Translational Oncology
www.transonc.com

Volume 11 Number 4 August 2018 pp. 1012–1022 1012

Biphasic Alteration of Butyrylcholinesterase (BChE) During Prostate Cancer Development

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Abstract

Butyrylcholinesterase (BChE) is a plasma enzyme that hydrolyzes ghrelin and bioactive esters, suggesting a role in modulating metabolism. Serum BChE is reduced in cancer patients. In prostate cancer (PC), the down-regulation is associated with disease recurrence. Nonetheless, how BChE is expressed in PC and its impact on PC remain unclear. We report here the biphasic changes of BChE expression in PC. *In vitro,* BChE expression was decreased in more tumorigenic PC stem-like cells (PCSLCs), DU145, and PC3 cells compared to less tumorigenic non-stem PCs and LNCaP cells. On the other hand, BChE was expressed at a higher level in LNCaP cells than immortalized but non-tumorigenic prostate epithelial BPH-1 cells. *In vivo,* BChE expression was up-regulated in DU145 xenografts compared to LNCaP xenografts; DU145 cell-derived lung metastases displayed comparable levels of BChE as subcutaneous tumors. Furthermore, LNCaP xenografts produced in castrated mice exhibited a significant increase of BChE expression compared to xenografts generated in intact mice. In patients, BChE expression was down-regulated in PCs (n = 340) compared to prostate tissues (n = 86). In two independent PC populations MSKCC (n = 130) and TCGA Provisional (n = 490), BChE mRNA levels were reduced from World Health Organization grade group 1 (WHOGG 1) PCs to WHOGG 3 PCs, followed by a significant increase in WHOGG 5 PCs. The up-regulation was associated with a reduction in disease-free survival (P = .008). Collectively, we demonstrated for the first time a biphasic alteration of BChE, its down-regulation at early stage of PC and its up-regulation at advanced PC.

Translational Oncology (2018) 11, 1012–1022

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Received 2 May 2018; Revised 31 May 2018; Accepted 11 June 2018

1936-5233/18
https://doi.org/10.1016/j.tranon.2018.06.003

Introduction

Prostate cancer (PC) is the most common male-specific malignancy in the developed world [1]. The disease develops from high-grade prostatic intra-epithelial neoplasia (HGPIN), to invasive carcinoma, and then to metastatic PC. Primary PCs can be approached by a variety of management plans depending on the severity of the disease, patient age and preference; this includes watchful waiting, radical prostatectomy (RP), and radiation. PCs are graded using Gleason score (GS) and GS-based World Health Organization (WHO) PC grading system (WHO grade group 1–5) which is also known as ISUP (the International Society of Urological Pathology) grade [2–4]. While a large proportion of GS6/WHO grade group 1 tumors are not life-threatening, higher grade PCs are associated with increased risk of disease progression. Approximately 30% of patients after RP will develop recurrent PC characterized by biochemical recurrence (BCR) with a rise in serum prostate-specific antigen (PSA) [5]. Progression to BCR is a major turning point in PC development as a large proportion of recurrent PCs will metastasize [6]. The standard treatment for metastatic PCs is androgen-deprivation therapy (ADT); inevitably the cancer mutates to escape the effect of ADT and develops into castration resistant prostate cancer (CRPC) [7]. PC formation and progression is regulated by complex factors, for which our knowledge remains limited.

Butyrylcholinesterase (BChE) is an appealing candidate in PC. BChE and acetylcholinesterase (AChE) are two choline esterases that share 65% of amino acid identity [8]. They have different tissue distributions and kinetics in hydrolyzing acetylcholine. AChE is expressed at high levels in brain, muscle, and erythrocytes, while BChE is abundant in liver, intestine, heart, kidney, and lung [9,10]. AChE hydrolyzes acetylcholine rapidly [9], which is consistent with its classic role in hydrolyzing acetylcholine in cholinergic synapses of the brain and autonomic nervous system [11]. Liver-produced BChE is secreted into serum [12]; the plasma BChE hydrolyzes butyrylcholine [12], succinylcholine [13], and ghrelin (the hunger hormone) [14–17]. The serum level of BChE is changed in numerous clinical conditions. It is elevated in increased levels of serum triglyceride and cholesterol as well as in insulin resistance [18,19] and is decreased in protein-energy malnutrition, stress, and inflammation [20]. This may reflect the metabolic alterations in these clinical conditions.

Inflammation and anorexia are associated with advanced cancer [21], supporting the observed common reduction of serum BChE in cancer patients. This down-regulation is correlated with cancer progression [19]. Lower levels of serum BChE were detected in patients with pancreatic cancer [22], colorectal carcinoma [23], oral cancer [24], head and neck squamous cell carcinoma [25], non-small-cell lung cancer [26], lung squamous cell carcinoma [27], papillary renal cell carcinoma [28], bladder cancer [29], and upper urinary track urothelial carcinoma [30]. In patients with PC, reductions in serum BChE are associated with decreases in BCR-free survival [31,32]. Nonetheless, the contributions of BChE to cancer development remain unclear, which may be attributable to the lack of knowledge on tumor-associated BChE. With respect to PC, this issue is also not settled, although there was a report for no alteration in the BChE protein in primary PCs (n = 11) compared to benign prostatic hyperplasia (BPH, n = 20) [33].

To investigate the above issue, we report here a thorough analysis of BChE expression in PC development. BChE expression was examined at both the mRNA and protein levels *in vitro*, *in vivo*, and in patients in the context of PC tumorigenesis and progression. We demonstrated for the first time a biphasic alteration of BChE level, in that the expression of BChE is down-regulated at early stage of PC and up-regulated at advanced PC. It is important to note that the up-regulation is associated with PC recurrence.

Materials and Methods

Cell Culture and Generation of DU145 Spheres or Prostate Cancer Stem-Like Cells (PCSLCs)

LNCaP, PC3, and DU145 cells were obtained from American Type Culture Collection (ATCC), and cultured in RPMI-1640 (LNCaP), F12 (PC3) and MEM (DU145) media supplemented with 10% FBS (Gibco) and 1% Penicillin–Streptomycin (Thermo Fisher Scientific). Generation and culture of DU145 spheres were performed based on our published conditions [34]. In brief, DU145 monolayer cells (non-PCSLCs) were seeded at a density of 5000 cells/mL in serum-free (SF) media (3:1 DMEM/F12 mixture) (Thermo Fisher Scientific) containing 0.4% bovine serum albumin (BSA) (Bioshop Canada Inc.). Cells were supplemented with 0.2× concentration of B27 minus Vitamin A (Thermo Fisher Scientific) and 10 ng/ml EGF (Sigma Aldrich) in T75 flasks. Typical spheres were formed from 10 to 12 days. Immortalized human prostate epithelial BPH-1 cells were kindly provided by Dr. Simon Hayward at Vanderbilt University Medical Center, Nashville, TN, USA [35].

