**Human Cancer Biology**

**Serum Glutamate Levels Correlate with Gleason Score and Glutamate Blockade Decreases Proliferation, Migration, and Invasion and Induces Apoptosis in Prostate Cancer Cells**

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**Abstract**

**Purpose:** During glutaminolysis, glutamine is catabolized to glutamate and incorporated into citric acid cycle and lipogenesis. Serum glutamate levels were measured in patients with primary prostate cancer or metastatic castrate-resistant prostate cancer (mCRPCa) to establish clinical relevance. The effect of glutamate deprivation or blockade by metabotropic glutamate receptor 1 (GRM1) antagonists was investigated on prostate cancer cells’ growth, migration, and invasion to establish biologic relevance.

**Experimental Design:** Serum glutamate levels were measured in normal men (n = 60) and patients with primary prostate cancer (n = 197) or mCRPCa (n = 109). GRM1 expression in prostatic tissues was examined using immunohistochemistry (IHC). Cell growth, migration, and invasion were determined using cell cytotoxicity and modified Boyden chamber assays, respectively. Apoptosis was detected using immunoblotting against cleaved caspases, PARP, and γ-H2AX.

**Results:** Univariate and multivariate analyses showed significantly higher serum glutamate levels in Gleason score ≥ 8 than in the Gleason score ≤ 7 and in African Americans than in the Caucasian Americans. African Americans with mCRPCa had significantly higher serum glutamate levels than those with primary prostate cancer or benign prostate. However, in Caucasian Americans, serum glutamate levels were similar in normal research subjects and patients with mCRPCa. IHC showed weak or no expression of GRM1 in luminal acinar epithelial cells of normal or hyperplastic glands but high expression in primary or metastatic prostate cancer tissues. Glutamate deprivation or blockade decreased prostate cancer cells’ proliferation, migration, and invasion and led to apoptotic cell death.

**Conclusions:** Glutamate expression is mechanistically associated with and may provide a biomarker of prostate cancer aggressiveness. *Clin Cancer Res*; 18(21); 5888–901. © 2012 AACR.

**Introduction**

Prostate cancer is estimated to account for 29% of all new cancers and is the second leading cause of cancer-related death in men in the United States in 2012 (1). Age, African-American race, and family history are unequivocal risk factors for prostate cancer (2). Focusing on early detection of prostate cancer has the capacity to decrease prostate cancer mortality. Serum prostate-specific antigen (PSA) is used for prostate cancer early detection, but its use is controversial because of its sensitivity and specificity is limited and it does not provide reliable prognostic information. These limitations have led investigators to consider new approaches, using systems biology, to identify biomarkers of prostate cancer aggressiveness. This goal could be reached by defining important interacting cellular networks and/or metabolic pathways that reflect tissue- and disease-specific phenotypes.

Metabolomics has emerged recently as a promising method for prostate cancer detection (3), one that may supplement, or even replace current PSA testing. Metabolomics profiling of prostate cancer offers the potential for early detection and discrimination of clinically aggressive tumors from indolent and nonaggressive diseases. Metabolic changes are affected by genetic and epigenetic changes and...
Translational Relevance

Aerobic glycolysis is a hallmark of proliferating normal and malignant cells. During glutaminolysis, glutamine is catabolized to glutamate and incorporated into the citric acid cycle and lipogenesis. Glutamate plays a substantial and overlapping role between metabolic and oncogenic signaling pathways. In this study, we found that serum glutamate levels were increased in patients with primary prostate cancer and directly correlated with Gleason score and prostate cancer aggressiveness. Compared with Caucasian Americans, serum glutamate levels were higher in African Americans with metastatic castrate-resistant prostate cancer (mCRPCa) than those with primary tumors. Immunohistochemical staining showed weak or no expression of metabotropic glutamate receptor 1 (GRM1) in normal prostate cells but overexpression in primary and metastatic prostate cancer. Glutamate deprivation or blockade with GRM1 antagonists reduced prostate cancer cells' growth, migration, and invasion and proved apoptogenic. Glutamate may provide a metabolic biomarker of prostate cancer aggressiveness, and glutamine metabolism or signaling may provide a target for therapeutic intervention.

A long list of environmental factors, which include diurnal variation, dietary habits, and lifestyle. The prostate is a unique target for metabolomics analysis both under normal conditions and upon neoplastic transformation. Extremely high amounts of citrate are produced and secreted by the prostate that reflects the unique state of anabolic and catabolic processes of secretory luminal cells. Citrate metabolism differs in prostate cancer compared with benign prostatic hyperplasia (BPH; ref. 4). Glutamate in secreted form is a phylogenetically conserved cell signaling molecule in addition to its intracellular activities in bioenergetics, biosynthetic pathways, maintaining amino acids and nucleotide pool, and metabolism (12). The glutamatergic system comprises the glutamate, glutamate receptors (GluR), and glutamate transporters. The GluRs are divided into 2 different categories, the ionotropic glutamate receptors (iGluR) and the metabotropic glutamate receptors (mGluRs; ref. 13). The iGluRs are gated ion channels with excitatory action (13). The mGluRs promote cellular signaling pathways via 7 transmembrane domain G-protein–coupled receptors (GPCR) and are subdivided into 3 groups and 8 subtypes: group I (mGluR1 and mGluR5), II (mGluR2 and mGluR3), and III (mGluR4, mGluR6, mGluR7, and mGluR8) based on their pharmacology, sequence homology, response to agonists, and downstream signaling. mGluRs are expressed in a variety of nonneuronal cell types, such as hepatocytes, melanocytes, keratinocytes, myocardial cells, pancreatic cells, and embryonic stem cells (14, 15). Recently, several studies have indicated the involvement of mGluRs in tumorigenesis. mGluRs are highly overexpressed in the nervous system, so initial discoveries of mGluRs in human tumors were reported in neuro-glial–derived tumors, such as gliomas, neuroblastoma, and medulloblastoma (14, 15). Glioma cells secreting high amounts of glutamate have higher growth rates than their isogenic parental cells (16). Glutamate antagonists decreased proliferation of a variety of cancer cells, which include colon adenocarcinoma, melanoma, lung carcinoma, thyroid carcinoma, breast carcinoma, astrocytoma, neuroblastoma, and rhabdomyosarcoma (17). The first proof for the involvement of mGluRs in nonneuronal tumorigenesis was obtained by Chen and colleagues (18, 19). A transgenic mouse line overexpressing mGluR1 (also known as GRM1) in melanocytes was predisposed to spontaneous melanoma development with 100% penetrance, short latency, and high metastatic potential (18, 19). In prostate cancer, mGluR1–5 mRNA expression was reported in PC-3 and LNCaP cells and mGluR6 and 8 were expressed only in LNCaP cells. Dihydrotestosterone (DHT) induced mGluR7 expression and inhibited mGluR8 expression in LNCaP cells (20). The clinical significance or biologic relevance of glutamate in prostate cancer has not been investigated.

