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*Original Article*

# Aberrant expression of multiple glycolytic enzyme genes is significantly associated with disease progression and survival outcomes in prostate cancers

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**Abstract:** Prostate cancer is the leading cause of cancer death after lung cancer in men. Recent studies showed that aberrant metabolic pathways are involved in prostate cancer development and progression. In this study, we performed a systemic analysis of glycolytic enzyme gene expression using the TCGA-PRAD RNAseq dataset. Our analysis revealed that among 25 genes, only four genes (HK2/GPI/PFKL/PGAM5) were significantly upregulated while nine genes (HK1/GCK/PFKM/PFKP/ALDOC/PGK1/PGAM1/ENO2/PKM) were downregulated in primary prostate cancer tissues compared to benign compartments. Among these 13 altered genes, four genes (ENO2/ALDOC/GPI/GCK) exhibited strong diagnostic potential in distinguishing malignant and benign tissues. Meanwhile, GPI expression exerted as a prognostic factor of progression-free and disease-specific survival. PFKL and PGAM5 gene expressions were associated with AR signaling scores in castration-resistant patients, and AR-targeted therapy suppressed their expression. In LuCap35 xenograft tumors, PFKL and PGAM5 expression was significantly reduced after animal castration, confirming the AR dependency. Conversely, GCK/PKLR genes were significantly associated with neuroendocrinal progression, representing two novel neuroendocrinal biomarkers for prostate cancer. In conclusion, our results suggest that GPI expression is a strong prognostic factor for prostate cancer progression and survival while GCK/PKLR are two novel biomarkers of prostate cancer progression to neuroendocrinal status.

**Keywords:** Prostate cancer, glycolysis, prognosis, gene expression, castration-resistant

## Introduction

Prostate cancer is the second leading cause of cancer death in men [1]. The American Cancer Society estimates that there will be about 288,300 new cases of prostate cancer and about 34,700 deaths from prostate cancer in the United States by 2023 [1]. The prognosis for a given newly diagnosed prostate cancer is largely related to the stage of the disease and the 5-year survival rate for metastatic diseases is only about 30% although it is about 99-100% for localized diseases [2].

Current clinical practices for diagnosis and prognosis of prostate cancer are heavily reliant on the serum level of prostatic specific antigen (PSA), an androgen-responsive gene produced by epithelial cells of the prostate [3]. It is well known that PSA screening has limitations in terms of sensitivity and specificity, leading to increased costs of testing, follow-up procedures, and treatment for false-positive results and overdiagnosis [3]. Therefore, it is urgent to find novel biomarkers for improved specificity and sensitivity.

Glycolysis, a 10-step process for breaking down the major nutrient glucose, is a constant metabolic pathway in living cells, especially in cancer cells [4, 5]. The glucose metabolic process occurs in the cytoplasm to produce pyruvate, which then enters the tricarboxylic acid cycle (TAC) within the mitochondria for further catabolic reaction through oxidative phosphorylation [6].

Glycolysis is usually regarded as two phases (5 steps each), preparatory and pay-off phases. The first five steps of Glycolysis only consume energy (2 ATP molecules) to convert the glucose into two three-carbon sugar phosphates. The second five steps produce the energy-rich molecules ATP and NADH. Under normoxia conditions, the pyruvate is catalyzed via the TAC to produce more ATP molecules in mitochondria. In contrast, under hypoxia conditions, pyruvate is fermented by lactate dehydrogenase (LDH) in the cytoplasm into lactate. However, cancer cells in solid tumors often display an enhanced lactate-producing activity, the so-called Warburg effect [7].

During the 10-step glycolysis flux, there are about 25 enzymes involved, depending on the tissue types and development stages. The first step has four isozymes of hexokinases (HK1/2/3 & GCK), of which GCK is mainly expressed in the liver. Recent studies showed that HK2 is involved in prostate cancer progression [8] and might be a potential therapeutic target for prostate cancer treatment [9]. The third step has three isozymes of phosphofructokinases (PKFL/PFKM/PFKP), encoded by three distinct genes in different tissues. The fourth step has three isoforms of Fructose-1,6-bisphosphate aldolase (ALDOA, ALDOB, and ALDOC), which are different in their electrophoretic and catalytic properties. These isozymes are developmentally regulated at the transcription level. The seventh step has two isozymes, PGK1 and PGK2, which catalyze the irreversible conversion of fructose 6-phosphate into fructose-1,6-bisphosphate. Previous studies suggested that PGK1 promotes the interactions between cancer and its microenvironment [10], as well as bone metastasis [10]. The step-8 glycolysis has four isozymes (PGAM1/2/4/5), of which PGAM1 was recently shown to promote angiogenesis and metastasis in prostate cancer [11] and PGAM2 suppression sensitized prostate cancer cells to Enzalutamide treatment [12]. The ninth step has four isozymes, ENO1-4, of which ENO2 has long been regarded as a biomarker of neuroendocrine prostate cancer [13]. The last step of glycolysis has two isozymes, PKM1/2 and PKLR, which is also a rate-limiting step in the glycolytic pathway and is expressed differently in different tissues. Recent reports showed that the isozyme PKM1 exerts a tumor-suppressive effect in prostate cancer [14] while PKM2 promotes tumor metastasis in prostate cancer models [15]. In addition, PKLR was linked to metabolism dysfunction during neuroendocrinal progression of prostate cancer [16, 17]. This data suggests that most glycolytic isozymes are involved in prostate cancer progression.

In this study, we sought to document the expression profiles of these genes encoding the glycolytic isozymes in prostate cancers during different progression stages. Our analysis revealed that GPI expression is the strongest prognostic factor for disease progression and patient survival while GCK and PKLR can serve as novel biomarkers of neuroendocrinal differentiation of castration-resistant prostate cancer.

## Materials and methods

### *Gene expression analysis of the TCGA-PRAD dataset*

Gene expression profiles were assessed at the mRNA levels in malignant and case-matched benign prostatic tissues using the RNA sequencing (RNA-seq) dataset from the TCGA project [18]. The RNA sequencing datasets in the format of fragments per kilobase per million (FPKM) were downloaded from the TCGA online portal and converted to the $\log_2$ value of Transcript Per Million reads (TPM) before statistical analysis. This TCGA-PRAD dataset contained 51 case-matched benign and malignant tissues, plus 449 cases of unmatched malignant tissues. Gene expression levels were compared between the case-matched pairs and different groups based on clinicopathological parameters. Receiver operating Characteristic (ROC) curve analysis was conducted to evaluate the diagnostic significance of gene expression in distinguishing malignant from benign tissues.

