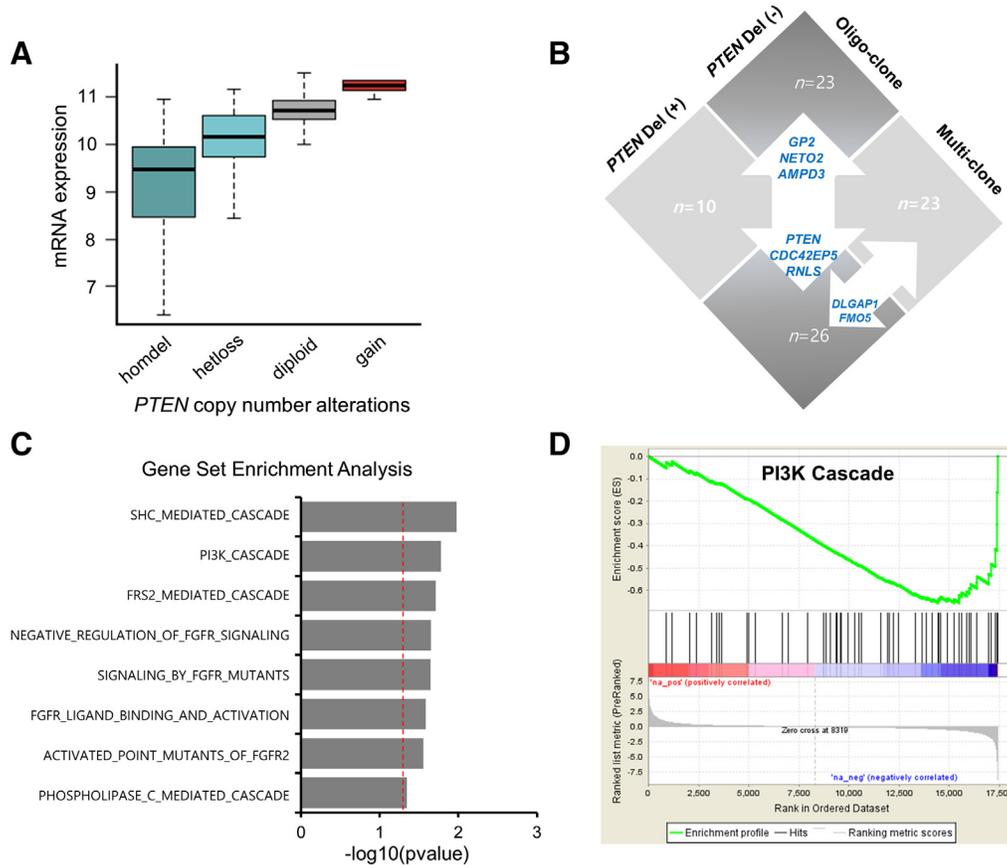


Figure 4. Gene expression and function associated with *PTEN* deletion and tumor heterogeneity. (A) Correlation between *PTEN* deletion and corresponding gene expression. Expression values are the log-transformed normalized read counts. (B) The result of DEG analysis for six possible combinations for *PTEN* deletion (+ and -) and clonality (oligo and multi). DEGs were found in only two tests: 1) *PTEN* Del (+) & multiclonal vs. *PTEN* Del (-) & oligoclonal, 2) *PTEN* Del (+) & multiclonal vs. *PTEN* Del (-) & multiclonal. (C) Enriched pathways associated with *PTEN* deletion and tumor heterogeneity. This result was obtained from gene set enrichment analysis for *PTEN* Del (+) & multiclonal vs. *PTEN* Del (-) & oligoclonal. (D) Enrichment of genes involved in the PI3K cascade pathway.

showed similar frequency and increased ITH. However, ITH was higher in heterozygous deletions than in homozygous.

A number of studies have suggested that tumor infiltrating immune cells can accelerate tumor invasion and metastasis [19–22]. However, the most recent study has demonstrated that progressing metastases showed the characterization of immune cell exclusion, while repressing and stable metastases exhibited infiltration of CD8+ T cells in ovarian cancers [23]. In our study, the activation score of CD8-positive T cell or cytotoxic T cell was negatively correlated with ITH, indicating that the pattern observed from our analysis is consistent with the characteristics of cancer progression. The heterogeneity of clones can vary depending on the immune-microenvironment of the surrounding tumor in prostate cancer.

We demonstrated that the expression of several genes was significantly associated with ITH potentially via *PTEN* deletion. These genes were *PTEN*, *CDC42EP5*, *RNLS*, *GP2*, *NETO2*, and *AMPD3*. Overexpression of Neuropilin and tolloid-like 2 (*NETO2*) has been found in many cancer types including proliferating hemangiomas and colorectal carcinoma [24–26] and thus could be considered as a potential biomarker in tumor progression. Recent study have suggested that adenosine monophosphate deaminase 3 (*AMPD3*) deletion suppresses the proliferation, migration, and invasion of gastrointestinal stromal tumor [27]. *AMPD3* expression is positively correlated with *ERG* overexpression and *PTEN* inactivation in prostate cancer

[28,29]. This result suggests that this abnormal alteration is tightly correlated with tumor heterogeneity and may be useful in the development of prognostic markers or novel drug targets in prostate cancer.

In conclusion, progression of ITH could foster tumor evolution in association with *PTEN* deletion, which is one of the key mechanisms in prostate cancer progression. Genes identified from ITH analysis could potentially serve as a biomarker promoting ITH or a therapeutic target.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tranon.2018.09.003>.

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

Acknowledgements

The study was supported in part by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B03034779).

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