In this study, we examined the association between serum glutamate levels and prostate cancer aggressiveness to establish clinical relevance. To determine the biologic relevance, the effect of glutamate deprivation or blockade was
investigated on prostate cancer cells’ growth, migration, and invasion.

Materials and Methods

Patients and samples

Primary prostate cancer cases investigated in this study refer to newly diagnosed, untreated, and clinically localized prostate cancer. Normal research subjects in this study are defined as men with no evidence of prostate cancer. Serum samples used to evaluate glutamate levels in normal individuals and men with primary tumor or metastatic castrate-resistant prostate cancer (mCRPCa), were obtained from the biospecimen core facilities at the Louisiana Cancer Research Consortium (LCRC) affiliated to Tulane Medical School and School of Medicine, Louisiana State University Health Sciences Center (LSUHSC, New Orleans, LA) and the tumor bank at the University of Washington Medical Center (UWMC, Washington) after informed consent and without any personal identifiers. Briefly, peripheral blood samples were collected by phlebotomy from consenting normal adult males or patients with primary or mCRPCa in the Urology Oncology clinics at the UWMC and LCRC-affiliated hospitals. All of the controls came from patients who presented at the University of Washington’s annual free PSA screening week. All patients were more than 40 years of age, and all stated that they did not have a history of prostate cancer. PSA values were below 4.0 ng/mL. Blood samples from UWMC were processed by centrifugation, and serum samples were stored in aliquots at −80°C within ≤4 hours of draw. Overall, all normal serum samples, 39% of primary prostate cancer, and 69% of mCRPCa samples were obtained from the UWMC. The remainder of serum samples was processed similarly and obtained from the biospecimen core facilities at LCRC. Serum samples were stored until used in the research study; no freeze-thaw cycles were incurred. We did not acquire fasting or postmeal status on any of the research subjects. Prostate cancer aggressiveness was defined using Gleason score, clinical stage, and PSA at diagnosis as: (i) high aggressive (Gleason sum ≥ 8, or PSA > 20 ng/mL, or Gleason sum = 7, and stage T3c–T4); (ii) low aggressive (Gleason sum < 7, stage T1–T2, and PSA < 10 ng/mL); or (3) intermediate aggressive (all other cases; ref. 21). The prostate cancer tissue specimens used for immunohistochemistry (IHC) were obtained from Accumax Array (Accurate Chemical & Scientific Corp.).

Ethical considerations

Ethical approval was obtained from the Institutional Review Boards at University of Washington Medical Center (Seattle, Washington), Tulane Cancer Center, Tulane University School of Medicine (New Orleans, LA), LSUHSC, School of Medicine (New Orleans, LA), and Roswell Park Cancer Institute (Buffalo, NY).

Determination of serum glutamate and creatinine levels

Serum glutamate levels were measured using BioVision’s glutamate assay kit (#K629-100) that provides sensitive detection method for glutamate in a variety of biologic samples and fluids including serum, urine, cells, and tissue extracts. The amount of glutamate is quantitated by measuring optical density at \( \lambda = 450 \text{ nm} \) in a microplate reader. Background reading was corrected by subtracting the value from the 0 glutamate control from all samples readings. Serum creatinine concentration was measured using the BioVision creatinine assay kit (#K625-100) and the manufacturer’s instructions. After plotting the independent glutamate and creatinine standard curves, glutamate and creatinine concentrations of the serum samples were calculated using the formula \( C = S_a/S_b \text{ nmol/μL}, \) or mmol/L, in which \( S_a \) is the sample amount of unknown (in nmol) from standard curve and \( S_b \) is the sample volume (μL) added to each well.

The minimum detectable amounts for serum glutamate or creatinine were 2 nmol/μL. The standard curve reproducibility was shown in 5 independent assays. The intra-assay variance was evaluated in 10 replicates for 3 serum samples of known concentrations on 1 plate. The interassay precision was tested in 3 independent assays for 3 control samples in triplicates. Stability of serum glutamate was determined by incubating aliquots of serum samples of known glutamate concentrations at −20°C or −70°C for 3, 6, 12, 18, 24, and 36 months.

Immunohistochemical analysis

The ABC detection system (Vector Laboratories Inc.) was used for immunostaining as described previously (22). Affinity purified, polyclonal rabbit antihuman-GRM1 antibody (at 1:250, Sigma) was used as the primary antibody. The incubation time for primary antibody was overnight at 4°C. The slides were counterstained with hematoxylin and mounted for examination. The negative control slides were treated in an identical manner except that the primary antibodies were omitted. Descriptive examinations of cell type–specific anti-GRM1 immunoreactivity in various tissue constituents, normal, hyperplastic, and neoplastic glands in the sections were completed by the study pathologists.

