Expression of Stress Response Protein Grp78 Is Associated with the Development of Castration-Resistant Prostate Cancer

Llana Pootrakul,1 Ram H. Datar,1 Shan-Rong Shi,1 Jie Cai,4 Debra Hawes,1 Susan G. Groshen,2 Amy S. Lee,3 and Richard J. Cote1,4

Abstract

Background: Induction of molecular chaperone Grp78 (78-kDa glucose-regulated protein) occurs in stress conditions that often characterize tumor microenvironments. We investigated the role of Grp78 in prostate cancer progression and the development of castration resistance, where cancer cells continue to survive despite the stress of an androgen-starved environment.

Experimental Design: Immunohistochemistry was done to examine Grp78 expression in 219 prostate cancers from patients with pathologic stage T3N0M0 disease (androgen ablation naive (untreated) and androgen ablation exposed (treated) ) and castration-resistant prostate cancer. Classification of tumors was based on intensity of Grp78 cytoplasmic immunoreactivity and percentage of immunoreactive tumor cells. The associations of Grp78 expression with prostate cancer recurrence (clinical and/or serum prostate-specific antigen) and survival were examined in the untreated stage T3N0M0 group. Grp78 expression was also analyzed in the androgen-dependent LNCaP and castration-resistant C42B cell lines.

Results: The percentage of tumor cells expressing Grp78 was strongly associated with castration-resistant status (P = 0.005). Increased Grp78 expression was consistently associated with greater risk of prostate cancer recurrence and worse overall survival in patients who had not undergone prior hormonal manipulation. Grp78 expression was also increased in the castration-resistant LNCaP-derived cell line C42B and in LNCaP cells grown in androgen-deprived conditions compared with LNCaP cells grown in androgen-rich media.

Conclusion: Our findings show that up-regulation of Grp78 is associated with the development of castration resistance, possibly in part by augmenting cell survival as previously suggested, and may serve as an important prognostic indicator of recurrence in a subset of patients with T3N0M0 disease.

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 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 castration resistance is through up-regulation 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 most well studied member of the GRP family is Grp78, a 78-kDa protein also recognized as immunoglobulin heavy-chain binding protein (BiP; ref. 3). Normal functions of Grp78, which resides in the endoplasmic reticulum (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 Ca2+ binding, and the regulation of trans-membrane ER stress inducers (4–6).

The involvement if Grp78 in enhanced cell survival is suggested by the remarkable elevation of GRP78 transcription rates under various stress conditions (2). Recently, Grp78 has been shown to directly interact with intermediates of the apoptotic pathway, blocking caspase activation, where Grp78 induction results in increased cell survival and inhibition of apoptosis (7–9). As an ER chaperone, Grp78 is a key component of the unfolded protein response, promoting cell...
survival under ER stress (10, 11). The inherent roles and antiapoptotic capabilities of Grp78 indicate a potential role in cancer progression. Suppression of Grp78 level by antisense in fibrosarcoma results in inhibition of tumor growth (12). 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 (13). The association between increased Grp78 and malignancy has previously been implicated in various cancer cell lines and tumors (10, 14–18). Grp78 serum reactivity has recently been identified in patient sera as a putative marker of castration resistance (14). We were interested in assessing the role of Grp78 in prostate cancer progression and the development of castration resistance.

Materials and Methods

Patient population. The recruitment and studies of patients described here have been approved by local institutional review boards (HS #006044). This study included tumor samples from 219 patients with prostate cancer, comprised of three distinct cohorts of patients. One hundred ninety-one patients were classified as pathologic stage T3N0M0 disease (19), and specimens were obtained through radical retropubic prostatectomy with bilateral pelvic lymph node dissection at the University of Southern California/Norris Comprehensive Cancer Center between 1982 and 1996. These patients were further subdivided according to treatment status. Treatment consisted of neoadjuvant androgen ablation therapy with 100 mg diethylstilbestrol two or four times per day for 3 days to 20 weeks before radical prostatectomy. The stage T3N0M0 untreated group included 164 patients who were not exposed to preoperative androgen ablation therapy. The group of 27 men comprising the stage T3N0M0 treated group had received neoadjuvant androgen ablation therapy, and these patients were considered responsive to androgen (20). Tumor samples were obtained from 28 patients with castration resistance who underwent hormone therapy with 1 mg diethylstilbestrol, and specimens were obtained through radical retropubic prostatectomy and bilateral pelvic lymph node dissection at the University of Southern California/Norris Comprehensive Cancer Center between 1982 and 1996. These patients were further subdivided according to treatment status. Treatment consisted of androgen ablation therapy with 100 mg diethylstilbestrol two or four times per day for 3 days to 20 weeks before radical prostatectomy. The stage T3N0M0 untreated group included 164 patients who were not exposed to preoperative androgen ablation therapy. The group of 27 men comprising the stage T3N0M0 treated group had received neoadjuvant androgen ablation therapy, and these patients were considered responsive to androgen (20). Tumor samples were obtained from 28 patients with castration resistance who underwent hormone ablation via orchectomy and systemic hormone therapy but continued to show increasing prostate-specific antigen (PSA). Between 1990 and 1992, these men underwent transurethral resection to relieve urinary obstruction at Ruhr University, Bochum, Germany. All tumor grading was in concordance with the Gleason system (21).