Collecting Primary Prostate Cancer

Prostate cancer tissues were obtained from St. Joseph's Hospital in Hamilton, Ontario, Canada under approval from the local Research Ethics Board (REB# 11–3472) and with patient consent.

Xenograft Tumor and Lung Metastasis

Generation of xenografts and lung metastasis was carried out according to our established procedure [36–38]. LNCaP (RPMI-1640), DU145 (MEM), and PC3 (F12) cells were resuspended in 0.1 ml cell culture medium/Matrigel mixture (BD) in their respective medium with 1:1 ratio, and implanted subcutaneously into the flanks of 6 weeks-old male NOD/SCID mice (The Jackson Laboratory). Tumors growth was measured weekly with calipers, and tumor volume (mm^3) were calculated using the formula $V = L \times W^2 \times 0.52$ with L and W being the longest and shortest diameters, respectively. Generation of LNCaP cell-derived xenografts in intact and castrated mice was performed following our published procedure [39]. In brief, LNCaP tumor cells were implanted as described above. Blood was collected biweekly *via* facial vein to monitor serum PSA with ELISA (Abcam). When tumor reached approximately 150 mm^3, mice received either castration (n = 5) or no surgery (n = 5). For the generation of lung metastasis, 10^6 DU145 cells were resuspended into 0.3 mL of PBS and injected through the tail vein of 6 weeks-old NOD/SCID mice (n = 4). Lungs were harvested at 10 weeks post-injection. All animal work was carried out according to experimental protocols approved by the McMaster University Animal Research Ethics Board.

Microarray Analysis

Total RNA was isolated from DU145 monolayer and DU145 PCSCs with TRIZOL (Life Technologies). Gene expression was examined using the Affymetrix Human Gene 1.0 ST microarrays, purchased through the University Health Network Microarray Centre (UHNMAC, www.microarrays.ca, Toronto, ON).

Procedures were carried out at UHNMAC according to the protocol detailed by Affymetrix. Functional analysis of differentially expressed genes was carried out using Ingenuity Pathway Analysis (Ingenuity) to determine top diseases and biological functions.

Nanostring Gene Expression

RNA was isolated from 10 μm sections of Formalin-fixed, Paraffin-embedded (FFPE) prostate cancer tissue with High Pure FFPET RNA isolation kit. Total RNA was sent to the Farncombe Metagenomics Facility (McMaster University) for nCounter Elements tags analysis. RNA probes for target gene was synthesized and raw gene counts from the nCounter system were imported into the nSolver software. Raw counts for respective gene tag and samples were normalized against positive controls and three endogenous reference genes: ATP-binding cassette, sub-family F member 1, glyceraldehyde-3-phosphate dehydrogenase and B-tubulin. Normalization was performed by dividing the raw counts of the target gene by the geometric mean of the positive controls followed by the geometric mean of the endogenous reference genes. The fold change was determined by the ratio of the normalized gene counts in the tumor and normal tissues.

Quantitative Real-Time PCR Analysis of BChE Expression

Total RNA was isolated using TRIZOL, and reversely transcribed into cDNA using superscript III (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was carried out with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) using SYBR-green (Thermo Fisher Scientific). All samples were run in triplicate. BChE (Forward): 5′-ACAGGC CAGCTTGTGCTATT-3′, BChE (Reverse): 5′-CAAAAGCCGAG GAAATTTTG-3′. β-Actin (Forward): 5′-ACCGAGCGCGGCTA CAG-3′, β-Actin (Reverse): 5′-CTTAATGTCACGCACGATTTCC-3′.

Immunohistochemistry (IHC)

IHC was performed on paraffin embedded and serially cut prostate cancer tissues obtained from St. Joseph's Hospital, Hamilton, Ontario, Canada. Slides were deparaffinized in xylene and cleared in an ethanol series. Antigen retrieval was performed in a food steamer for 20 minutes using sodium citrate buffer (pH = 6.0). Tissues were blocked for 1 hour in PBS containing 1% BSA and 10% normal goat serum (Vector Laboratories). BChE (1:250, Sigma Aldrich) antibodies were incubated overnight at 4°C. Tissues were subsequently incubated in secondary antibody biotinylated goat anti-rabbit IgG, and Vector ABC reagent (Vector Laboratories) according to the manufacturer's instructions. Secondary antibody-only was used as negative control. Washes were performed with PBS and slides were counterstained with hematoxylin (Sigma Aldrich). Image analysis was performed using ImageScope software (Leica Microsystems Inc.). Staining intensity values derived from ImageScope were converted to an HScore using the formula [HScore = (% Positive) × (intensity) + 1]. The HScore was normalized through background subtraction and averaged amongst approximately 10 images per tissue sample.

Cut-Off Point Estimation

Cut-off point of BChE mRNA expression in separation of recurrent tumor from those without BCR was determined using Maximally Selected Rank Statistics (the *Maxstat* package) in R.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test and one-way ANOVA followed by Tukey's and Bonferroni's multiple comparisons tests with SPSS. Kaplan–Meier surviving curves and log-rank test were performed using the R survival package. A value of $P < .05$ is considered statistically significant.

Results

Down-Regulation of BChE During PC Tumorigenesis

Reductions of serum BChE in patients with PC have been observed [31,32]. Although there was a report for non-changes of BChE protein expression in primary PCs (n = 11) compared to BPH (n = 20) [33], the status of BChE expression in PC needs further investigation. To address this issue, we examined BChE mRNA expression in prostate cancer stem-like cells (PCSLCs) which we have isolated from DU145 cells [34]. In comparison to DU145 monolayer (non-PCSLC) cells, BChE mRNA expression was dramatically reduced in PCSLCs based on the analyses of cDNA microarray (Figure 1*A*) and real-time PCR (Figure 1*B*). As PCSLCs are associated with 100 fold elevation in tumorigenesis compared to non-PCSLCs [34], the above observations indicate a down-regulation of BChE during PC development.