Cell Culture, reagents, and Western blot analysis

Androgen-independent (PC-3, DU145, MDA-PCa2b, VCaP, and 22Rv1) and androgen-sensitive (LNCaP) prostate cancer cell lines were purchased from American Type Culture Collection and maintained in their individually specified medium (23). E006AA cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (23). Noncompetitive GRM1 antagonists (riluzole and BAY36-7620) were purchased from Tocris (24). Customized glutamine- and glutamate-free RPMI-1640 or DMEM, dialyzed FBS, and GlutaMAX-I (a dipeptide, l-alanine-l-glutamine) were purchased from Life Technologies. GlutaMAX was used as a substitute for glutamine in cell growth, which cannot be degraded or converted into glutamate. Cells were cultured in complete media up to 75% confluency, washed with cold PBS, and whole-cell lysates were extracted as described previously (23). GRM1 was detected using a rabbit polyclonal antibody against human GRM1 (Cat # 19955-1-AP at 1:1,000; Proteintech Group, Inc.)

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with 400 µL basal medium containing 10% FBS as chemoattractant. Riluzole or BAY36-7620 was included at equimolar concentrations (0–50 µmol/L) to upper and lower compartments. After 48 hours for PC-3 or 24 hours for DU145, the nonmigratory cells on the upper surface were removed using a cotton swab, and the cells on the lower surface were fixed and stained with Diff-Quick solution (Dade Behring). Migrated or invaded cells in transwell filters were counted from 10 randomly selected fields in at least 4 independent wells. The experiment was repeated 3 times independently.

Statistical design and data analysis
All analyses were conducted using SAS 9.3 (SAS Institute) with a nominal significance level of 0.05. The serum samples were stratified by clinical status of the research subjects (normal, primary prostate cancer, or mCRPCa) with patient demographics (e.g., age, race, etc.) reported as mean and SD for continuous variables, and as frequencies and relative frequencies for categorical variable (Supplementary Tables S1 and S2). Patients with primary prostate cancer were divided into 3 subgroups of aggressiveness (low aggressiveness, intermediate aggressiveness, and high aggressiveness) using clinical criteria as described in “Materials and Methods.” The demographic variables were compared between normal research subjects and men with primary prostate cancer or mCRPCa using one-way ANOVA and an extension of Fisher exact test for continuous and categorical variables, respectively. Age, PSA, and Gleason scores were categorized as: 40–49, 50–59, 60–69, 70–79; ≤4, 4.1–10, ≥10.1; and ≤6, 7, ≥8, respectively.

Serum glutamate levels were compared for variables of interest and among the research subjects using one-way ANOVA, with Tukey-adjusted post hoc pairwise comparisons. All serum samples measurements were log-transformed before statistical analysis. Simple linear regression models were used to assess the association between research subject’s serum glutamate and clinical status (normal, primary prostate cancer with low-, intermediate-, or high-aggressiveness score, mCRPCa) while adjusting for demographic covariates. Tukey-adjusted pairwise comparisons were made between clinical status of research subjects and their demographic strata when appropriate. The covariate-adjusted models considered a single demographic covariate (with and without interactions) as well as multiple demographic covariates, as this information is not applicable or available for normal research subjects and patients with mCRPCa. Only research subjects with primary prostate cancer were considered when Gleason score and stage were included as covariates. All model assumptions were verified graphically using quantile–quantile (QQ) and residual plots.

Results

Serum glutamate levels in normal and prostate cancer research subjects
The research subjects’ race, Gleason scores, PSA values, age, and clinical aggressiveness were described in Supplementary Table S1. Serum creatinine values were within the
expected range and did not differ significantly among research subject subgroups. Serum PSA levels were found to be significantly ($P < 0.001$) different among normal individuals ($0.556 \pm 0.03$), patients with primary prostate cancer ($10.38 \pm 2.19$), and patients with mCRPCa ($32.73 \pm 51.43$). The average age was found to be significantly higher ($P < 0.001$) in research subjects with mCRPCa ($70.2 \pm 1.0$) than in those with primary prostate cancer ($62.0 \pm 0.6$) or normal research subjects ($61.4 \pm 1.2$). Serum glutamate levels were not significantly different among different age groups of normal research subjects (Supplementary Table S2, $P = 0.1735$).

The entire study cohort was analyzed regardless of age and race. When the normal research subjects ($n = 60$) were used for comparison, serum glutamate levels significantly increased in men with primary prostate cancer ($n = 197$; $P = 0.003$) but returned to normal levels in men with mCRPCa ($n = 109$; $P = 0.002$; Fig. 1A, left). When the patients with primary prostate cancer were divided into low, intermediate, and high aggressiveness, glutamate levels were significantly different as: (i) between normal research subjects and intermediate ($P < 0.001$) or high aggressiveness ($P < 0.001$); (ii) between low aggressiveness group and intermediate ($P < 0.002$) or high aggressiveness ($P < 0.001$) groups; (iii) between intermediate aggressiveness group and mCRPCa ($P < 0.001$), and (iv) between high aggressiveness and mCRPCa ($P < 0.001$; Fig. 1A, middle). Pairwise comparisons indicated a significant association between glutamate levels and Gleason score ($P < 0.001$). Glutamate levels differed significantly between Gleason score 6 ($n = 30$; $P < 0.001$) and Gleason score 7 ($n = 72$; $P < 0.001$) or Gleason score $\geq 8$ ($n = 30$; $P < 0.001$). Glutamate levels were higher in Gleason $\geq 8$ than the Gleason 7 groups, but the difference did not reach statistical significance ($P < 0.064$; Fig. 1A, right). Univariate analyses, after adjusting for PSA or stage, revealed no significant association between serum glutamate levels and any of the 3 aggressiveness categories in the pool of research subjects with primary prostate cancer (Table 1).