Patient follow-up. Evaluations of the T3N0M0 patients were done at 1, 2, and 6 months postoperatively, at 6-month intervals for 5 years following surgery, then yearly. Biopsy was used to assess clinical recurrence of prostate cancer, and metastatic disease was determined according to bone scan or alternative clinical findings. PSA recurrence was designated to patients showing serum PSA levels ≥0.4 ng/mL on two consecutive tests. Median follow-up in the untreated group of 164 patients was 12.7 years with a range of 1.6 to 20 years. Median age was 67 years, ranging from 47 to 81 years.

Immunohistochemistry. Formalin-fixed 5-μm sections were taken from paraffin-embedded prostate cancer specimens and cell lines and mounted on poly-L-lysine–coated slides. The slides were deparaffinized in xylene, washed with 100% ethanol, followed by rehydration in 95% ethanol, 3% hydrogen peroxide in absolute methanol was used to quench endogenous peroxidase. Antigen retrieval was done using citrate buffer (pH 6) and microwaving for 30 minutes (22) followed by cooling at room temperature for 15 minutes. Subsequent steps in immunohistochemistry protocol follow as described above.

Immunoreactivity assessment of clinical samples. All slides were interpreted by two pathologists (S.R.S. and D.H.) who were blinded to all clinical data. Tumor sections were categorized based on two criteria: (a) percentage of tumor cells showing cytoplasmic immunoreactivity and (b) intensity of cytoplasmic immunostaining. For assessment according to percentage of cytoplasmic reactivity, tumors were classified as showing low Grp78 expression (≤50%) or high Grp78 expression (>50%). For intensity of cytoplasmic immunoreactivity, tumors were classified as having low Grp78 expression (≤1), moderate Grp78 expression (≥2), or high Grp78 expression (≥3). Grp78 status was assigned as negative to cases with <10% Grp78 immunoreactivity or weak (≥1) staining. All other cases were assigned positive Grp78 status. Upon identification of focal areas where Grp78 expression levels were markedly intense, tumors were further categorized by percentage of cells showing intense (≥3) Grp78 immunoreactivity (<5%, low Grp78; ≥5%, high Grp78). Due to the heterogeneity of Grp78 immunoreactivity, scoring corresponds with an overall evaluation of the entire tissue section. Lymphocytes, which are highly immunoreactive with anti-Grp78, were used as internal positive controls.

Cell culture. Androgen-responsive LNCaP and androgen-resistant LNCaP-derived C42B cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) with 50 units/mL penicillin, 50 units/mL streptomycin, and 10% FCS (Mediatech, Inc., Herndon, VA). For preparation of androgen-depleted medium, FCS and RPMI 1640 were replaced by 10% dextran/charcoal-stripped serum (Omega Scientific, Inc., CA) and phenol-free RPMI 1640 (Invitrogen), as previously described in the literature (23). All cell lines were maintained in a humidified incubator at 5% CO₂ and 37°C.