### *Analysis of gene expression with biochemical relapse*

The RNAseq data from 292 early-onset prostate cancers (diagnosis ≤ 55 years) were obtained from the cBioportal platform [19, 20]. The mRNA expression levels were calculated as a value of [$\log_2$ value + 1] of the RPKM (Reads per kilobase of transcript per Million reads).

*Assessment of patient survival outcomes*

Patient survival outcomes were assessed using the TCGA-PRAD dataset with the Kaplan-Meier curve approach. We used the minimum *p*-value approach to set up the cutoff value for splitting patients into high or low subgroups [21]. A time-dependent prediction of patient survival was performed to determine the prognostic value of aberrantly expressed genes according to a previous report [22].

*Gene expression analysis in LuCaP35 xenografts after castration*

Gene expression changes after castration in human prostate cancer LuCaP35 xenograft model was downloaded from NCBI GEO dataset GDS4120 [23]. Briefly, LuCaP35 xenograft tumors were established in athymic nude mice. After castration for 4 weeks, xenograft tumors were harvested for RNA extraction using the RNeasy Mini Kit (QIAGEN). Gene expression profiling was carried out with the GeneChip human genome U133 Plus 2.0 array (Affymetrix) [23].

*Gene expression analysis in castration-resistant and neuroendocrinal differentiated tumors*

Gene expression data were downloaded from the cBioportal SU2C/PCF dataset, which was derived from 332 specimens of 323 patients with metastatic castration-resistant prostate cancer (mCRPC) including 41 NEPC samples [24]. Gene expression (FPKM polyA value) was compared in groups with or without androgen receptor signaling inhibitor (ARSI) usage and NEPC features [24]. A Spearman correlation analysis was conducted between gene expression and NEPC score [25], AR score [25], and AR-V7 expression (capture AR-v7 spliced reads per million).

*Data presentation and statistical analysis*

All quantitative data were presented as the MEAN with the SEM (standard error of the mean) and analyzed using the R-package (version 4.2.1) and GraphPad Prism software (version 9.1.0). Comparisons for case-matched pairs were conducted with the Wilcoxon signed rank test. Group comparisons were performed using the Mann-Whitney U test coupled with the Wilcoxon rank sum test. Patient survival outcomes were analyzed and visualized using the survival (version 3.3.1), survminer, and ggplot2 (version 3.3.6) in the R-package. The hazard ratio (H.R.) was evaluated using the Log-rank test. The ROC curve analysis was conducted using the pROC (1.18.0) or timeROC (version 0.4) and visualized with ggplot2 (version 3.3.6) from the R-package.

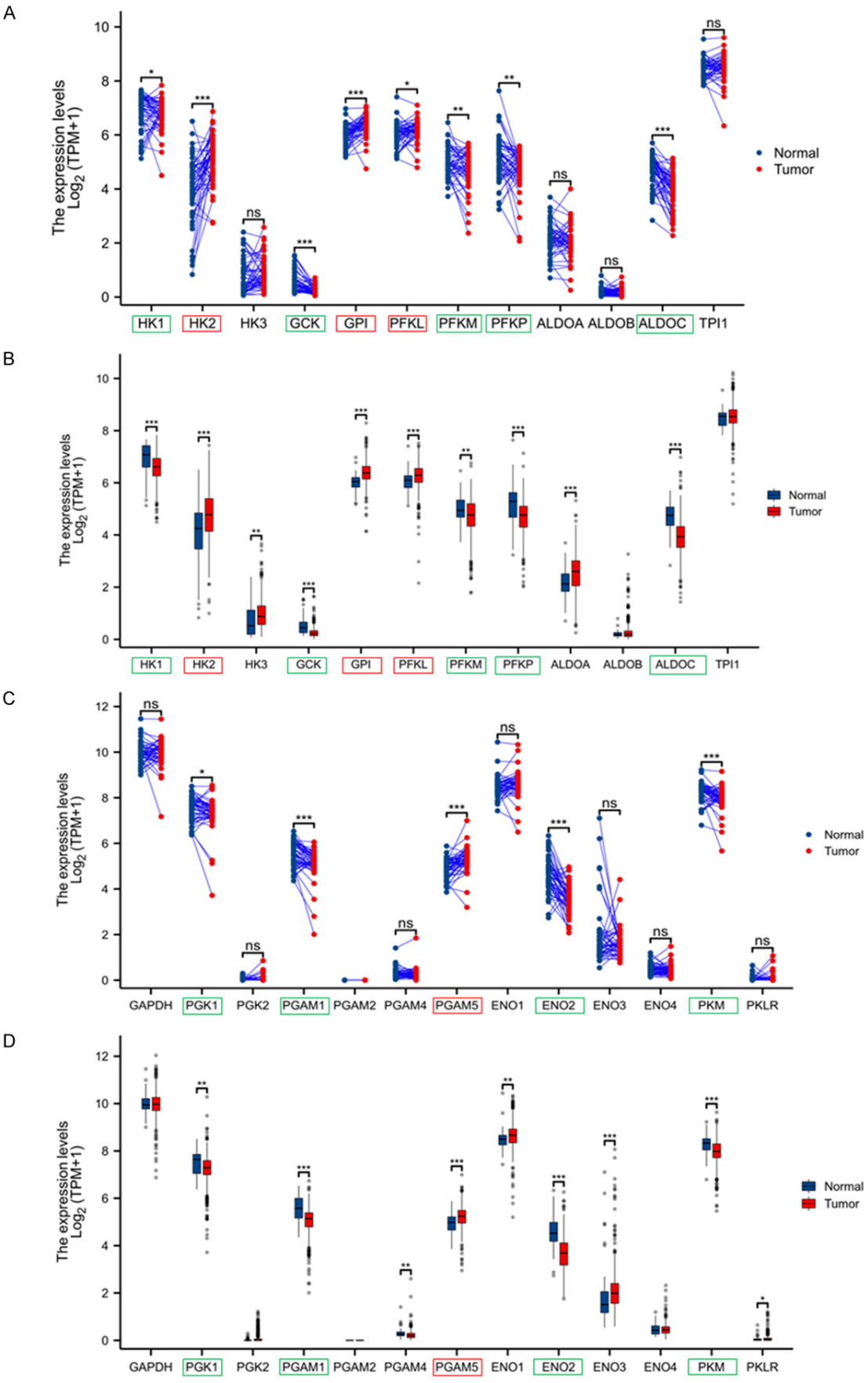
## Results

*Multiple glycolytic enzyme genes were aberrantly expressed in primary prostate cancers*