To further investigate this notion, we determined BChE mRNA expression in 7 pairs of PC and prostate tissues using Nanostring technology. The PC tissues had 60–80% of cancer. In 5/7 pairs of tissues, PTEN expression was decreased, while ERG expression was increased (indicative of TMPRSS2-ERG fusion) (Table 1). Down-regulation of PTEN and up-regulation of ERG due to TMPRSS2-ERG fusion occur frequently in PC [40,41], thus validated these tissues for the analysis of PC-associated gene expression. In comparison to the matched prostate tissues, BChE mRNA expression was reduced in 5/7 tumor tissues (Table 1), supporting a down-regulation of BChE in PCs.

To further examine BChE down-regulation in PC, we retrieved BChE mRNA expression data from the Grasso [42] and Taylor [43] datasets in the Oncomine™ database (Compendia Bioscience, Ann Arbor, MI). In both cohorts, a significant decrease in BChE mRNA expression was detected in PC (n = 190) compared to non-tumor prostate tissues (n = 57) (Figure 1, *C* and *D*); it was not clear whether BChE level was further reduced in metastatic PCs (Figure 1, *C* and *D*). We also extracted the BChE mRNA expression data from the MSKCC dataset [44], and demonstrated a significant decrease of BChE mRNA expression in PC (n = 150) compared to prostate tissues (n = 29) (Figure 1*E*). The down-regulation discriminates PC from normal prostate tissues with an area under curve (AUC) value of 0.77 (Figure 1*F*). Collectively, we provide a comprehensive set of *in vitro* and clinical evidence for a down-regulation of BChE expression in PC cells during tumorigenesis.

Heterogeneous Alteration of BChE During PC Progression

The observed reduction of BChE in PC compared to non-tumor prostate tissues above (Table 1; Figure 1, *C-E*) reveals that the down-regulation is associated with PC formation. We then determined whether the decrease is also associated with PC progression; this possibility is suggested by the robust down-regulation of BChE in DU145 PCSLCs (Figure 1, *A* and *B*). This concept is in line with BChE reduction in response to CNTN1 overexpression in DU145 and C4–2 PC cells (Figure 2, *A* and *B*). CNTN1 promotes PC progression [36].

To further investigate this association, we examined BChE expression in a set of PC cell lines, BPH-1, LNCaP, DU145, and

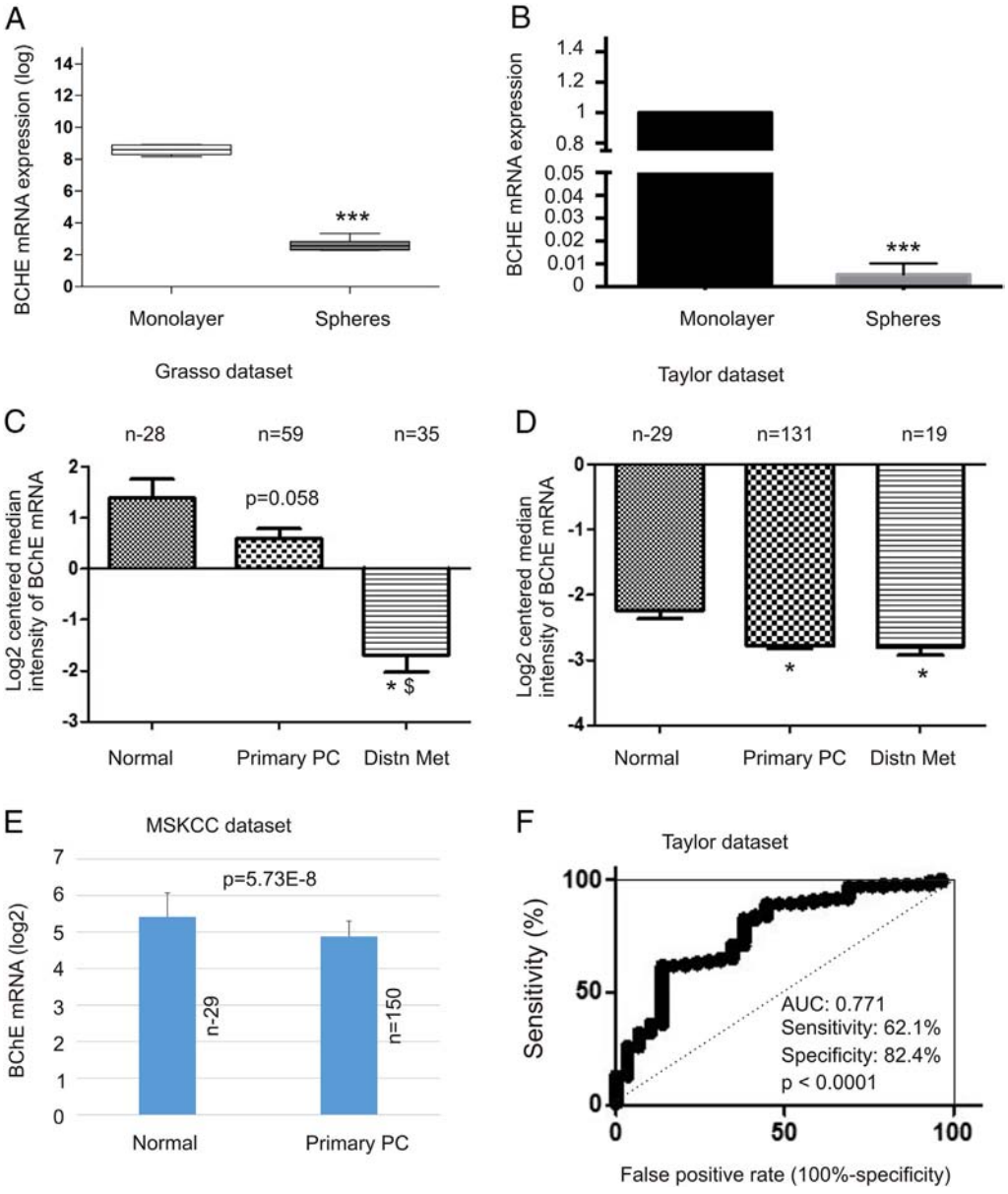

Figure 1. down-regulation of BChE in prostate cancer. (A) DU145 monolayer and sphere cells were analyzed by DNA microarray (3 repeats) for BChE mRNA. The average levels of BChE mRNA (log2) are graphed. ***$P < .001$ by a two-tailed Student's *t*-test. (B) Real-time PCR analysis of BChE mRNA expression in DU145 monolayer cells and sphere cells. Experiments were repeated three times. BChE mRNA levels were normalized to actin and expressed in fold change to DU145 monolayer cells; means ± standard deviation (SD) are graphed. *** $P < .001$ in comparison to DU145 monolayer cells by a two-tailed Student's *t*-test. (C-E) Data was extracted from the Grasso (C), Taylor (D) datasets within Oncomine™ (Compendia Bioscience, Ann Arbor, MI), and the MSKCC dataset within cBioPortal (E). Means ± SD are graphed; * $P < .05$ by a two-tailed Student's *t*-test in comparison to Normal; $ $P < .05$ in comparison to primary PC (two-tailed Student's *t*-test). (F) A receiver-operating characteristic (ROC) curve of primary PCs *versus* normal prostate tissues. AUC: area under the curve.