Automated cellular imaging. Immunostaining and evaluation of immunostained cell lines were carried out in triplicate, where immunoreactivity was assessed using ACIS II (Clarient, Inc., Aliso Viejo, CA; refs. 24–27). The ACIS II system consists of a computer-assisted bright-field microscope (x4, x10, x20, x40, and x60 objectives) coupled to a SONY 3-chip CCD camera. This fully automated system creates a reconstructed image of an immunohistochemistry-stained slide and uses wavelength-specific technology to detect color differences between objects. Immunostained slides of cytospun cell lines were scanned at ×4 magnification followed by image capture, transformation to pixels, and quantification by hue (color), saturation (color purity), and luminosity (brightness). Five regions of interest at ×4 magnification were manually selected for each sample slide, and brown color (3,3’-diaminobenzidine chromogen) was assessed by ACIS software, which counts pixels based on 256 levels of color intensity. Representative areas were analyzed for intensity and percentage of cells positive for brown color.

Western blot analysis. For Western blot analysis, cell lysates from LNCaP and C42B cells were prepared by lysing in 1 mL ice-cold radioimmunoprecipitation assay buffer. Equal amounts of total protein from each sample were subjected to SDS-PAGE in a 7.5% Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA). Following electrophoresis, the proteins were transferred to a pure nitrocellulose membrane (Bio-Rad Laboratories). The membrane was then incubated in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) followed by overnight incubation with primary rabbit polyclonal anti-Grp78 antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution in PBS. Incubation with biotinylated horse anti-rabbit secondary antibody at 1:2,000 dilution was followed by avidin-biotin conjugate (Vector Laboratories, Inc., Burlingame, CA). Chromogen of 0.03% diaminobenzidine was then applied, with hematoxylin counterstaining. Negative controls consisting of diluent with no antibody and positive prostate cancer controls with heterogeneous immunoreactivity were used in all experiments.

Cultured prostate cancer cells (LNCaP and C42B) were harvested, cytospun on poly-l-lysine–coated slides at 250,000 per slide, and formalin fixed. Antigen retrieval was done using citrate buffer (pH 6) and microwaving for 5 minutes (22) followed by cooling at room temperature for 15 minutes. Subsequent steps in immunohistochemistry protocol follow as described above.

Human Cancer Biology

Clin Cancer Res 2006;12(20) October 15, 2006 5988 www.aacrjournals.org
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Grp78 expression in localized prostate cancer. Immunohistochemistry was employed to evaluate Grp78 protein levels in tumors from 164 stage T3N0M0 untreated and 27 stage T3N0M0 treated prostate cancer patients. In the untreated group, 120 of 164 cases (73%) showed high Grp78 expression by percentage of cytoplasmic immunoreactivity (>50% stained tumor cells), as shown in Fig. 1 and Table 1. Of the 27 cases in the treated group, however, 18 (67%) cases showed high Grp78 percent immunoreactivity (Fig. 1; Table 1). According to intensity of Grp78 immunoreactivity, 91 of 164 (55%) untreated cases showed moderate to high expression of Grp78 (Fig. 1; Table 1). In the treated group, 14 of 27 (52%) tumors showed moderate to high Grp78 expression (Fig. 1; Table 1). For percent immunoreactivity and intensity, the differences between the untreated and treated groups did not reach statistical significance (P = 0.484 and P = 0.913).

We identified focal areas of cells showing intense Grp78 immunoreactivity (≥5%; ≥3 intensity) in both the untreated and treated stage T3N0M0 cases (Fig. 1B and C). In the untreated cases, 36% (59 of 164) versus 44% (12 of 27) of treated cases were classified as positive for this intense focal immunoreactivity.

Grp78 expression in castration-resistant prostate cancer. Of the 28 castration-resistant tumors immunostained for Grp78, 28 (100%) showed high Grp78 expression by percent cytoplasmic immunoreactivity (Fig. 1; Table 1). Compared with the untreated and treated stage T3N0M0 cases, Grp78 expression according to percentage of immunoreactive tumor cells was significantly increased in castration resistance (P = 0.005). This elevation in Grp78 expression remained significant even when comparing castration-resistant cases to the untreated and treated groups separately (P = 0.002 and P < 0.001). When Grp78 expression was examined in castration-resistant tumors by intensity of cytoplasmic immunoreactivity, 22 of 28 (79%) cases showed moderate to high expression (Fig. 1; Table 1). Compared with the stage T3N0M0 cases, the number of tumors showing moderate to high intensity Grp78 expression in the castration-resistant group was significantly greater than both the untreated group (P = 0.033) and the treated group (P = 0.053).

