# Expression of Stress Response Protein GRP78 is Associated with the Development of Castration-Resistant Prostate Cancer

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## Introduction

Resistance to castration therapies persists as the predominant challenge in the treatment of advanced prostate cancer. Androgen dependent prostate cancer is characterized by the ability of cancer cells to undergo apoptosis in response to hormone depletion. The transition to castration-resistant prostate cancer (CR) requires the survival of tumor cells in such conditions, which may be attributed to a number of molecular mechanisms resulting in the evasion of apoptosis. One potential cellular survival mechanism in CR is through upregulation of stress response pathways, which confers protection to cells when they are subject to adverse conditions.

The glucose-regulated proteins (GRP) were initially identified as such in transformed chick embryo fibroblasts growing in glucose-deprived medium [1, 2]. The best examined member of the GRP family is GRP78, a 78-kDa protein also recognized as immunoglobulin heavy-chain binding protein (BiP) [3]. Normal functions of GRP78, which resides in the ER lumen, include proper folding and assembly of other polypeptides leading to formation of functional proteins, retention of unassembled precursors to the ER, targeting misfolded protein for degradation, ER $Ca^{2+}$ binding, and the regulation of trans-membrane ER stress inducers.

As an ER chaperone, GRP78 is a key component of the unfolded protein response, promoting cell survival under ER stress. The inherent roles and anti-apoptotic capabilities of GRP78 indicate a potential role in cancer progression. Elevation of GRP78 in the microenvironment of tumors due to nutrient deprivation or hypoxia confers survival advantage to cancer cells and leads to resistance to therapeutics. The association between increased GRP78 and malignancy has previously been implicated in various cancer cell lines and tumors [4-8]. GRP78 serum reactivity has recently been identified in patient sera as a putative marker of CR [4]. **We were interested in assessing the prospective role of GRP78 in prostate cancer progression and the development of CR**.

## Immunohistochemical Analysis of GRP78 in Prostate Cancer: GRP78 is Significantly Increased in Castration Resistant Disease

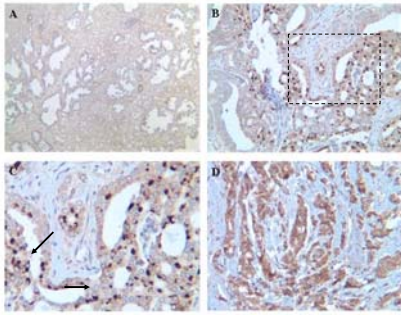


Figure 1: **GRP78 expression in prostate cancer. A)** Tumor from untreated $T_3N_0M_0$ group showing >50% of tumor cells with moderately (2+) intense GRP78 cytoplasmic immunoreactivity; original magnification x200; **B)** Tumor from treated group showing strong (3+) focal GRP78 cytoplasmic immunoreactivity; original magnification x200; **C)** Inset from (B); subpopulations of prostate cancer cells demonstrate intense GRP78 cytoplasmic immunoreactivity (arrows); treated group had a greater number of cases (44%) with at least 5% of tumors cells demonstrating this intense GRP78 immunoreactivity than did the untreated group (36%); **D)** Tumor from castration-resistant group showing strong (3+) intensity cytoplasmic immunoreactivity; original magnification x200.

**Table 1. GRP78 Expression (Immunoreactivity) in Untreated $T_3N_0M_0$, Treated $T_3N_0M_0$, and Castration Resistant Prostate Cancer**

| | Percent Tumor Cells Reactive [%] | | | Intensity [%] | | |
|---|---|---|---|---|---|---|
| Tumor | Low (≤50%) | High (>50%) | p Value* | Low (1+) | Moderate/Strong (2-3+) | p Value* |
| Untreated | 44 [27] | 120 [73] | 0.002 | 73 [45] | 91 [55] | 0.033 |
| Treated | 9 [33] | 18 [67] | <0.001 | 13 [48] | 14 [52] | 0.053 |
| CR | 0 [0] | 28 [100] | | 6 [21] | 22 [79] | |

*p-values represent significant differences from CR group

## Effect of Androgen Deprivation on Expression of GRP78 in LNCaP and PC-3 cells

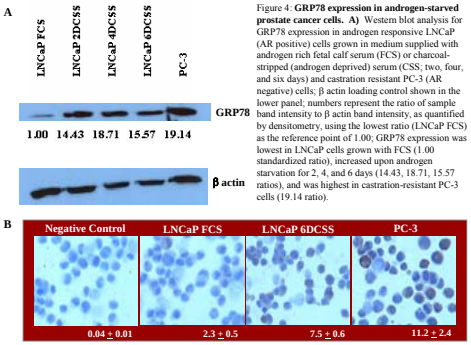


Figure 4: **GRP78 expression in androgen-starved prostate cancer cells. A)** Western blot analysis for GRP78 expression in androgen responsive LNCaP (AR positive) cells grown in medium supplied with androgen rich fetal calf serum (FCS) or charcoal-stripped (androgen deprived) serum (CSS; two, four, and six days) and castration resistant PC-3 (AR negative) cells; β actin loading control shown in the lower panel; numbers represent the ratio of sample band intensity to β actin band intensity, as quantified by densitometry, using the lowest ratio (LNCaP FCS) as the reference point of 1.00; GRP78 expression was lowest in LNCaP cells grown with FCS (1.00 standardized ratio), increased upon androgen starvation for 2, 4, and 6 days (14.43, 18.71, 15.57 ratios), and was highest in castration-resistant PC-3 cells (19.14 ratio).