Table 1. Nanostring Analysis of Gene Expression in Primary Prostate Cancer tissues

Genes	P1[a]	P2[a]	P3[a]	P4[a]	P5[a]	P6[a]	P7[a]
BCHE*	N	−5.9	N	−3.4	−1.5	−3.2	−1.5
TMPRSS2-ERG[b]	+16.2	+30.2	N[c]	N	+17	+25.3	+27.3
PTEN[b]	−1.4	−1.4	N	N	−1.4	−1.3	−2.6

[a] Patients 1–3 (GS6), patients 4–6 (GS7, 4 + 3 for P4,5, and 3 + 4 for P6), and P7 (GS4+ 4).
[b] TRPRSS2-ERG and PTEN were used as positive controls for up-regulated and down-regulated genes in PC.
[c] No alterations.
* $P < .01$ in comparison to benign tissues (two-tailed Student's *t*-test).

PC3 lines. BPH-1 is an immortalized but non-tumorigenic prostate epithelial cells [35]; LNCaP cells were derived from lymph node metastasis, while DU145 and PC3 cells were isolated from brain and bone metastasis, respectively. This sample collection thus includes some major steps in PC progression, including immortalization, early stage metastasis (lymph node), and distance metastasis (brain and bone). Unexpectedly, LNCaP cells display the highest level of BChE expression, followed by DU145 cells with both PC3 and BPH-1 expressing the lowest level of BChE (Figure 2*C*).

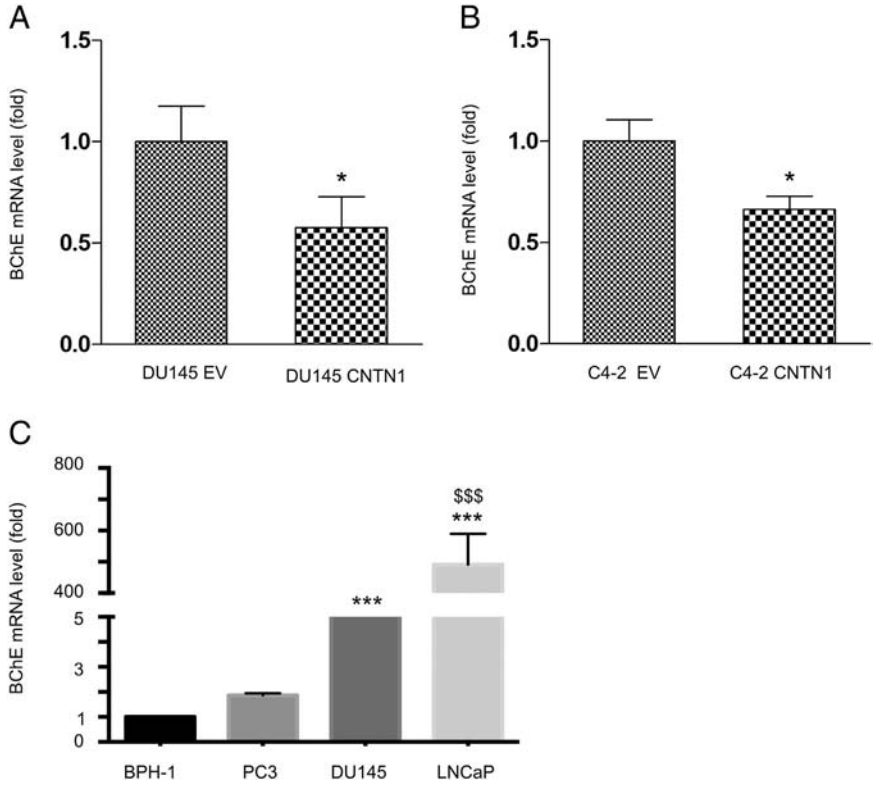

Figure 2. Changes in BChE expression in PC cell lines. (A, B) The indicated empty vector (EV) and contactin 1 (CNTN1) stable lines were previously established [36]. BChE mRNA levels in these cell lines were determined using real-time PCR and normalized to actin. BChE mRNA levels were presented as fold change to the respective EV lines. Means ± SD are graphed. * $P < .05$ in comparison to the EV lines by a two-tailed Student's *t*-test. (C) BChE mRNA levels in immortalized prostate epithelial cells (BPH-1), PC3, DU145, and LNCaP cells were determined using real-time PCR, normalized to actin, and presented as fold change to the BChE mRNA level in BPH-1 cells. Experiments were repeated three times; means ± SD are graphed. *** $P < .001$ in comparison to BPH-1 cells by a two-tailed Student's *t*-test; $$$ $P < .001$ in comparison to DU145 cells by a two-tailed Student's *t*-test.

We then investigated the association of BChE expression with PC progression *in vivo*. Xenograft tumors were produced using either DU145 monolayer cell or sphere (PCSLC) cells. Unlike the down-regulation of BChE in DU145 spheres (Figure 1, *A* and *B*), the xenografts (n = 4) generated from sphere cells exhibit comparable level of BChE as xenografts (n = 3) produced by monolayer cells (Figure 3*A*). Furthermore, lung metastases (Supplementary Figure 1; n = 4) were produced using DU145 monolayer cells; the same cell-generated lung metastases expressed likely higher BChE compared to xenografts (n = 3) derived from DU145 monolayer cells (Figure 3*A*). Additionally, we also produced subcutaneous xenografts using LNCaP, PC3, and DU145 cells. While LNCaP cells expressed a significantly higher level of BChE than DU145 cells *in vitro* (Figure 2*C*), the reverse was observed in xenografts (Figure 3*B*). We further examined BChE expression in PC treated with and without castration. LNCaP cell-based xenografts were produced in NOD/SCID mice; animals were either castrated (n = 5) or non-castrated (n = 5) when tumors grew to approximately 150mm^3. Castration was associated with an initial decline in serum PSA for 3 weeks, followed with elevations by 4 weeks, indicative of CRPC status [39]. In comparison to xenografts generated in intact mice, LNCaP xenograft tumors produced in castrated mice showed a significant higher level of BChE (Figure 3*C*). Collectively, these results demonstrate complex alterations of BChE expression in PC progression.