Furthermore, when Grp78 expression was examined as a combined measure of percentage of overall immunoreactive tumor cells and intensity (Grp78 status), Grp78 expression remained significantly elevated in the castration-resistant group when compared with both the untreated group (P = 0.018) and the treated group (P = 0.037).

In vitro expression of Grp78 corroborates clinical observations. Our cell line model consisted of LNCaP-derived castration-resistant C42B cells and androgen-dependent LNCaP cells grown in medium with FCS or in androgen-deprived conditions where FCS was replaced with charcoal-stripped serum. We found that C42B cells and LNCaP cells maintained in medium with charcoal-stripped serum (androgen depleted) for 6 days showed prominent cytoplasmic Grp78 immunoreactivity compared with LNCaP cells grown with FCS, which showed
faint cytoplasmic Grp78 immunostaining (Fig. 1E). Quantitation of Grp78 cytoplasmic immunoreactivity by AICS II computer imaging of five representative areas on each sample slide showed that C42B cells had a mean of 84.0% immunoreactive tumor cells; LNCaP cells grown in charcoal-stripped serum for 6 days showed a mean of 64.2% reactive tumor cells; and LNCaP cells grown in FCS were found to have an average of 24.5% tumor cells showing cytoplasmic reactivity to Grp78 antibody. The ACIS II system reported a mean of 1.2% reactive tumor cells for the negative control LNCaP FCS cells excluding primary antibody. Intensity of each sample analyzed by ACIS II was also found to be greater in C42B and 6-day hormone-starved LNCaP cells (data not shown) than in LNCaP cells grown in FCS. As shown in Fig. 2 and Table 2, these results were corroborated by Western blot analysis of cell lysates prepared from LNCaP cells grown with FCS; LNCaP cells grown with charcoal-stripped serum for 2, 4, and 6 days; and C42B cells. Comparison of Grp78 protein levels, expressed as band intensity ratios, showed that Grp78 expression in LNCaP cells was lowest in cells grown with FCS (1.00 standardized ratio), increased upon androgen starvation for 2 and 4 days (3.63 and 2.63 ratios), even further increased upon 6 days of hormone depletion (8.37 ratio), and was highest in castration-resistant C42B cells (13.94 ratio).

**Association of Grp78 expression with prostate cancer recurrence and survival.** To evaluate Grp78 as a potential marker of prostate cancer progression, we examined the association of Grp78 expression with cancer recurrence risk and overall survival in untreatedstage T3N0M0 patients. Treated cases were stratified by age, PSA level, and Gleason grade. We examined the associations between Grp78 expression and prostate cancer recurrence and survival in untreated stage T3N0M0 patients ($n = 164$, Table 3). At median follow-up of 12 years in the stage T3N0M0 untreated cohort, the probability of remaining recurrence free in cases expressing low Grp78 (<5% cells with intense immunoreactivity to Grp78) was 64% versus 54% in those expressing high (≥5% cells with intense immunoreactivity) levels of Grp78 (Fig. 3). Stratification of Grp78 expression in the stage T3N0M0 untreated cohort, by the standard clinical variables (multivariable analyses adjusting for age, PSA measurements, and Gleason score), consistently showed that the risk of recurring or dying was greater for patients with tumors that expressed high levels of Grp78 (≥5% of tumor cells with ≥3 intensity) compared with patients with tumors that expressed low levels of Grp78, even after adjusting for these known predictors of outcome. Thus, as shown in Table 3, the relative risks, which compare patients with high Grp78 expression in tumors with those with low Grp78 expression in tumors, were not changed substantially after stratification. Although these trends did not achieve statistical significance at the 0.05 level, they are consistent across strata, for both recurrence and survival. Grp78 expression proved to be significant, however, among particular subsets of patients. Interestingly, we observed that in the untreated stage T3N0M0 patients who were below the median age of 67 ($n = 80$) at diagnosis, increased Grp78 immunoreactivity (≥5% cells expressing high levels of Grp78) was significantly associated with increased risk of clinical and/or PSA recurrence (Fig. 4; Table 3). In these cases, the probability of remaining recurrence free in cases expressing low Grp78 was 61% versus 45% in those expressing high levels of Grp78, at follow-up year 12. The median recurrence-free interval for patients with low versus high Grp78 expression was 14.5 versus 8.7 years.