**B)** Immunostaining of GRP78 in LNCaP cells grown in FCS, LNCaP cells grown in androgen-depleted medium for six days (6DCSS), and PC-3 cells. ACIS II-assisted computer imaging analysis shows that PC-3 (11.2% tumor cells reactive) and LNCaP cells grown in CSS for 6 days (7.5% tumor cells reactive) showed higher GRP78 cytoplasmic immunoreactivity than cells grown in FCS (2.3% tumor cells reactive); percentages given as mean value of 5 representative areas ± standard deviation. Negative control with no primary antibody shown in the left panel.

## Materials and Methods

- **Patient Population: Stages in the Development of CR**
  - **Untreated group** (n=164): Primary tumors from patients with pathological stage $T_3N_0M_0$ disease without pre-op androgen ablation therapy; expected to respond to anti-androgen therapy
  - **Treated group** (n=27): Primary tumors from patients with pathological stage $T_3N_0M_0$ disease with pre-op androgen ablation therapy (DES); considered responsive to anti-androgen therapy; range of treatment 3 days to 20 weeks
  - **Castration-resistant group** (n=28): Primary tumors from patients with advanced prostate cancer resistant to hormone therapy
- **Immunohistochemistry (IHC)**
  - Tissue: 5μm sections from formalin-fixed, paraffin embedded prostate cancer tissue
  - Pretreatment: Antigen retrieval with citrate buffer (pH=6), microwave 30 minutes (15 minutes high power, 15 minutes medium power)
  - Primary antibody: Polyclonal anti-GRP78 (Santa Cruz Biotech)
  - Detection system: Three-step ABC Kit (Vector Laboratories)
  - Counterstain: Hematoxylin
- **Scoring Criteria**
  - Classification of tumors by:
    - Intensity (1+, weak; 2+, moderate; 3+, strong)
    - Percent of tumor cells (≤50%, low; >50%, high) with positive GRP78 cytoplasmic immunoreactivity
    - Presence of focal intense immunoreactivity (<5% of tumor cells with 3+ intensity, negative; ≥5% of tumors cells with 3+ intensity, positive)
- **Cell Line Model (IHC, Western Blot)**
  - Androgen responsive: LNCaP cells (express androgen receptor, AR) grown in medium supplied with fetal calf serum (FCS; androgen-rich)
  - Androgen deprived: LNCaP cells grown in medium supplied with charcoal-stripped serum (CSS; androgen-depleted) for 2, 4, 6 days
  - Castration-resistant: PC-3 cells (do not express AR)

## GRP78 Expression is Associated with Prostate Cancer Recurrence in Younger Patients

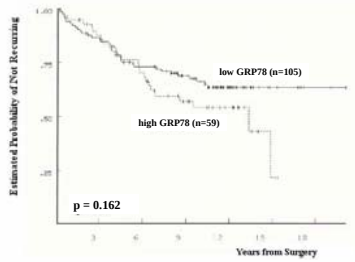


Figure 2: **Probability of recurrence-free (clinical and/or PSA) status in 164 patients with stage $T_3N_0M_0$ prostate cancer, based on levels of GRP78 immunoreactivity.** Untreated stage $T_3N_0M_0$ patients who demonstrated high GRP78 expression (≥5% of tumor cells with strong immunoreactivity) had greater probability of prostate cancer recurrence as compared to those who demonstrated low GRP78 expression (<5% of tumor cells with strong immunoreactivity); tick marks represent patients with no evidence of disease at last follow-up; the p value was obtained using the logrank test.

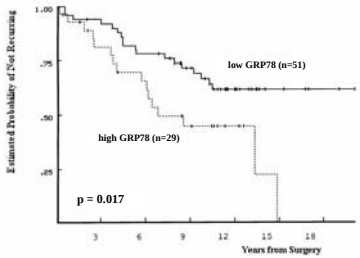


Figure 3: **Probability of recurrence-free (clinical and/or PSA) status in 80 patients with stage $T_3N_0M_0$ prostate cancer stratified by median age, based on levels of GRP78 immunoreactivity.** Untreated stage $T_3N_0M_0$ patients were stratified by age, where patients under the cohort median age of 67 years (n=80) who demonstrated high GRP78 expression (≥5% of tumor cells with strong immunoreactivity) had significantly greater probability of prostate cancer recurrence as compared to those who demonstrated low GRP78 expression (<5% of tumor cells with strong immunoreactivity); tick marks represent patients with no evidence of disease at last follow-up; the p value was obtained using the logrank test.

## Conclusions/Future Directions

Our findings show that upregulation of GRP78 is significantly associated with the development of CR. Interestingly, GRP78 expression is relatively unchanged in androgen responsive tumors on initial exposure to anti-androgen therapy, however, greater levels of strong focal immunoreactivity were identified in the treated $T_3N_0M_0$ group as compared to the untreated group. Thus, GRP78 may act in part through augmentation of cell survival on exposure to anti-androgen therapy. Increased GRP78 is further associated with worse outcome in patients not previously exposed to anti-androgen therapy, particularly in younger patients, and may serve as an important prognostic indicator for recurrence in a subset of patients with $T_3N_0M_0$ disease. These clinical results are supported by our *in vitro* findings, which demonstrate that GRP78 is upregulated in androgen responsive cell lines on exposure to androgen deprivation, and is strongly expressed in castration-resistant cells.

Although the precise role of GRP78 in the development of CR is unclear, increased GRP78 expression may confer a survival advantage through a number of prospective courses, including its molecular chaperone functions, and inhibition of the apoptotic pathway [9]. Additional studies are currently being done to analyze GRP78 association with key signaling molecules during the progression of CR. Analysis of these interactions can result in devising novel therapies targeted towards GRP78 and GRP78-linked molecules for rational therapeutic management of CR.

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