The association between prostate cancer aggressiveness and serum glutamate were explored among primary prostate cancer research subjects while adjusting for potential confounders. Age, PSA, and stage did not seem to be confounders in the single and 2 covariate (confounder) models; they were nonsignificant and did not change the direction or significance of the relationship between serum glutamate levels and prostate cancer aggressiveness. However, the inclusion of Gleason score seemed to overpower the relationship between serum glutamate levels and prostate cancer aggressiveness. The Gleason score seemed to drive the difference between serum levels within the primary prostate cancer research subjects (Tables 1 and 2).

Serum glutamate levels in Caucasian Americans versus African Americans

The adjusted analysis involving race indicated that patient clinical status (primary or mCRPCa; $P < 0.001$), race ($P = 0.029$), and interaction ($P = 0.029$) effects were significant (Table 1). The pairwise comparisons of race within each clinical subgroup of research subjects indicated that a significant difference exists in patients with mCRPCa ($P < 0.037$). Supplementary Fig. S1 shows that this difference is the result of the significant interaction term, where serum glutamate decreases for Caucasians from the high aggressiveness to mCRPCa but increase for African Americans.

Among Caucasian Americans, pairwise comparisons indicated that significant differences in serum glutamate existed between research subjects with primary prostate cancer and mCRPCa ($P < 0.030$; Fig. 1B, left), between normal research subjects and those with prostate cancer of high aggressiveness ($P < 0.004$), between low and high aggressiveness subgroups ($P = 0.001$), and between mCRPCa and high aggressiveness subgroups ($P < 0.001$; Fig. 1B, middle). Among Caucasian American research subjects with primary prostate cancer, a significant difference was found between research subjects with Gleason score $\leq 6$ and Gleason score 7 ($P < 0.002$) and between Gleason score $\leq 6$ and Gleason score $\geq 8$ ($P < 0.001$; Fig. 1B, right).

Among African Americans, pairwise comparisons indicated that serum glutamate levels differed between normal research subjects and primary prostate cancer ($P < 0.026$) and mCRPCa ($P < 0.016$; Fig. 1C, left). A significant difference in serum glutamate was found in African Americans between the normal research subjects and intermediate aggressiveness subgroup ($P < 0.014$) and between normal and both high aggressiveness subgroup ($P < 0.027$) and patients with mCRPCa ($P < 0.038$; Fig. 1C, middle). Among African-American research subjects with primary prostate cancer, significant differences in serum glutamate levels were found between research subjects with Gleason score $\leq 6$ or Gleason score $\geq 8$ ($P < 0.02$; Fig. 1C, right). The serum glutamate levels differed significantly between the races only in the research subjects with mCRPCa ($P = 0.037$; Supplementary Fig. S1).

Immunohistochemical staining of metabotropic glutamate receptor 1 in benign and malignant prostate tissues

Specificity of anti-GRM1 immunoreactivity was shown by deleting the primary antibody or replacing it with a non-immune serum (data not shown). Basal cells exhibited nuclear staining, whereas no staining was found in luminal acinar epithelial cells of BPH or normal prostate ($n = 10$; Fig. 2A). Endothelial cells, infiltrating macrophages, and stromal cells exhibited intense nuclear staining (Fig. 2A, mesenchymal cells). Intense cytoplasmic and nuclear staining was observed in proliferating epithelial cells of glands with basal cell hyperplasia (Fig. S2). Moderate to intense cytoplasmic staining was noted in high-grade prostate intraepithelial neoplasia (HGPIN; Fig. 2A). To further determine the GRM1 expression during prostate cancer progression and metastasis, we carried out additional immunohistochemical staining in primary ($n = 15$) and metastatic prostate cancer tissues ($n = 6$). Moderate to intense cytoplasmic staining with perinuclear enhancement was noted in prostate cancer with Gleason scores 6 and 7.

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Figure 1. Box and whiskers plot of serum glutamate levels in normal individuals and patients with primary or metastatic castrate-resistant prostate cancer (PCa) of Caucasian- or African-American origin. Serum glutamate levels were measured as described in "Materials and Methods." The study cohorts were analyzed according to their clinical status as normal research subjects, primary PCa, or metastatic castrate-resistant PCa (left). All patients with primary PCa were divided according to their aggressiveness score based on Gleason and PSA (middle) or according to Gleason score (right). A, the entire study cohorts. B, the entire study cohorts of Caucasian Americans. C, the entire study cohorts of African Americans. Actual P values are indicated on individual graphs. The distribution of log-transformed serum measurements was compared among groups using one-way ANOVA with Tukey pairwise comparisons. The box frame defines the 25 to 75 percentiles, the ends of error-bar, such as whiskers, depict the minimum and maximum values, and the line within the box marks the median value.
Enhancement was also observed in malignant acinar cells (Fig. 2A). Intense cytoplasmic staining with perinuclear enhancement was also observed in malignant acinar cells of Gleason score 8 (4+4), 9, and 10 (Fig. 2A) tumors. Prostate cancer cells invading into extraprostatic adipose and muscular tissues exhibited intense cytoplasmic staining (Fig 2A). Intense nuclear staining was observed at tumor-infiltrating inflammatory interfaces in inflammatory cells, endothelial cells, and isolated macrophages (Fig 2A). Intense cytoplasmic staining was found in multilayer prostate cancer cells infiltrating around nerves (Fig. 2A). Intense cytoplasmic staining with perinuclear enhancement was detected in prostate cancer cells metastatic to bone and the abdominal wall (Fig. 2A and Supplementary Fig. S3). GRM1 staining seemed to be distributed in both nuclear and cytoplasmic compartments. The nuclear staining of GRM1 was heterogeneous.