**Discussion**

Based on previous studies identifying the role of Grp78 in cell survival and cancer progression (10, 14, 18), we hypothesized that expression of Grp78 may be associated with prostate cancer progression and the development of castration resistance. In the current study, we examined the expression of Grp78 in three cohorts of prostate cancer patients, uniquely designed to represent successive stages in the development of castration resistance. This included men who were not exposed to hormone

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**Table 1.** GRP78 expression (immunoreactivity) in untreated T3N0M0, treated T3N0M0, and castration-resistant prostate cancer

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Percentage tumor cells reactive (%)</th>
<th>Intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (&lt;50%)</td>
<td>High (&gt;50%)</td>
</tr>
<tr>
<td>Untreated$^*$</td>
<td>44 (27)</td>
<td>120 (73)</td>
</tr>
<tr>
<td>Treated$^*$</td>
<td>9 (33)</td>
<td>18 (67)</td>
</tr>
<tr>
<td>CR$^*$</td>
<td>0 (0)</td>
<td>28 (100)</td>
</tr>
</tbody>
</table>

$^*$P values represent significant difference from castration-resistant group.

1Tumors from stage T3N0M0 prostate cancer patients who have not undergone preoperative androgen ablation therapy.

2Tumors from stage T3N0M0 prostate cancer patients who have undergone preoperative androgen ablation therapy.

3Tumors from patients with castration-resistant prostate cancer.

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**Fig. 2.** Grp78 expression in prostate cancer cells during brief and prolonged androgen starvation. Western blot analysis for Grp78 expression in LNCaP cells grown in FCS and charcoal-stripped serum (2, 4, and 6 days) and castration-resistant C42B cells. β-Actin loading control (bottom). Numbers are the ratio of sample band intensity to β-actin band intensity, using the lowest ratio (LNCaP FCS) as the reference point of 1.00.
therapy, men who were exposed and considered responsive to hormone therapy, and men who were exposed and resistant to hormone therapy. Our results suggest that Grp78 expression is up-regulated during the transition from localized prostate cancer to metastatic castration resistance. Thus, it is possible that overexpression of Grp78 may confer resistance to apoptosis in stress conditions such as anti-androgen therapy and may be an integral component of castration-resistant growth. Clonal selection has been implicated in the development of castration resistance (28). This is corroborated by identification of focal areas of cells showing intense Grp78 immunoreactivity (>5%, ≥3 intensity) in both the untreated and treated stage T3N0M0 cases (Fig. 1B and C) where a greater percentage of treated cases (44%) versus untreated cases (36%) were classified as positive for this intense focal immunoreactivity. We have also provided corroborating in vitro evidence, using our cell line model that mimics clinical development of castration resistance, of increased Grp78 expression in castration-resistant growth, further suggesting the feasibility of Grp78 as a novel therapeutic target for prostate cancer.

It has been postulated that Grp78 induction occurs in response to cellular stress is involved in escape from apoptosis and contributes to drug resistance (7–9, 29). Recent evidence also suggests that Grp78 may contribute to the metastatic potential of prostate cancer cells via α-2-macroglobulin–mediated signaling (30). Our findings accordingly showed significantly higher expression of Grp78 in metastatic castration resistance when compared with localized stage T3N0M0 prostate cancer. This result is also consistent with those reported by Mintz et al. (14) that show strong Grp78 epitope immunoreactivity in bone marrow metastases from castration-resistant patients versus weak immunostaining in normal prostate and higher serum immunoreactivity to Grp78 in castration resistance compared with locally advanced prostate cancer.

<table>
<thead>
<tr>
<th>Grp78 expression* (n)</th>
<th>Recurrence-free survival, relative risk</th>
<th>Overall survival, relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (n = 164)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grp78 &lt; 5%</td>
<td>105</td>
<td>1.00</td>
</tr>
<tr>
<td>Grp78 ≥ 5%</td>
<td>59</td>
<td>1.43</td>
</tr>
<tr>
<td>Adjusted for age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤67</td>
<td>29/51</td>
<td>2.22</td>
</tr>
<tr>
<td>&gt;67</td>
<td>30/54</td>
<td>0.87</td>
</tr>
<tr>
<td>Stratified</td>
<td></td>
<td>1.47</td>
</tr>
<tr>
<td>Adjusted for PSA k (ng/dL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>4/9</td>
<td>5.87</td>
</tr>
<tr>
<td>4-10</td>
<td>10/24</td>
<td>1.45</td>
</tr>
<tr>
<td>10-20</td>
<td>15/18</td>
<td>1.44</td>
</tr>
<tr>
<td>&gt;20</td>
<td>13/17</td>
<td>1.11</td>
</tr>
<tr>
<td>Stratified</td>
<td></td>
<td>1.33</td>
</tr>
<tr>
<td>Adjusted for Gleason score</td>
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<td></td>
</tr>
<tr>
<td>2-4</td>
<td>1/7</td>
<td>NA</td>
</tr>
<tr>
<td>5-6</td>
<td>20/43</td>
<td>1.06</td>
</tr>
<tr>
<td>7-10</td>
<td>38/55</td>
<td>1.30</td>
</tr>
<tr>
<td>Stratified</td>
<td></td>
<td>1.24</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