We analyzed all genes encoding glycolytic enzymes and their isoforms participating in the 10-step process at the mRNA level using the TCGA-PRAD dataset. Our analysis revealed that among 25 genes, 10 genes encoding for the isozymes of HK3/GCK/ALDOA/ALDOB/PGK2/PGAM2/PGAM4/ENO4/PKLR were expressed at very low levels in both malignant and benign prostatic tissues, indicating that they were not important isozymes in primary prostate cancer. Four genes HK2/GPI/PFKL/PGAM5 showed a significantly higher mRNA level in malignant tissues compared to benign compartments (**Figure 1A**, **1B**). In contrast, nine genes HK1/GCK/PFKM/PFKP/ALDOC/PGK1/PGAM1/ENO2/PKM showed a significantly lower mRNA level in malignant tissues compared to benign tissues (**Figure 1C**, **1D**).

To determine the clinical significance of these altered gene expression profiles, we conducted a diagnostic receiver operating characteristic (ROC) curve analysis. Our results showed that there were three genes (ENO2/GPI/GCK) with a significantly higher AUC value (≥ 0.75, **Figure 1E**, **1F**), representing a potential biomarker value for clinical diagnosis [26]. We labeled these altered genes based on their role during the glycolytic process (**Figure 1G**).

We then further analyzed the correlation of these four genes with clinicopathological parameters. As shown in **Figure 2A-C**, GPI and ALDOC expression was significantly increased in patients with higher PSA levels, but only GPI expression was significantly associated with disease progression and disease-specific survival, although increased ENO2 expression was associated with progression-free interval (**Figure 2B**), lymph node invasion (**Figure 2D**)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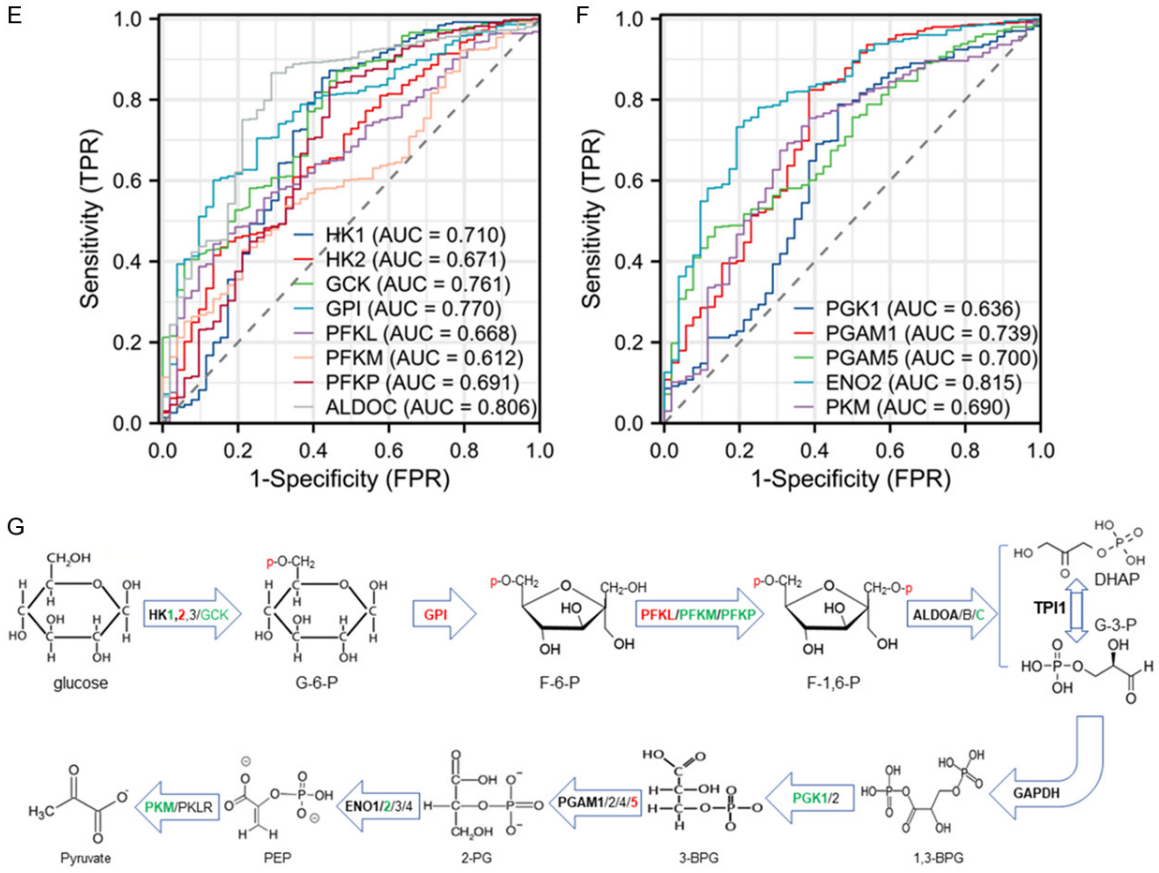


**Figure 1.** Multiple glycolytic enzyme genes were aberrantly expressed in primary prostate cancers. (A-D) Gene expression was compared among the 51 cases of case-matched benign and malignant prostate tissue pairs (A, C) and between 51 benign and 500 malignant prostate tissues (B, D). The *p*-values were derived from the Wilcoxon signed rank test (paired comparison) and the Wilcoxon rank sum test (group comparison). *P < 0.05; **P < 0.01; ***P < 0.001. (E, F) Receiver Operating Characteristic (ROC) curve analysis was conducted for the diagnostic performance of gene expression in prostate cancer tissues. AUC: Area under the ROC curve. (G) The predominant isozymes in prostate tissues (bold font), upregulated genes (red font), or downregulated genes (green font) were highlighted in the 10-step diagram of glycolysis.

and Gleason scores (**Figure 2E**). In contrast, reduced GCK expression was associated with lymph node invasion (**Figure 2D**). Their expression levels were not significantly associated with tumor stage, distal metastasis, and post-surgery residual tumors (Figure S1). These data strongly suggest that GPI expression has more weight in related disease progression and survival.