The above heterogeneity in terms of BChE expression *in vitro* and *in vivo* could also be demonstrated in primary PCs. In our examination of 8 Gleason score (GS) 6–7 and 16 GS8–10 PCs (Supplementary Table 1), a general higher level of the BChE protein expression was detected in PIN lesions and GS6–7 PC (Figure 4*A*); the difference was statistically significant (Figure 4*B*). However, the high-grade PC population has tumors expressing a high level of BChE (Supplementary Table 1) and intra-tumor regions with high BChE expression were also observed in high-grade PCs (Figure 4*A*, see patient #19). Consistent with the nuclear expression of BChE in xenografts, its predominant nuclear distribution was detected in primary PCs (Figure 4*A*). Collectively, the above results suggest a complex relationship between BChE expression and PC progression.

Biphasic Alteration of BChE Expression Following PC Progression

To further examine BChE expression during PC progression, we retrieved BChE gene expression data from the TCGA Provisional dataset (n = 490) within the cBioPortal database along with Gleason score-based PC grades. The GS grade system has been further defined to the WHO grade group 1–5. We first converted the GS grades into WHO grade group (GS6 = WHO grade group 1, GS3+ 4 = WHO grade group 2; GS4+ 3 = WHO grade group 3, GS8 = WHO grade

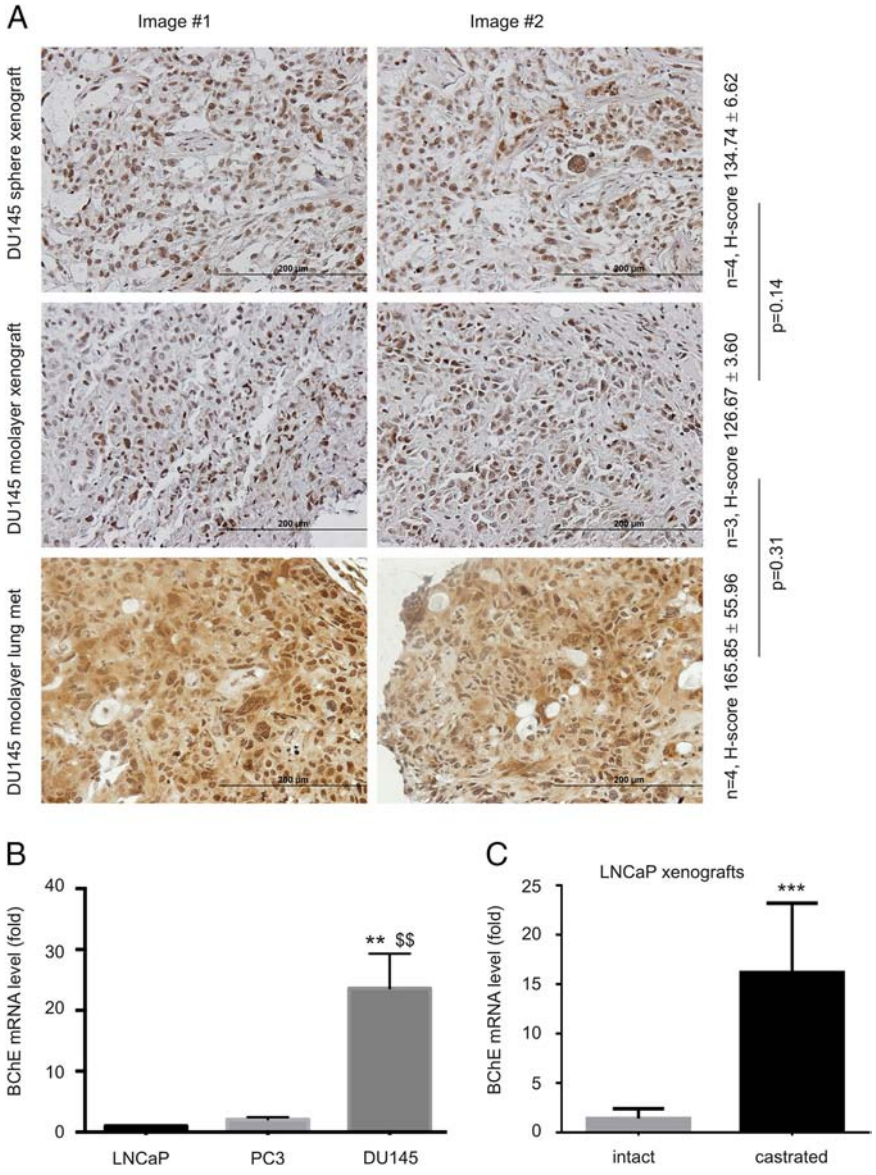

Figure 3. Examination of BChE expression in xenograft tumors. (A) Subcutaneous (s.c.) xenograft tumors were produced using either DU145 sphere cells or monolayer cells. Lung metastases were generated using DU145 monolayer cells *via* tail vein injection. BChE protein expression in these tumors was examined using immunohistochemistry (IHC) staining. Two images of typical individual tumors were presented. BChE staining was quantified using Hscores; means ± SD were included along with the *P* values determined by a two-tailed Student's *t*-test. (B) S.C. xenografts were derived from the indicated cell lines (n = 5 per cell line). BChE mRNA expression in the xenografts was determined using real-time PCR, normalized to actin, and presented as fold change to LNCaP cell-produced xenografts. Means ± SD are graphed; $^{**}P < .01$ and $\$\$ P < .01$in comparison to LNCaP and PC3 xenografts by a two-tailed Student's *t*-test, respectively. (C) LNCaP xenograft tumors were produced in intact (n = 5) or castrated (n = 5) mice. BChE mRNA expression was determined using real-time PCR, normalized to actin, and presented as fold change to the BChE mRNA level detected in xenograft tumors in intact mice. Means ± SD are graphed; *** $P < .001$ by a two-tailed Student's *t*-test.

group 4, and GS9–10 = WHO grade group 5), and observed a trend of decline in BChE expression following PC progression from WHO grade group 1 to WHO grade group 3 (Figure 5*A*). A significant reduction of BChE expression in WHO grade group 3 and WHO grade group 4 PCs in comparison to WHO grade group 1 tumors was demonstrated (Figure 5*A*); this supports BChE down-regulation in PC *vs* prostate tissues (Figure 1, *C–E*). Intriguingly, a significant elevation of BChE expression occurs following PC progression from WHO grade groups 3 and 4 PCs to WHO grade group 5 tumors (Figure 5*A*). In addition to Student's *t*-test used above to analyze BChE expression in different PC grades, we also performed multiple group comparisons using one-way ANOVA ($P = .0057$). The significant up-regulation of BChE in WHO grade group 5 compared to WHO grade group 3 was confirmed by post-hoc analysis ($P < .01$ by Tukey's multiple comparisons test and $P < .05$ by Bonferroni's multiple comparisons test). Similar BChE expression pattern was also detected in an independent and smaller MSKCC dataset [44] (Figure 5*B*). Additionally, while gene expression in the TCGA Provisional dataset was determined with RNA sequencing, mRNA in the MSKCC dataset was profiled by cDNA microarray. The