### Table 1. Single covariate adjusted analysis of serum glutamate levels in normal individuals, patients with primary or metastatic castrate-resistant prostate cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal Glu (μmol/L ± SE) (n = 60)</th>
<th>Primary prostate cancer (Aggressiveness subgroup) Glu (μmol/L ± SE)</th>
<th>MCRPCa Glu (μmol/L ± SE) (n = 109)</th>
<th>P valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gleason scoreb, c</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>≤6 (n = 94)</td>
<td>N/Aa</td>
<td>51.7 ± 1.8 (n = 80)</td>
<td>60.0 ± N/Aa (n = 1)</td>
<td>N/Aa Aggressiveness subgroupd: 0.222</td>
</tr>
<tr>
<td>7 (n = 73)</td>
<td>N/A (n = 0)</td>
<td>65.8 ± 2.8 (n = 13)</td>
<td>55.9 ± 4.8 (n = 18)</td>
<td>Gleason: 0.007</td>
</tr>
<tr>
<td>≥8 (n = 30)</td>
<td>N/A (n = 0)</td>
<td>75.7 ± 5.0 (n = 30)</td>
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<tr>
<td>PSAb (ng/mL)</td>
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<td></td>
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<tr>
<td>≤4 (n = 119)</td>
<td>49.3 ± 1.4 (n = 60)</td>
<td>68.0 ± 6.4 (n = 6)</td>
<td>60.9 ± 11.5 (n = 3)</td>
<td>Clinical statusf: &lt;0.001</td>
</tr>
<tr>
<td>4.1–10 (n = 100)</td>
<td>N/Aa</td>
<td>63.6 ± 3.7 (n = 16)</td>
<td>69.9 ± 14.5 (n = 6)</td>
<td>PSA: 0.252</td>
</tr>
<tr>
<td>≥10.1 (n = 139)</td>
<td>N/Aa</td>
<td>68.0 ± 4.5 (n = 92)</td>
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<tr>
<td>Stageb</td>
<td></td>
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<tr>
<td>T1 (n = 47)</td>
<td>N/Aa</td>
<td>69.4 ± 5.9 (n = 14)</td>
<td>73.9 ± 10.5 (n = 7)</td>
<td>N/Aa Aggressiveness subgroup: &lt;0.001</td>
</tr>
<tr>
<td>T2 (n = 113)</td>
<td>49.5 ± 1.9 (n = 54)</td>
<td>64.9 ± 7.1 (n = 15)</td>
<td></td>
<td>Stage: 0.179</td>
</tr>
<tr>
<td>T3 (n = 37)</td>
<td>N/A (n = 0)</td>
<td>69.4 ± 5.1 (n = 27)</td>
<td></td>
<td>Int: 0.591</td>
</tr>
<tr>
<td>Race</td>
<td></td>
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</tr>
<tr>
<td>Whites (n = 222)</td>
<td>50.3 ± 1.7 (n = 10)</td>
<td>70.7 ± 6.5 (n = 19)</td>
<td>50.6 ± 1.7 (n = 99)</td>
<td>Clinical statusf: &lt;0.001 Race: 0.029</td>
</tr>
<tr>
<td>Blacks (n = 144)</td>
<td>46.8 ± 2.3 (n = 43)</td>
<td>67.4 ± 4.7 (n = 30)</td>
<td>72.6 ± 9.4 (n = 10)</td>
<td>Int: 0.029</td>
</tr>
</tbody>
</table>

Abbreviations: Glu, glutamate; Int, interaction term; Med, medium; N/A, not available or not applicable.

a In each scenario a linear regression model was used to evaluate the association between patient clinical status and log-transformed serum glutamate while adjusting for different patient characteristics and their interaction. All model assumptions were checked graphically using quartile–quartile and residual-plots. Data are presented as mean ± SE.

b Only patients with primary prostate cancer were included in the analysis. PSA data was not available for all research subjects in the mCRPCa subgroup.

cInteraction term not considered given the frequency of sparse cell counts.

dCannot compute the SE based on a single observation. PSA value for this research subject was 28.0 ng/mL.

eThe variable of interest was not applicable or analyzed for the specified research subjects.

fNo research subject was identified in the low-aggressiveness subgroup for the variable of interest.

gComparison made among aggressiveness subgroups of patients with primary prostate cancer.

hClinical status refers to comparison made among all normal research subjects, aggressiveness subgroups of patients with primary prostate cancer, and patients with mCRPCa.
Table 2. Multiple covariate adjusted analysis of serum glutamate levels in patients with primary and metastatic castrate-resistant prostate cancer