*GPI expression is highly related to patient survival outcomes*

We then analyzed patient survival outcomes related to GPI expression. Using the Kaplan-Meier curve approach, our results showed that higher levels of GPI expression were significantly associated with both progression-free interval (**Figure 3A**) and disease-specific survival (**Figure 3B**). We then calculated a 10-year prediction using the time-dependent ROC analysis approach. Among the four highly altered genes, GPI exerted the highest predicting value for the progression-free interval (ROC = 0.76, **Figure 3C**) and disease-specific survival (ROC = 0.979, **Figure 3D**). These data indicate that GPI expression is a potential novel prognostic factor for prostate cancer patients.

*GPI and seven other glycolytic enzyme genes were highly expressed in patients with disease relapse*

Disease relapse is a critical issue in managing cancer patients. We then analyzed the expression levels of these glycolytic enzyme genes in

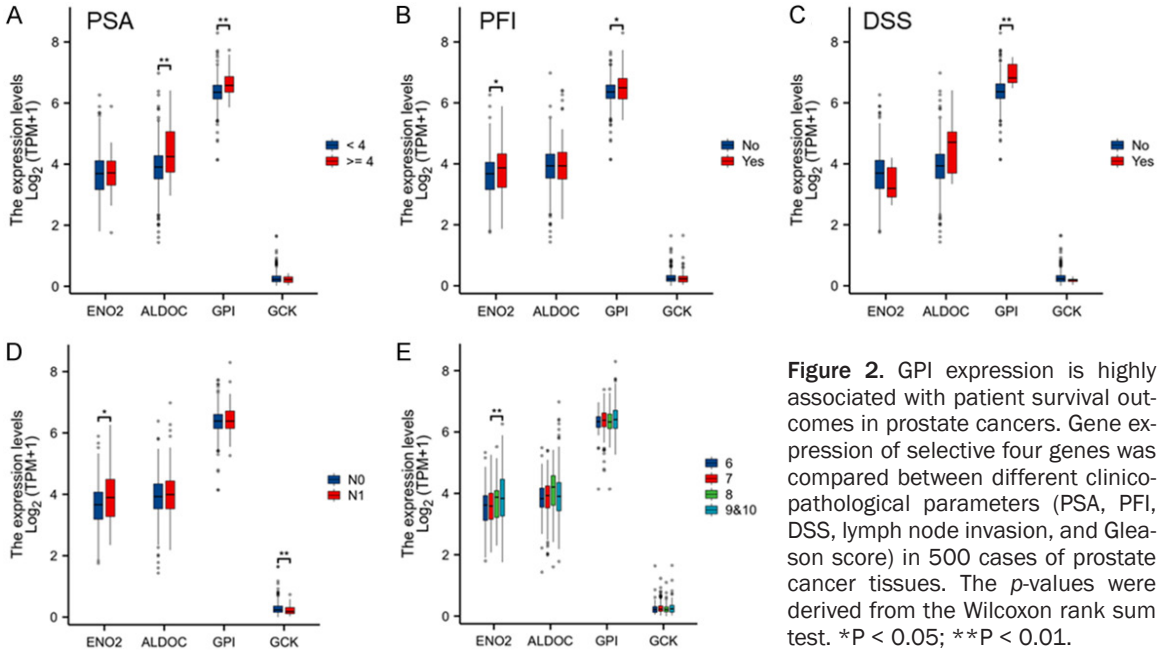


**Figure 2.** GPI expression is highly associated with patient survival outcomes in prostate cancers. Gene expression of selective four genes was compared between different clinicopathological parameters (PSA, PFI, DSS, lymph node invasion, and Gleason score) in 500 cases of prostate cancer tissues. The *p*-values were derived from the Wilcoxon rank sum test. *P < 0.05; **P < 0.01.

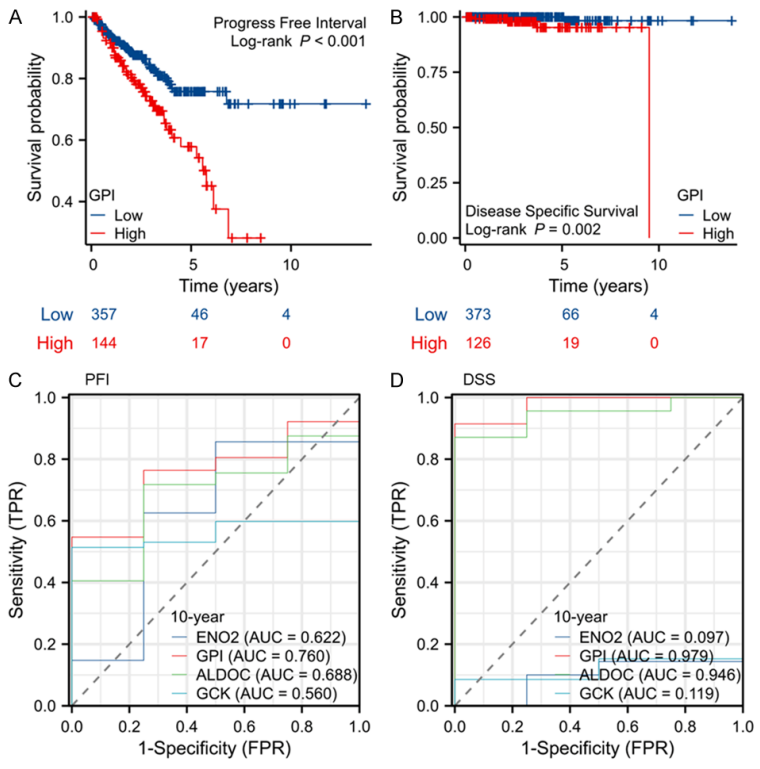


**Figure 3.** Higher GPI expression is associated with poor patient survival outcomes in prostate cancers. A, B. Patient survival outcomes were analyzed concerning GPI expression using the Kaplan-Meier curve coupled with the minimum *p*-value cut-off approach. C, D. The time-dependent ROC analysis of gene expression as a prognostic factor. AUC: Area under the ROC curve.

the primary tumor tissues with or without relapse after initial treatment. Our analysis found a significant upregulation of 8 genes in patients with disease relapses compared to those without relapses (**Figure 4A-C**), including GPI/HK3/PFKL/ALDOA/ALDOB/ENO2/ENO3/PKM. These altered genes were marked on the 10-step glycolytic scheme (**Figure 4D**).