*Percentage (<5% or ≥5%) of tumor cells with ≥3 intense immunoreactivity represents Grp78 expression.

†Hazard ratios were calculated as a measure of relative risk.

‡Number of patients with ≥5% tumor cells with ≥3+ Grp78/number of patients with <5% tumor cells with ≥3 Grp78.

§PSA values were not available for 54 patients who were excluded from PSA-stratified analyses.

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Table 2. Quantification of protein expression from Western blot analysis of Grp78

<table>
<thead>
<tr>
<th>Cell line sample</th>
<th>Average intensity of Grp78 band (I)*</th>
<th>Average intensity of β-actin band (Iβ)*</th>
<th>Ratio I/Iβ,*</th>
<th>Standardized ratio I/Iβ,*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP FCS</td>
<td>23.39</td>
<td>142.58</td>
<td>0.16</td>
<td>1.00</td>
</tr>
<tr>
<td>LNCaP 2DCSS</td>
<td>52.84</td>
<td>91.36</td>
<td>0.58</td>
<td>3.63</td>
</tr>
<tr>
<td>LNCaP 4DCSS</td>
<td>37.59</td>
<td>90.27</td>
<td>0.42</td>
<td>2.63</td>
</tr>
<tr>
<td>LNCaP 6DCSS</td>
<td>139.37</td>
<td>103.78</td>
<td>1.34</td>
<td>8.37</td>
</tr>
<tr>
<td>C42B</td>
<td>169.61</td>
<td>75.90</td>
<td>2.23</td>
<td>13.94</td>
</tr>
</tbody>
</table>

*All measurements taken as average band intensity with data units of absorbance.

†Standardized ratio calculated using lowest ratio I/Iβ, as reference point of 1.00.

‡LNCaP cells grown in medium supplemented with 10% fetal calf serum (FCS).

§LNCaP cells grown for 2, 4, or 6 days in androgen-depleted conditions of medium supplemented with 10% charcoal-stripped serum (CSS).

|||
Interestingly, we found that Grp78 expression is relatively unchanged in androgen-responsive tumors on initial exposure to anti-androgen therapy. However, we identified focal areas of cells showing intense Grp78 immunoreactivity in both the untreated and treated stage T$_2$N$_0$M$_0$ cases, where a greater percentage of treated cases versus untreated cases were classified as positive for this intense focal immunoreactivity. Craft et al. has previously established in their xenograft prostate tumor model the existence of castration-resistant cells, even before hormone ablation (28). It is possible that the focal areas of prostate tumor, which strongly express Grp78, are comprised of clones that have been conferred a survival advantage by overexpressing Grp78, which may potentially develop into the more homogeneous staining cells in castration-resistant prostate cancer that show strong Grp78 immunoreactivity. Thus, our results are suggestive of clonal selection, whereby androgen withdrawal allows for selective survival and proliferation of castration-resistant cells that existed upon initiation of therapy (31).

Using our previously established cell line model of castration resistance development (32), we showed the occurrence of increased Grp78 expression in conditions of androgen deprivation and even greater Grp78 expression in established castration-resistant cells. Furthermore, the application of quantitative methods, such as densitometry for Western blot analysis and ACIS II automated image analysis for immunohistochemistry, allowed for objective assessment of relative Grp78 levels. Given the subjective nature of immunohistochemical reactivity assessment, results from the novel ACIS II system showing increased Grp78 in castration-resistant growth compared with androgen-dependent growth provided numerical validation of our clinical findings.