<table>
<thead>
<tr>
<th>Adjusted variable</th>
<th>Variable of interest</th>
<th>Primary prostate cancer (Aggressiveness Subtype) Glu (µmol/L ± SE)</th>
<th>MCRPCa Glu (µmol/L ± SE)</th>
<th>P valuesa</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Low               Intermediate                     High</td>
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<tr>
<td>Race</td>
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</tr>
<tr>
<td>Whites</td>
<td>≤6</td>
<td>48.2 ± 2.4        47.9 ± 1.8       71.5 ± N/Ab</td>
<td>N/A</td>
<td>N/A</td>
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<td></td>
<td>≥7</td>
<td>71.5 ± N/Ab       63.4 ± 3.7       56.2 ± 5.2</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>Blacks</td>
<td>≤6</td>
<td>54.4 ± 2.5        63.3 ± 7.2       66.8 ± 3.7</td>
<td>N/A</td>
<td>79.1 ± 9.1</td>
</tr>
<tr>
<td></td>
<td>≥7</td>
<td>N/A               64.8 ± 3.7       57.7 ± 7.4</td>
<td>N/A</td>
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</tr>
<tr>
<td>PSA (ng/mL)</td>
<td>≤4.0</td>
<td>47.9 ± 2.4        60.6 ± 4.3       86.9 ± 12.8</td>
<td>69.1 ± 5.9</td>
<td>60.9 ± 11.5</td>
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<td>4.1–10</td>
<td>49.7 ± 4.2        62.3 ± 6.2       58.5 ± 7.8</td>
<td>N/A</td>
<td>114.5 ± N/Ab</td>
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<tr>
<td></td>
<td>≥10.1</td>
<td>54.4 ± 3.2        64.2 ± 4.6       64.8 ± 4.1</td>
<td>N/A</td>
<td>49.1 ± 1.7</td>
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<tr>
<td>Age (y)</td>
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<tr>
<td></td>
<td>40–49</td>
<td>58.3 ± 5.6        59.9 ± 0.8       71.9 ± 15.4</td>
<td>71.2 ± 5.4</td>
<td>70.1 ± 14.8</td>
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<td></td>
<td>50–59</td>
<td>51.0 ± 3.0        46.7 ± 7.5       68.0 ± N/Ab</td>
<td>N/A</td>
<td>114.5 ± N/Ab</td>
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<td></td>
<td>60–69</td>
<td>50.9 ± 2.8        49.9 ± 1.0       71.2 ± 5.4</td>
<td>N/A</td>
<td>79.5 ± 10.5</td>
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<td></td>
<td>70+</td>
<td>51.8 ± 5.1        56.1 ± 7.3       N/A</td>
<td>N/A</td>
<td>71.9 ± 9.7</td>
</tr>
<tr>
<td>PSA (ng/mL)</td>
<td>≤4.0</td>
<td>50.6 ± 2.4        61.1 ± 6.6       61.0 ± N/Ab</td>
<td>N/A</td>
<td>N/A</td>
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<td>4.1–10</td>
<td>71.5 ± N/Ab       68.0 ± 6.4       64.9 ± N/Ab</td>
<td>N/A</td>
<td>90.6 ± 12.4</td>
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<td></td>
<td>≥10.1</td>
<td>52.6 ± 2.5        48.5 ± 2.5       N/A</td>
<td>N/A</td>
<td>57.7 ± 9.0</td>
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| Abbreviations: Glu, glutamate; Int, interaction term; Med, medium; N/A, not available or not applicable.

In each scenario a linear regression model was used to evaluate the association between aggressiveness subgroups of research subjects with primary prostate cancer or clinical status of the research subject and log serum glutamate while adjusting for different combinations of patient characteristics. All model assumptions were checked graphically using quartile–quartile and residual-plots.

bCannot compute the SE based on a single observation.

cClinical status refers to comparison made among all normal research subjects, aggressiveness subgroups of patients with primary prostate cancer, and patients with mCRPCa.

dComparison made among aggressiveness subgroups of patients with primary prostate cancer.

GRM1 expression in prostate cancer cell lines

An affinity purified anti-GRM1 antibody detected a strong band at approximately 85 kDa (Fig. 2B) and 3 other weakly expressing bands at approximately 40, 60, and 170 kDa, which were close to the predicted bands based on GRM1 molecular weight described by the manufacturer.
Parallel immunoblotting with anti-GRM1 antibody preabsorbed by its immunogenic peptide confirmed specificity (Fig. 2B).

GRM1 expression in androgen-sensitive LNCaP cells was less than androgen-independent PC-3, DU145 and androgen-independent, and bone metastatic prostate cancer cell lines (i.e., MDA-PCa 2b and VCaP). GRM1 expression in the E006AA cell line obtained from an organ-confined Gleason 6 score prostate cancer from an African American was less than bone-metastatic MDA-PCa2b cells (Fig. 2B). GRM1 expression in 22Rv1 cells was higher than LNCaP cells.
Prostate cancer cells growth in glutamate-free culture condition

Proliferation of PC-3 cells was decreased by 42%, DU145 cells by 63%, and LNCaP cells by 68%, as compared with their isogenic control cell types after culturing 3, 5, or 7 days in customized glutamine- and glutamate-free culture media supplemented with 10% dialyzed FBS and 2 mM glutamine (Fig. 3A). The media were refreshed every 48 hours. Cell proliferation was measured by adding 20 μL MTS solution per well for 1 hour and measuring the absorbance at 490/630 nm. Bars, mean ± SEM, P < 0.001, Student t tests were used to compare cell growth between glutamate-free and complete medium at each day. One-way ANOVA test with Bonferroni corrections was used to compare values among different days for each cell line. Effect of GRM1-antagonist on PCa cells growth. Cells were seeded as described earlier and were incubated in their complete medium in the presence or absence of riluzole at 10, 25, or 50 μM for 2, 4, or 6 days with media refreshment every 48 hours, followed by cell proliferation assay as described earlier. Data represented the average of 3 independent experiments ± SEM. Statistical significance (P < 0.0001) between the control and treatment groups was evaluated by one-way ANOVA test with Bonferroni adjustment. C, induction of apoptosis by riluzole. Cells were cultured in their complete medium in the presence or absence of riluzole as described earlier. Whole-cell lysate were extracted and immunoblotting was conducted on 30 μg protein using antibodies against cleaved caspases-9, -7, and -3, PARP, and phospho-H2AX. Anti-GAPDH antibody was used for control loading. Experiment was repeated twice independently.