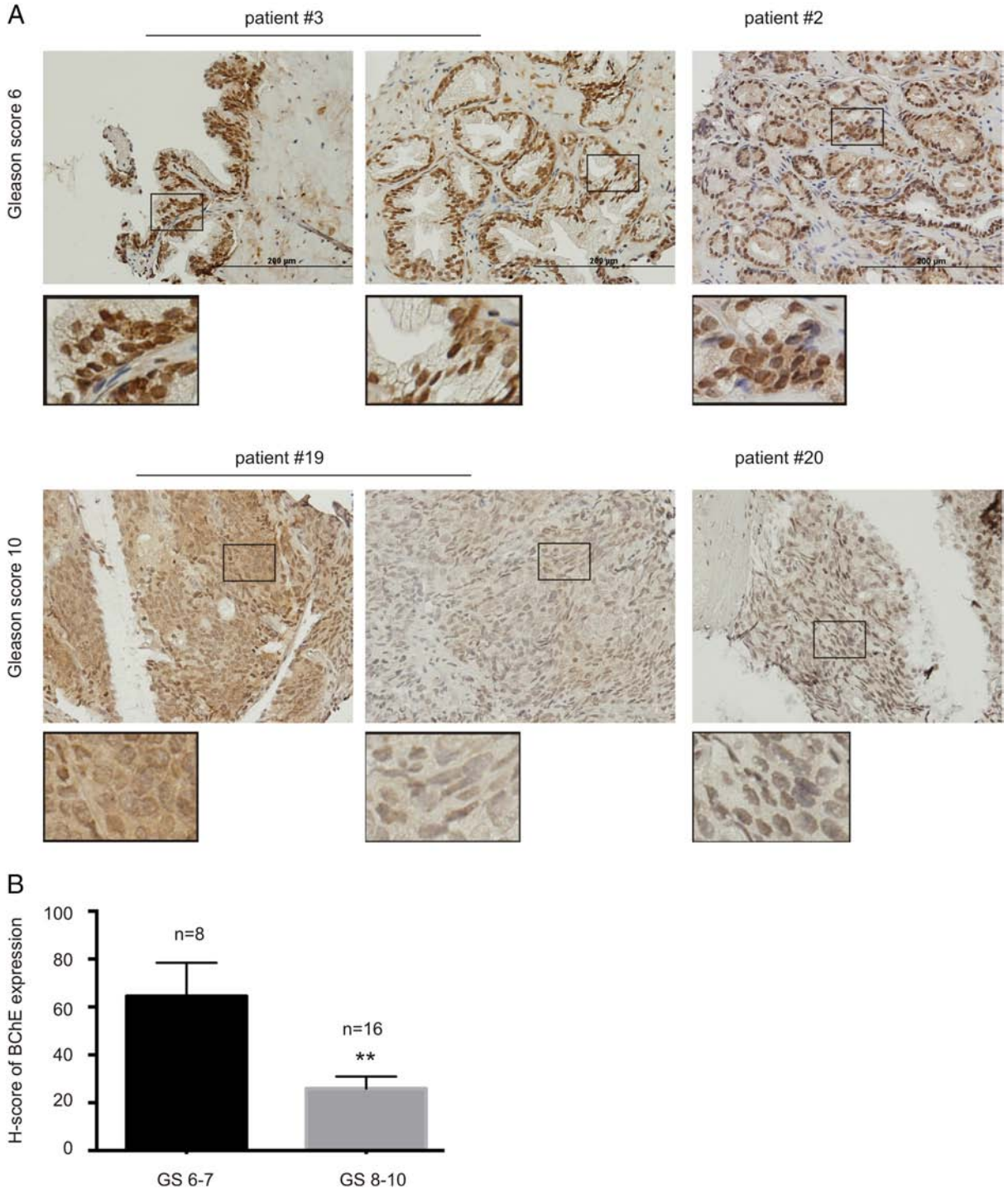

Figure 4. BChE protein expression in primary PCs. Low grade (GS ≤ 7, n = 8) and high-grade PCs (GS ≥ 8, n = 16, see Supplementary Table 1 for details) were stained for BChE using IHC. Typical images for two low-grade PCs and 2 high-grade PCs are shown (A). The staining was quantified using Hscores, means ± SD were graphed; ** $P < .01$ in comparison to low-grade PCs by a two-tailed Student's *t*-test (B).

demonstration of similar BChE alterations in two independent datasets with different approaches for gene expression supports the authenticity of BChE expression changes in PC. The biphasic change is likely specific for BChE. While AChE is another major choline esterase and shares 65% of amino acid identity with BChE [8], its expression is steadily up-regulated following PC progression (Supplementary Figure 2). Taken together, we demonstrate a biphasic alteration of BChE mRNA expression in PC (n = 720, Figure 5).

BChE Up-Regulation in PC is Associated with PC Recurrence

To examine the impact of BChE expression on PC progression, we determined a potential association of BChE expression and PC