*Aberrantly changed genes after biochemical relapse in prostate cancers*

Androgen deprivation therapy (ADT) is the first-line treatment for metastatic prostate cancers. However, biochemical relapse (BCR), in other words, serum PSA level increase, often occurs within two years. Especially for early-onset patients, BCR event is a poor prognosis indicator [19]. We analyzed the gene expression profiles using the DKFZ dataset on the cBioportal platform [19]. Our analysis revealed that 6 glycolytic genes were highly upregulated in patients with BCR event, including GPI/PFKL/ALDOA/TPI1/GAPDH/ENO1 (**Figure 5A-F**), of which GPI/PFKL/ALDOA were also upre-

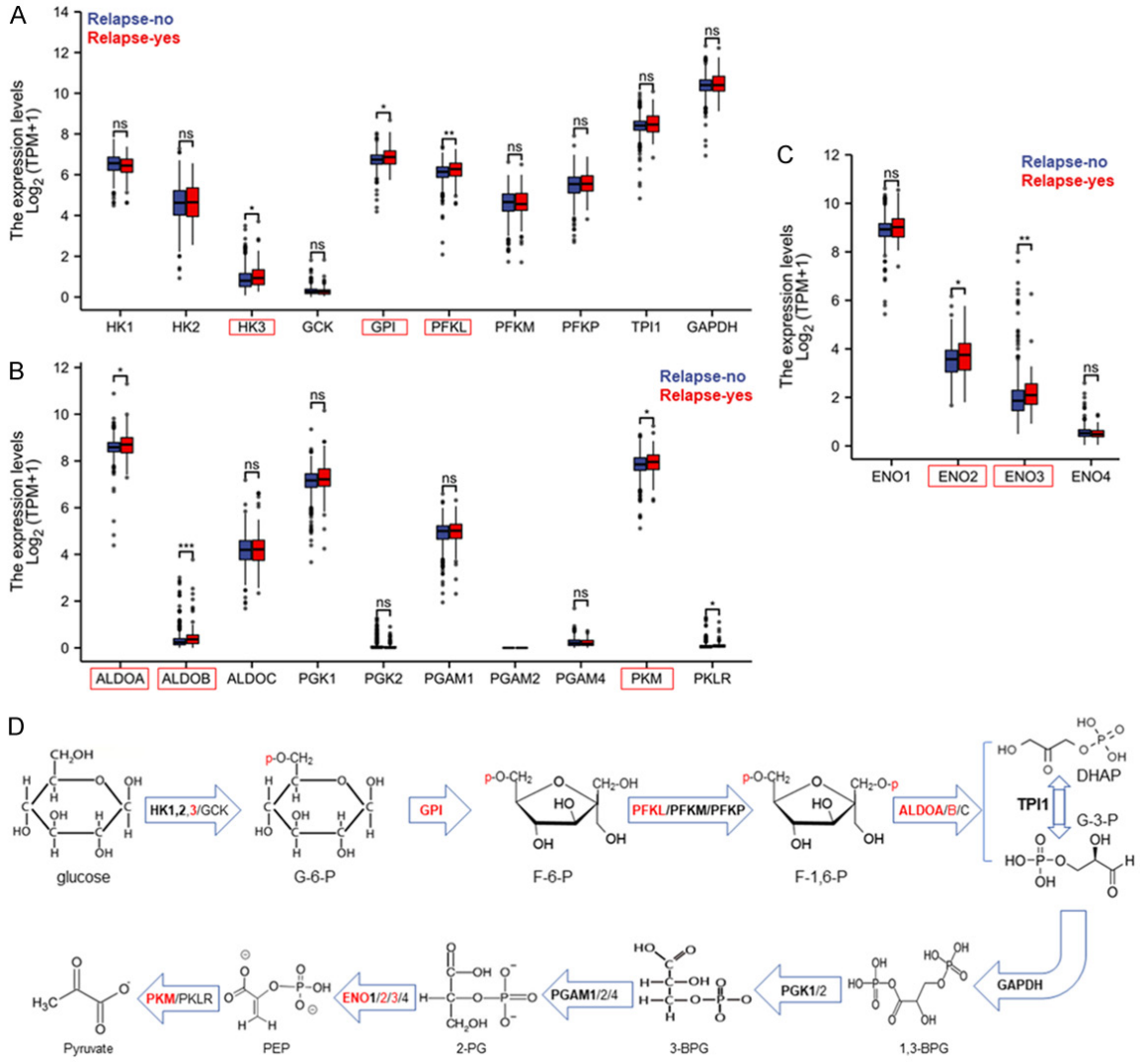


**Figure 4.** Multiple glycolytic genes are highly upregulated in patients with disease relapse. A-C. Gene expression profiles derived from the TCGA-PRAD dataset were compared in patients (n = 500) with or without disease relapse. The *p*-values were derived from the Wilcoxon rank sum test. *P < 0.05; **P < 0.01; ***P < 0.001; ns, no significance. The isozyme expression pattern was summarized in the 10-step diagram. D. The predominant isozymes are in bold font; upregulated genes are in red font.

gulated in patients with clinical relapsed diseases (**Figures 4D**, **5G**). This data indicates that GPI and PFKL upregulation might play an important role during prostate cancer progression.