Inhibition of prostate cancer cell proliferation by GRM1 antagonists

To evaluate the potential of intracellular glutamate levels on prostate cancer cells growth, we used riluzole that functions in part as the glutamate release inhibitor (14, 15). Inhibition of glutamate release in a cell type-, dose-, and time-dependent manner decreased prostate cancer cells growth (Fig. 3B). LNCaP cells were the most sensitive among the prostate cancer cell lines investigated. LNCaP cells growth was reduced by 54% (day 2) and by 85% (day 6) compared with their control cell type at the same incubation period (Fig. 3B, right). Among all prostate cancer cells, DU145 cells were least sensitive to riluzole (Fig. 3B, middle). Proliferation of DU145 cells was decreased by 14% (day 2) and by 18% (day 6). However, PC-3 growth was decreased by 10% (day 2) and by 40% on day 6 (Fig. 3B, left).
To show GRM1 responsiveness as a functional glutamate receptor, independent studies were conducted using BAY36-7620 as a noncompetitive GRM1 antagonist at different concentrations and for a period of 2 to 6 days. As in the riluzole study, cells were maintained in their complete medium containing nondialyzed FBS and 2-glutamine (2 mmol/L). BAY36-7620 showed similar growth inhibitory effect on the 3 prostate cancer cell lines and LNCaP cells were found to be the most sensitive cell line (Supplementary Fig. S4).

**Glutamate blockade induces apoptotic cell death**

The expression of cleaved (active) forms of the initiator caspase-9 and its active downstream effectors (caspases-7 and -3) were determined in the cells grown in the presence of riluzole for 4 or 6 days. The expression of cleaved caspase-9, -7, and -3 increased in a cell type–specific and dose-dependent manner in all cells investigated (Fig. 3C). In general, riluzole induced a cell type–specific increase in the expression level of initiator and effector caspases.

The intensity of cleaved PARP expression increased in a dose-dependent manner in all prostate cancer cell lines. H2AX, a member of H2A histone family is phosphorylated on serine residue 139 (known as γ-H2AX) after the activation of the caspase cascade and as a result of apoptotic DNA fragmentation (26). Cell lysates showed a dose-dependent increase in phospho-H2AXSer139 levels in LNCaP cells (Fig. 3C). An increase in γ-H2AX levels was also detected at 50 µmol/L riluzole in PC-3 and DU1145 cells. Accumulation of intracellular glutamate levels secondary to riluzole treatment seemed to be apoptogenic.

**Glutamate blockade decreases prostate cancer cells migration and invasion**

Addition of riluzole or BAY36-7620 to both upper and lower compartments of transwell filters decreased cell motility and invasion in a dose-dependent manner in PC-3 and DU1145 cells (Fig. 4A and B). Riluzole reduced migration of PC-3 cells by 53% and DU1145 cells by 76% and invasion of PC-3 by 65% and DU1145 by 82% (Fig. 4A and B). BAY36-7620 decreased migration of PC-3 cells by 67%, DU1145 cells by 88% and invasion of PC-3 by 83% and DU1145 by 96% (Fig. 4A). Similar data was obtained using a wound assay (Supplementary Fig. S5A–S5C).

**Discussion**

Serum glutamate levels positively correlated with Gleason score and prostate cancer aggressiveness. Immunohistochemical staining showed weak or no expression of GRM1 in luminal acinar cells of normal or benign glands but high expression levels in primary and metastatic prostate cancer tissues. Glutamate deprivation or blockade with GRM1 antagonists decreased prostate cancer cells growth, migration, and invasion and led to apoptotic cell death. Glycolysis and glutaminolysis are major contributing metabolic pathways of tumorigenesis. Glutamine is the most abundant serum amino acid and a necessary metabolic precursor for other amino acids and nucleotides.

Glutamine deprivation decreased cell proliferation, DNA synthesis, and protein synthesis rates in a cell type–dependent manner in hepatocellular carcinoma and several breast and colon adenocarcinoma cells (27). Similarly, the importance of glutamate signaling in cancer has been revealed by antiproliferative activity of glutamate antagonists in different types of human tumor cells, such as melanoma, colon adenocarcinoma, breast carcinoma, astrocytoma, and lung carcinoma (17).

Humoral response profiling of serum samples by Taylor and colleagues revealed elevated levels of nitrogen metabolism during prostate cancer progression (10). Concomitant validation of increased levels of the constituents of nitrogen metabolism pathway was determined further by using high-throughput quantitative profiling of relative levels of metabolites in BPH (n = 16) and organ-confined prostate cancer (n = 12) tissues. This study revealed significantly increased levels of glutamate in the prostate cancer tissues (P = 0.0003) compared with BPH. Further examination of metabolomic changes during prostate cancer progression by the same group verified elevated levels of glutamate in a majority of prostate cancer tissues as compared with benign glands (11).
The excess amount of glutamate in prostate cancer tissues and patients' serum may be explained by: (i) increased rate of glutaminolysis or glutamine addiction in proliferating prostate cancer cells and (ii) carboxypeptidase function of prostate-specific membrane antigen (PSMA) overexpressed by prostate cancer cells. While "glutamine addiction" serves as a nonspecific metabolic hallmark of malignant and proliferating cells, it is likely that the known enzymatic activity of PSMA may have a key contributing role in glutamate production by prostate cancer cells. PSMA is a type II transmembrane glutamate carboxypeptidase, and glutamate is the major constant product of its enzymatic activity in prostate cancer cells (28, 29). PSMA is overexpressed in prostate cancer and high PSMA expression is associated with advanced tumor stage, poor prognosis, and high risk of biochemical recurrence (28, 30). PSMA-generated glutamate signaling may serve as a nonandrogenic regulator of prostate cancer growth (31). Therefore, PSMA may have critical functions in metabolomics profiling during prostate carcinogenesis and progression.