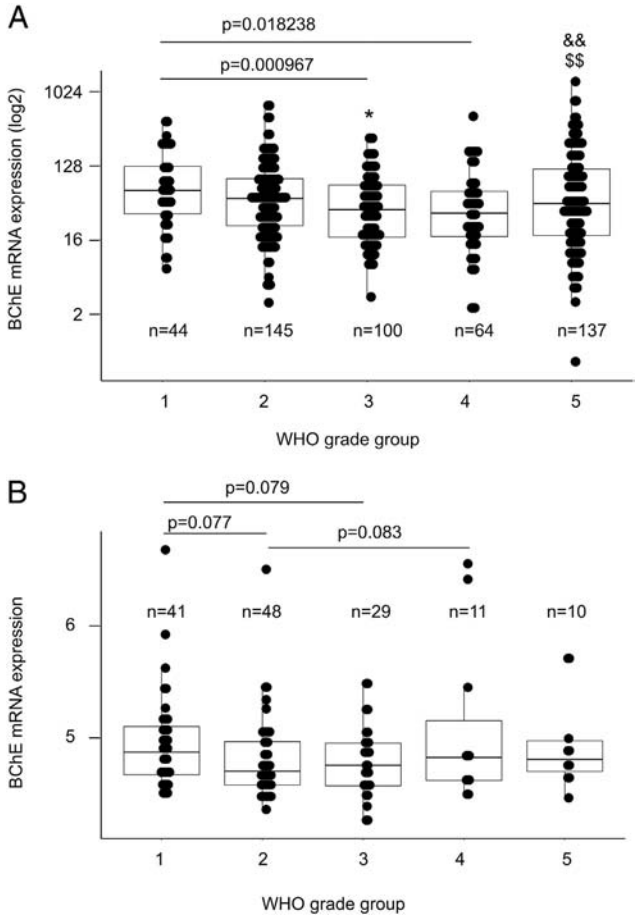

Figure 5. Biphasic alterations of BChE expression in PC. BChE mRNA data was retrieved from the TCGA Provisional (A) and MSKCC (B) datasets within cBioPortal. Means and dot distributions of BChE mRNA expression in individual WHO grade group 1–5 were graphed. Statistical analyses were performed using a two-tailed Student's *t*-test. The *P* values for the indicated comparisons are shown. * $P < .05$ in comparison to WHO grade group two PCs (A); $$ $P < .01$ in comparison to WHO grade group 3 PCs (A); && $P < .01$ in comparison to WHO grade group 4 PCs (A). The data was also analyzed by one-way ANOVA and post-hoc analysis using Tukey's multiple comparison test and Bonferroni's multiple comparison test. For one-way ANOVA, $P = .0057$ (A) and $P = .1688$ (B). For the TCGA dataset (A), * $P < .05$ for the comparison of WHO grade group 5 PCs and WHO grade group 3 PCs by both Tukey's multiple comparison test and Bonferroni's multiple comparison test.

recurrence. We extracted BChE mRNA expression data and the follow-up data on PC recurrence (BCR) from the MSKCC dataset. The cut-off point was first estimated to separate the recurrent PCs from non-recurrent PC using the Maximally Selected Rank Statistics available from the *Maxstat* package in R (Figure 6*A*). PCs with BChE expression above the cut-off point display a significantly higher level of BChE expression compared to those expressing BChE below the cut-off point (Figure 6*B*). We then assigned a binary code "1" (cut-off point-positive) to PCs with BChE expression > the cut-off point score and "0" (cut-off point-negative) to PCs with BChE expression ≤ the cut-off point score. This demonstrated that cut-off point-positive PCs were associated with a significant reduction in disease free survival (DFS) (Figure 6*C*).

We further investigated the impact of BChE expression on PC recurrence using the large TCGA cohort (n = 490). The BChE expression data and the recurrence information were retrieved and analyzed following the above system. However, a cut-off point could not be defined to classify PCs with elevated risk of recurrence (data not shown). The BChE expression data determined by RNA sequencing in this dataset shows high variability (Figure 5*A*), which might be a contributing factor for its inability to stratify tumors for risk of recurrence. To solve this issue, we derived a subpopulation (n = 245) consisting of tumors with BChE expression > the Median level of BChE expression. We then estimated the cut-off point and analyzed DFS in PCs positive or negative for the cut-off point. In comparison to tumors negative for the cut-off point, the cut-off point-positive PCs displayed a reduction in DFS (Figure 6*D*). These results are in accordance with the observed effect of elevations in BChE expression on PC recurrence in the MSKCC dataset (Figure 6*C*).

Discussion

BChE has been and remains a very appealing biomarker in cancer diagnosis owing to the common reduction of plasma BChE in multiple cancer types and the general association of the decrease with poor prognosis [19]. This concept is becoming even more attractive by the recent demonstration that BChE hydrolyzes ghrelin [15,17], thus regulating metabolism. Alteration of metabolism is a major contributor of tumorigenesis and cancer progression [45]. In line with this knowledge, decreases in serum BChE were associated with PC progression in terms of Gleason score advancement, bone metastasis, and recurrence following radical prostatectomy [31,32]. Nonetheless, reductions of serum BChE may reflect the general deterioration of patient's health condition and not directly indicate a role of BChE in tumorigenesis and development.

We performed the first thorough examination of BChE expression in prostate cancer at both the protein and mRNA levels using *in vitro*, *in vivo*, and patient-related approaches. Intriguingly, BChE expression displays a biphasic alteration following PC development. *In vitro*, a reduction of BChE expression was associated with PC progression, evident by its down-regulation in DU145 PCSLCs compared to DU145 non-PCSLCs, as well as in DU145 and PC3 cells compared to LNCaP cells. These observations were consistent with the reported BChE down-regulation in the CRPC-derivative of LNCaP C4–2 cells compared to LNCaP cells [46]. However, we also observed a reverse pattern, *i.e.* significantly elevations of BChE expression in tumorigenic LNCaP and DU145 cells over the immortalized non-tumorigenic prostate epithelial BPH-1 cells. This reverse pattern is supported by a recent report in which ectopic expression of an AR type 3a variant was found to inhibit LNCaP cell proliferation and reduce BChE expression [47]. *In vivo,* BChE expression in xenograft tumors does not reflect the status of BChE expression in their parental cell *in vitro,* suggesting a complex role of BChE in PC tumorigenesis. This notion is consistent with the BChE expression in more than 600 patients examined which demonstrated: a significant down-regulation of BChE expression in PCs *vs* prostate tissues, a significant decrease in BChE in WHO grade group 3 PCs compared to WHO grade group 1 PCs, and a significant up-regulation of BChE in WHO grade group 5 PCs. To our best knowledge, this is the first demonstration of biphasic alterations of BChE expression in human cancers in general and PC in particular. Nonetheless, a decrease in BChE expression was reported in colorectal carcinoma [48] and BChE up-regulations were observed in ovarian cancer [49] as well as head and neck squamous cell