*Multiple genes are strongly associated with castration-resistant and neuroendocrinal progression*

At the late stage, prostate cancer patients progressed into castration-resistant status (also known as CRPC), exhibiting treatment resistant to anti-AR treatment, about 20-30% of CRPC patients eventually progressed into neuroendocrinal status (also known as NEPC). Both CRPC and NEPC are lethal types of metastatic prostate cancer with no means to cure. We performed a correlation analysis and found that PFKL and PGAM5 were positively associated with AR score and AR-V7 expression (two critical CRPC factors) but negatively associated with NEPC score (**Table 1**). In contrast, HK3/GCK/PKLR genes were positively associated with NEPC score but negatively associated with CRPC factors (AR score and AR-V7 expression), similar to the well-known NEPC marker ENO2 (**Table 1**) [27]. Further analysis showed that

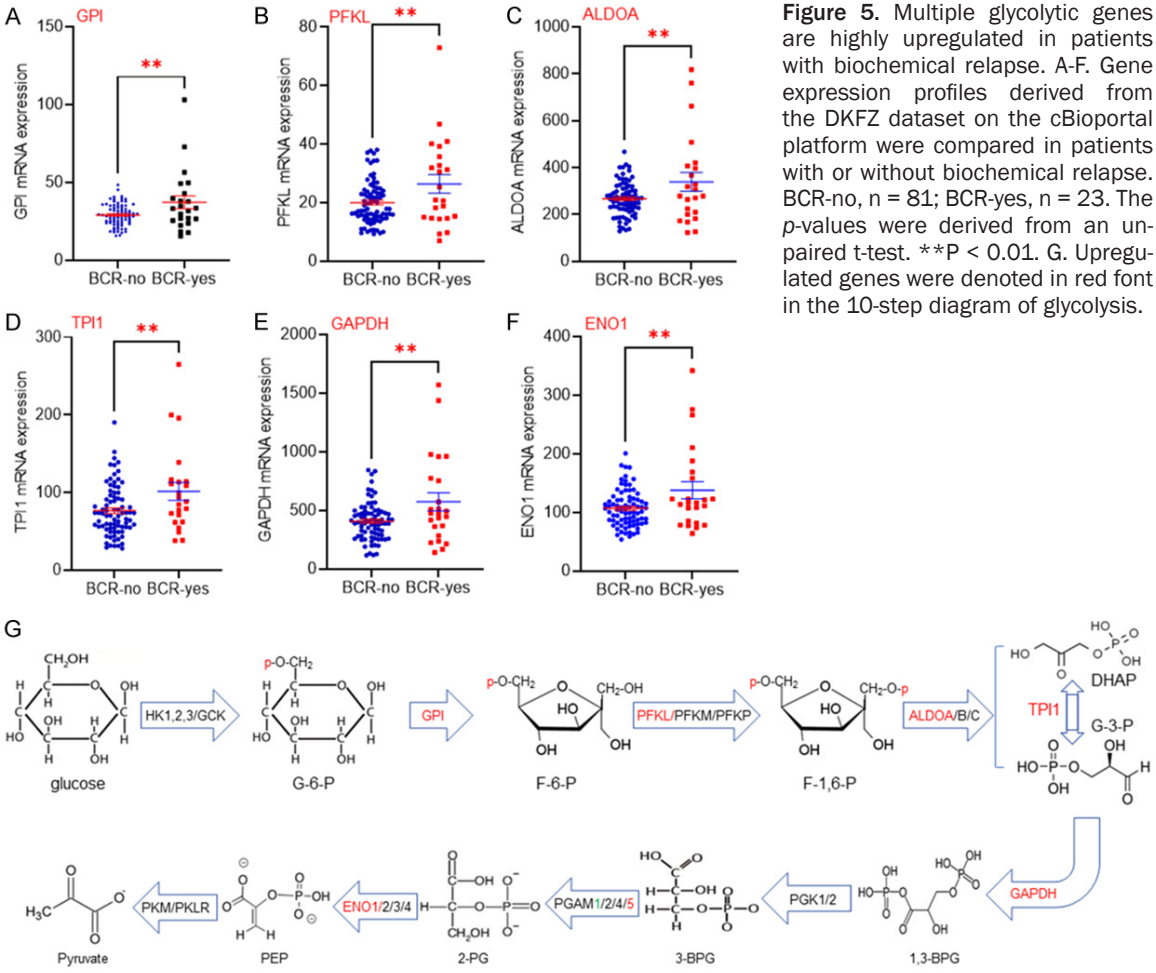


**Figure 5.** Multiple glycolytic genes are highly upregulated in patients with biochemical relapse. A-F. Gene expression profiles derived from the DKFZ dataset on the cBioportal platform were compared in patients with or without biochemical relapse. BCR-no, n = 81; BCR-yes, n = 23. The *p*-values were derived from an unpaired t-test. **P < 0.01. G. Upregulated genes were denoted in red font in the 10-step diagram of glycolysis.

PFKL and PGAM5 expression was suppressed in patients treated with AR inhibitors (**Figure 6A**, **6B**). Similarly, PFKL and PGAM5 expression was also significantly reduced in LuCaP35 xenograft tumors after animal castration (**Figure 6C**, **6D**), confirming an androgen-dependent mechanism in the regulation of PFKL/PGAM5 expression in prostate cancer. Meanwhile, ENO2/GCK/PKLR but not HK3 genes were highly increased in tumors with NE features compared to adenocarcinomas (**Figure 6E-H**), indicating that GCK/PKLR might be used as a novel NEPC biomarker (**Figure 6I**).

## Discussion

In this study, we conducted a systematic analysis of gene expression encoded for the glycolytic process in prostate cancer tissues. From the TCGA-PRAD RNAseq dataset derived from primary tumor tissues, our results showed that HK2/GPI/PFKL/PGAM5 gene expression was upregulated in prostate cancers, of which GPI upregulation in primary prostate cancer tissues was tightly correlated with progression-free progression and disease-specific survival, representing a novel prognostic factor. Like ENO2 as a well-known NEPC biomarker, GCK and PKLR were highly expressed in castration-resistant prostate cancers that bear neuroendocrine differentiation features, indicating their potential as novel NEPC biomarkers.

Glucose-6-phosphate isomerase (GPI) is a glycolytic enzyme that catalyzes the second step of glycolysis by interconverting glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) in the cytoplasm, and it also exhibits additional function as an extracellular cytokine autocrine motility factor (AMF) [28]. It has been shown that AMF/GPI activates small Rho-like GTPases and subsequently induces actin fiber rearrangement, leading to increased mobility [29]. Higher expression of AMF/GPI was associated