Analysis of the glutamate receptor, GRM1, in prostate cancer cell lines showed a cell type–dependent expression. GRM1 expression was higher in metastatic or androgen-independent prostate cancer cell lines (e.g., DU145, MDA-PCa2b, VCaP, and 22RV1) than in the primary (i.e., E006AA) or androgen-sensitive LNCaP cell lines. GRM1 overexpression might be an adaptive change hypersensitizing prostate cancer cells to extracellularly available glutamate or could confer oncogenic activity due to its postreceptor signaling activation. Aberrant expression of GRM1 was found sufficient for oncogenic transformation of melanocytes in transgenic mice (18, 19). Overexpression of several members of mGlurRs has been reported in other cancers (32–34). Activation of mGlurR-triggered signaling pathways may occur in response to free circulating glutamate, glutamate released by tumor cells (autocrine loop), or stromal cells in the tumor microenvironment (paracrine loop). The data presented here also support an intracrine regulatory role for glutamate in prostate cancer cell growth, migration, and invasion.

Intense nuclear staining was detected in basal cells of normal glands and in endothelial cells, infiltrating macrophages, and stromal cells at the tumor–inflammatory interface or tumor microenvironment. Moderate to intense cytoplasmic staining was detected in HGPIN, which is considered to be a precursor lesion to prostate cancer. While "glutamine addiction" serves as a nonspecific metabolic hallmark of malignant and proliferating cells, it is likely that the known enzymatic activity of PSMA may have a key contributing role in glutamate production by prostate cancer cells. PSMA is a type II transmembrane glutamate carboxypeptidase, and glutamate is the major constant product of its enzymatic activity in prostate cancer cells (28, 29). PSMA is overexpressed in prostate cancer and high PSMA expression is associated with advanced tumor stage, poor prognosis, and high risk of biochemical recurrence (28, 30). PSMA-generated glutamate signaling may serve as a nonandrogenic regulator of prostate cancer growth (31). Therefore, PSMA may have critical functions in metabolomics profiling during prostate carcinogenesis and progression.

First evidence for a link between glutamate receptor mGluR1 (GRM1) and tumorigenesis was provided by an accidental discovery in which spontaneous melanoma in a transgenic mouse line (TG-3) was discovered to be due to multiple tandem insertions of the transgene into intron 3 of GRM1 leading to aberrant expression of GRM1 in melanocyte and the development of melanoma at a high rate and young age (18, 19). This was verified in a subsequent transgenic mouse line, TG(GRM1)(Etv) (E), in which GRM1 expression was regulated by a melanocyte-specific promoter, dopachrome tautomerase (36). Different pharmacologic agents have been used as GRM1 (i.e., mGluR1) agonist or antagonists. Riluzole is an U.S. Food and Drug Administration (FDA)–approved GRM1-blocking agent and well-tolerated oral medicine for the treatment of amyotrophic lateral sclerosis that has been used to prevent glutamate release (37). LY367385 or BAY36-7620 is known as competitive or noncompetitive GRM1 antagonist, respectively (24, 38). Riluzole, BAY36-7620, or LY367385 prevent the growth promoting effect and activation of signaling pathways by glutamate in a variety of cancer cell types including melanoma and breast carcinoma (38, 39).

Prostate cancer cell proliferation was significantly reduced in a time-dependent manner in glutamate-depleted medium or in the presence of GRM1 antagonists (Fig. 3A and B and Supplementary Fig. S4). These data indicated that...
prostate cancer cells’ growth depended on GRM1-signaling. Riluzole inhibition of glutamate release and GRM1-signaling in prostate cancer cells cultured in complete growth medium not only decreased growth rate, but also led to apoptotic cell death, shown by increased expression levels of cleaved caspase-9, -7, and -3 and PARP. These results were confirmed by the demonstration that phospho-H2AXSer139 levels (known as γ-H2AX) appeared early during apoptosis. Initiation of DNA double-strand breaks following caspase and apoptotic endonuclease activation induces phosphorylation of H2AX histone at Ser139 (26).

The inhibitory effect of glutamate blockade on prostate cancer cell proliferation and migration may be at least partially mediated by the PI3K/Akt/mTOR pathway (6). The PI3K/Akt/mTOR function as the main regulator of aerobic glycolysis in malignant cells. Activation of the PI3K/Akt/mTOR pathway in cancer cells triggers many of the metabolic activities that support cellular biosynthesis, such as: (i) increased surface expression of nutrient transporters leading to increased uptake of glucose and amino acid, (ii) activated Akt increases glycolysis and lactate production and induces a “Warburg effect” in cancer or even proliferating nontransformed cells (40), (iii) activation of PI3K and Akt induce lipogenesis and stimulate lipogenic genes expression in a variety of cell types (41), and (iv) mTOR serves as a major regulator of protein translation.

A cell type–specific response to glutamate blockade suggests differential expression levels for GRM1 in different prostate cancer cells or their dependence on glutamate signaling. Excess intracellular glutamate and/or inhibition of GRM1 signaling by riluzole treatment may have deleterious effects on prostate cancer cells growth, migration, and invasion, which cannot be salvaged by an intact extracellularly activated GRM1 signaling. In addition, accessibility to a certain amount of glutamate is essential for prostate cancer cells growth that may signify “glutamine addiction.” However, more than this necessary amount may lead to “glutamate toxicity” and activation of various interactive apoptotic signaling pathway leading to prostate cancer cell-killing. Intracellular glutamate as a metabolic intermediate, metabolic byproduct, or metabolic regulator of interactive multiple signaling pathways may play a major role in malignancy. Glutamate via a combination of intracrine, autocrine, and paracrine pathways may contribute to prostate cancer growth and aggressiveness. Our data suggest that glutamate together with other predictive or prognostic factors may be a useful metabolic marker for early detection and clinical discrimination of aggressive tumors from nonaggressive tumors. Glutamate-initiated signaling pathways may provide novel therapeutic opportunities for prostate cancer. Additional work is needed to ascertain the clinical and therapeutic implications of glutamate dependence in prostate cancer.

Disclosure of Potential Conflict of Interest
The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or National Cancer Institute. No potential conflicts of interest were disclosed.

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References


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