In this study, we observed that increased Grp78 expression in prostate tumors is associated with greater relative risk of recurrence and worse overall survival. This pattern was still observed following stratified analyses. Furthermore, Grp78 expression may have important prognostic value in distinct subsets of patients, particularly in those for whom favorable prognosis is indicated by low PSA levels or Gleason grade, as suggested by Table 3. Our results also suggest that increased Grp78 expression may be predictive of prostate cancer recurrence among patients who are diagnosed at relatively earlier age. These and further confirmatory studies may potentially indicate more aggressive treatment in patients who show high levels of Grp78, particularly for younger patients who may be better able to undergo such therapies.

A number of other stress-induced proteins have been implicated in the development of castration resistance. Heat shock protein Hsp70, which shares 60% homology with Grp78, and other stress-response proteins, such as Hsp27 and Hsp90, seem to contribute substantially to prostate cancer progression and the development of castration resistance, where suppression of these proteins is inhibitory to castration-resistant growth (33–38). This is indicative of the potential role of Grp78 in castration resistance development and as an additional therapeutic target, which, if inhibited, may allow cells to become susceptible to environmental stresses.

A significant clinical problem in prostate cancer is that patients who are initially responsive to androgen ablation therapy often develop castration resistance, despite recent advances in cancer therapy. One of the most common molecular defects associated with castration resistance is a deregulated androgen receptor (AR) pathway, resulting in constitutively active cell proliferative machinery. A better understanding of the complex contributing molecular mechanisms underlying the development of castration resistance is necessary so that the most appropriate therapeutic targets may be identified. Feldman et al. proposed five molecular pathways through which prostate cancer cells may undergo transition from androgen-dependent to castration-resistant growth (39). Among these pathways are mechanisms that result in downstream AR.

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**Fig. 3.** Probability of recurrence-free (clinical and/or PSA) status in 164 patients with stage T$_2$N$_0$M$_0$ prostate cancer, based on levels of Grp78 immunoreactivity. Untreated stage T$_2$N$_0$M$_0$ patients showed greater probability of prostate cancer recurrence with higher Grp78 expression. Tick marks represent patients with no evidence of disease at last follow-up. $P$ was obtained using the log-rank test.

**Fig. 4.** Probability of recurrence-free (clinical and/or PSA) status in 80 patients with stage T$_2$N$_0$M$_0$ prostate cancer stratified by median age, based on levels of Grp78 immunoreactivity. Untreated stage T$_2$N$_0$M$_0$ patients were stratified by age, where patients under the cohort median age of 67 years ($n = 80$) showed greater probability of prostate cancer recurrence with higher Grp78 expression. Tick marks represent patients with no evidence of disease at last follow-up. $P$ was obtained using the log-rank test.
activation, or those in which alternative signals are up-regulated and bypass the necessity to activate AR-regulated transcription. Although the precise role of Grp78 in the development of castration resistance is unclear, increased Grp78 expression may confer a survival advantage through a number of prospective courses, including its molecular chaperone functions in assisting proper protein folding, inhibition of the apoptotic pathway, and contribution to growth and invasive potential of prostate cancer cells via cell surface receptor functions (7, 29, 40, 41). The intrinsic functions of Grp78 indicate a potential role in the AR bypass pathway of castration resistance development, although we believe that Grp78 signaling may contribute to a multitude of molecular mechanisms resulting in castration-resistant growth. Our studies suggest that up-regulation of Grp78 stress-response protein plays a role in the progression of localized prostate cancer to castration resistance and promotes Grp78 as a potential prognostic marker and therapeutic target for prostate cancer. Based on these and previous results showing Grp78 mediation of signal transduction (42), we plan to assess the chaperone function as well as cell membrane-resident signaling activity of Grp78 during castration resistance–associated molecular changes. Additional studies are currently being done to analyze Grp78 association with key signaling molecules during the progression of castration resistance, which may lead to downstream deregulation of AR activity, such as members of the epidermal growth factor receptor family, potentially establishing Grp78 as a link between AR activation and AR bypass pathways. Analysis of these interactions can result in devising novel therapies targeted towards Grp78 and Grp78-linked molecules for rational therapeutic management of castration-resistant prostate cancer.

**References**


Expression of Stress Response Protein Grp78 Is Associated with the Development of Castration-Resistant Prostate Cancer

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