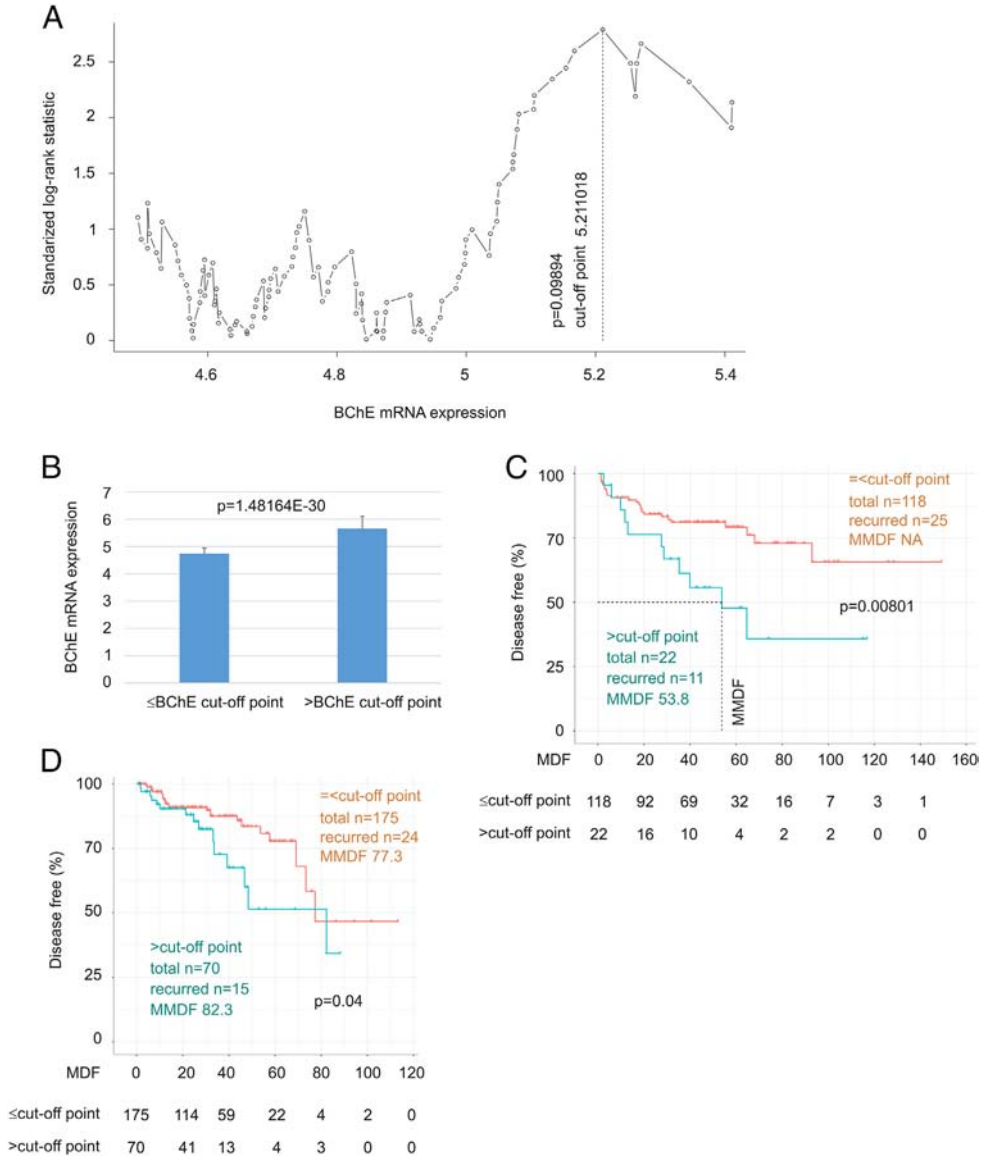

Figure 6. Up-regulation of BChE is associated with a reduction of disease free survival in PC. (A-C) BChE mRNA data from the MSKCC dataset (cBioPortal) was estimated for a cut-off point to separate recurrent PC from non-recurrent PC using Maximally Selected Rank Statistics (the Maxstat package) in R. The vertical dot-line shows the cut-off point and the associated *P* value (A). Mean BChE mRNA level in PCs expressing less than or equal to the cut-off point was compared to that in PCs with BChE mRNA expression above the cut-off point. The *P* value was determined using a two-tailed Student's *t*-test (B). Kaplan–Meier survival analysis of two groups of PC classified according to the cut-off point. MMDF: median months disease free; NA: not available as MMDF being not reached. Numbers of patient at risk at the start of the indicated follow-up period were included. Kaplan–Meier and log-rank test were carried out with the R *survival* Package (C). (D) BChE mRNA data was retrieved from the TCGA Provisional dataset (cBioPortal). PCs were group based on the Median level of BChE mRNA expression. The group (n = 245) above the Median was estimated for a cut-off point of separating recurrent from non-recurrent tumors, followed by analysis of PC recurrence in PCs classified according to the cut-off point. Numbers of patient at risk at the start of the indicated follow-up period were included. Kaplan–Meier and log-rank test were performed using the R *survival* Package.

carcinoma [25]. In light of our research here, it will be interesting to determine whether biphasic alterations in BChE expression also occur in these cancers.

Importantly, up-regulation of BChE is associated with PC recurrence. Similar results in other cancer types were also reported. Up-regulation of BChE is associated with a reduction in OS in ovarian cancer [49,50] and head and neck squamous cell carcinoma [25]. Additionally, in a chemical-induced rat model of hepatocellular carcinoma, BChE was induced in poorly differentiated tumor cells [51]. Collectively, while the role of BChE reductions in the early phases of PC tumorigenesis remains unclear, evidence supports its up-regulation being associated with PC recurrence.

The mechanisms underlying the biphasic alteration of BChE expression in PC and its functional impact on PC are not clear. Based on the steadily up-regulation of AChE following the advancement of WHO grade group in PC, it is tempting to propose that BChE may inhibit PC tumorigenesis at early stages through catalyzing non-acetylcholine substrates and that BChE enzymatic activity towards a different substrate facilitates PC progression at late stages. This possibility is appealing as cancer metabolism may differ at different

stages of progression. Alternatively, BChE may affect PC through mechanisms independent of its enzymatic activity. Despite these uncertainties, our study clearly demonstrates the different roles of PC-associated BChE from serum BChE in PC. It will be intriguing to investigate the functional impact of BChE in PC in future. In view of the general reduction of serum BChE in multiple cancer types, our findings will likely be applicable to other cancers.

Finally, the potential roles of BChE in PC and other cancer types should be evaluated in the context of BChE deficient mice [52] and humans [53,54], both being healthy. This genetic evidence indicates that loss of BChE can be compensated or alternatively BChE may not be a major oncogenic factor.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.06.003.

Acknowledgements

The results shown here are in part based upon data generated by the TCGA Research Network (http://cancergenome.nih.gov/). D.T. is supported by an Award from Teresa Cascioli Charitable Foundation Research Award in Women's Health and grants from Canadian Cancer Society (grant #: 319412) and Cancer Research Society.

Conflict of Interest Statement

All authors declare no conflict of interest.

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