**Table 1.** Spearman Correlation Analysis

| Items | NEPC score | AR score | AR-v7 |
|---|---|---|---|
| HK1 | ns | 0.18* | 0.28** |
| HK2 | ns | 0.13* | ns |
| HK3 | 0.37**** | (-0.45)**** | (-0.24)** |
| GCK | 0.24**** | (-0.50)**** | (-0.25)** |
| GPI | ns | 0.17** | ns |
| PFKL | (-0.29)**** | 0.28**** | 0.28*** |
| PFKM | ns | 0.31**** | ns |
| PFKP | ns | ns | ns |
| ALDOA | (-0.22)*** | 0.25**** | ns |
| ALDOB | 0.25**** | (-0.20)*** | ns |
| ALDOC | ns | ns | ns |
| TPI1 | (-0.16)* | 0.15* | ns |
| GAPDH | (-0.13)* | 0.15* | ns |
| PGK1 | ns | ns | ns |
| PGK2 | ns | ns | ns |
| PGAM1 | ns | ns | ns |
| PGAM2 | ns | (-0.16)* | ns |
| PGAM4 | ns | ns | ns |
| PGAM5 | (-0.23)*** | 0.35**** | 0.28*** |
| ENO1 | ns | ns | ns |
| ENO2 | 0.45**** | (-0.33)**** | (-0.33)*** |
| ENO3 | 0.25*** | (-0.27)**** | ns |
| ENO4 | ns | 0.13* | 0.27** |
| PKM | ns | ns | ns |
| PKLR | 0.28**** | (-0.31)**** | ns |

*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

with tumor development and poor progression in multiple human cancers including gastric cancer [30], musculoskeletal tumors [28, 31], and clear cell RCC [32]. A recent study showed that GPI expression confers treatment resistance under androgen deprivation and hypoxia conditions in human prostate cancer [33]. Under the hypoxia condition, GPI shifts the AR-mediated pentose phosphate pathway (PPP) to the hypoxia-induced glycolysis pathway during Enzalutamide treatment. Therefore, GPI suppression sensitizes CRPC cells to Enzalutamide therapy in vitro and in vivo [33]. In our study, we discovered that GPI is upregulated in prostate cancer, which is associated with disease progression and patient survival. Our data is in line with the recent discovery that GPI promotes treatment resistance in CRPC [33]. However, it is not known if AMF/GPI promotes prostate cancer progression by acting as an extracellular cytokine in addition to metabolic reprogramming under hypoxia conditions.

Glucokinase (GCK) is the fourth hexokinase that converts glucose to glucose-6-phosphate (G6P), the first step in glucose metabolism pathways. In contrast to the other three hexokinases, GCK is not suppressed by its product G6P but remains active while glucose is abundant [34]. GCK is mainly expressed in the liver and pancreas [35], but at a very low level in prostate tissues, as shown in **Figure 1A**. Currently, there is a paucity of GCK expression or activity in prostate cancers [35]. In this study, we found that GCK expression was extremely low in primary prostate cancer tissues, but largely increased in neuroendocrinal differentiated CRPC tumors, the same as the NEPC biomarker ENO2. Although GCK expression was not responsive to anti-AR treatment in patients or castration in animals, its expression was negatively correlated with AR activity score and AR-V7 expression. These data suggest that GCK upregulation might be involved in the neuroendocrinal progression of prostate cancer independent of AR suppression.

Pyruvate kinase L/R (PKLR) is one of the pyruvate kinase isozymes, the critical kinases that catalyze the last step of glycolysis [36]. It is highly expressed in the liver and kidney tissues, as well as bone marrow, duodenum, and small intestine at a relatively low level [36]. In prostate tissues, we found that PKLR expression was extremely low in both malignant and benign prostate tissues. In our study, however, PKLR expression was not associated with clinicopathological parameters, which is not in line with a recent report [16]. We also found that PKLR expression was positively correlated with NEPC score but negatively correlated with AR activity score, the same as ENO2 and GCK. Our finds were in line with two recent reports [16, 17], which demonstrated the critical role of PKLR in promoting the neuroendocrinal progression of castration-resistant prostate cancer.

In conclusion, our studies discovered that GPI upregulation was associated with progression-free interval and disease-specific survival as a putative prognostic factor. Our results also showed that GCK/PKLR expression was highly upregulated in neuroendocrinal differentiation in CRPC tissues, similar to the well-known NEPC biomarker ENO2. These data suggest that GCK/PKLR might be used as novel NEPC biomarkers.

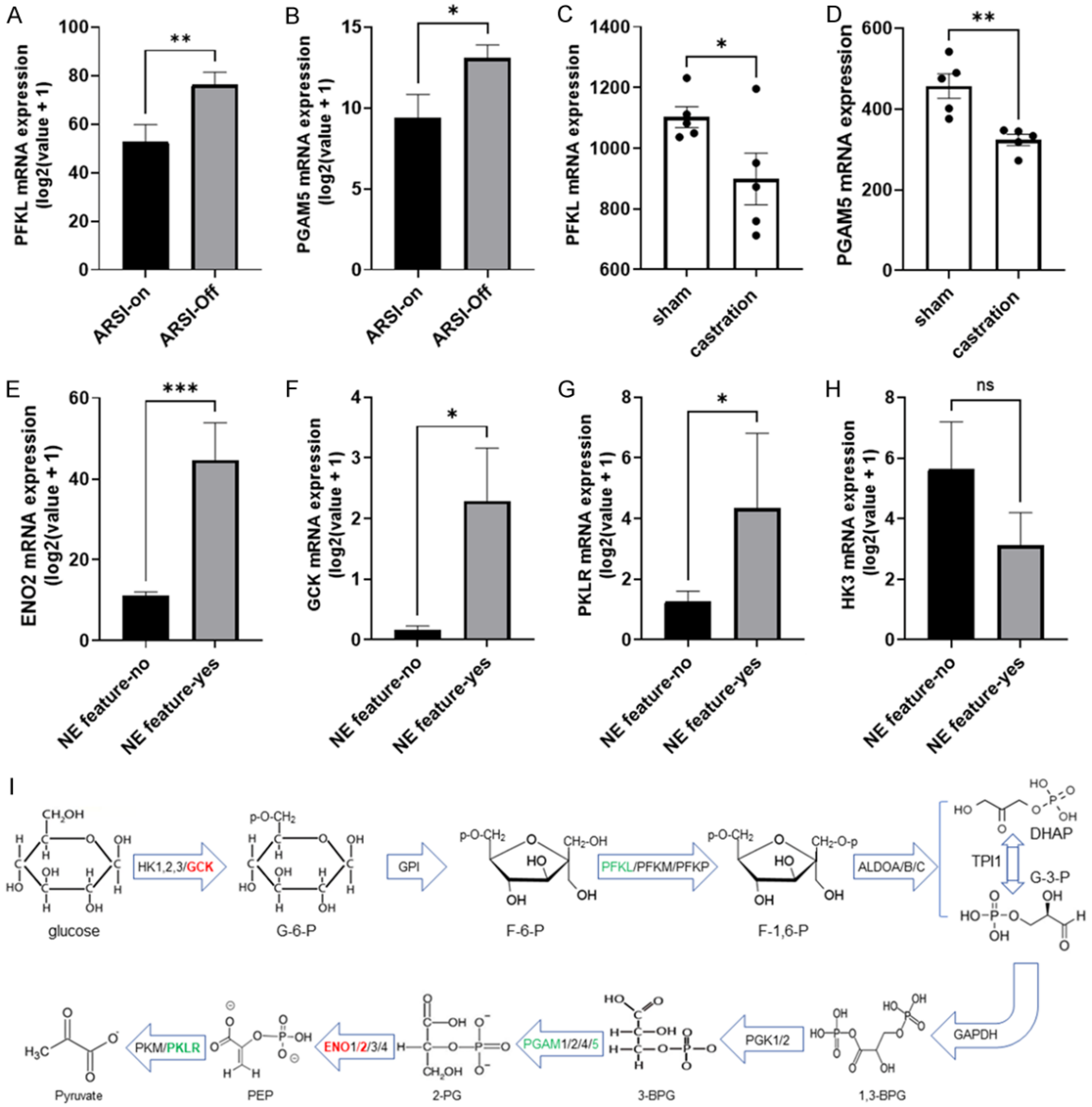


**Figure 6.** PFKL/PGAM5 is AR-dependent and GCK/PKLR are novel NEPC biomarkers. A, B. Gene expression profiles generated on prostate cancer tissues with or without ARSI treatment were downloaded from the SU2C/PCF RNAseq dataset on the cBioportal platform. ARSI-on, n = 9; ARSI-off, n = 47. The *p*-values were derived from Welch's test. *P < 0.05; **P < 0.01. C, D. Gene expression profiles generated on prostate cancer LuCap35 xenografts with or without castration were obtained from NCBI GEO dataset GDS4120. The *p*-values were derived from an unpaired t-test. n = 5. *P < 0.05; **P < 0.01. E-H. Gene expression data were obtained from the SU2C/PCF dataset. NE feature-no, n = 210; NE feature-yes, n = 22. The *p*-values were derived from Welch's test. *P < 0.05; ***P < 0.001; ns, no significance. I. Upregulated genes in CRPC and NEPC patients were denoted in red font in the 10-step diagram of glycolysis.

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## Disclosure of conflict of interest

None.

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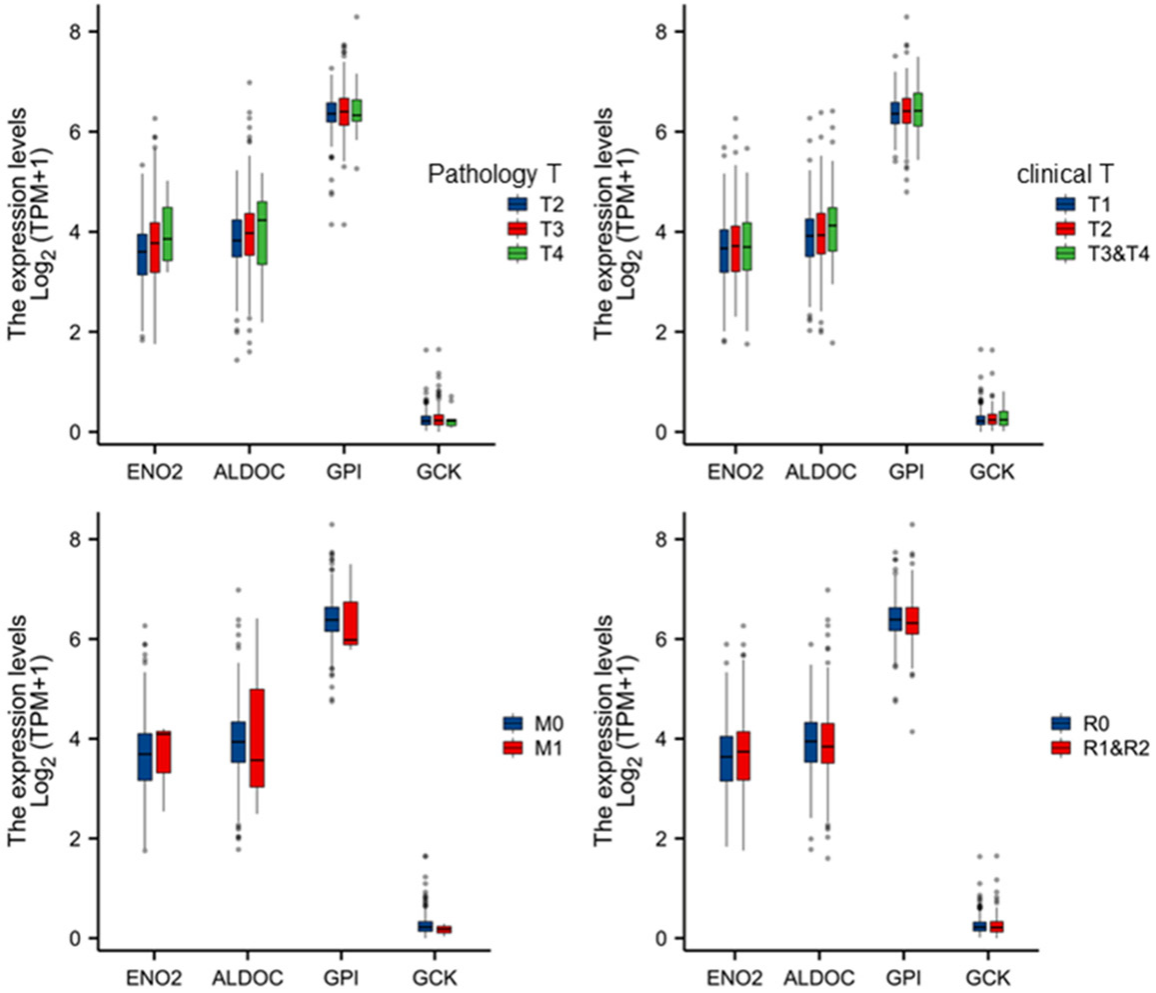


**Figure S1.** Gene expression in association with clinicopathological parameters. Gene expression profiles were downloaded from TCGA-PRAD dataset as described in the legend of **Figure 2**.