

# Relationship Between Changes in Quality of Life and Grading of Genitourinary Toxicity After Brachytherapy with I-125 Alone for Localised Prostate Cancer

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## Research article

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# Abstract

**Background** A rapid increase in human papilloma virus (HPV)-positive oropharyngeal squamous cell carcinoma (OPSCC) is a global trend. Although HPV-positive patients have a more favorable prognosis, distant metastases occur, warranting new, systemic treatment options. The aim of this study was to investigate the effect of combining proteasome or MDM2 inhibitors with cisplatin on an HPV-positive oropharyngeal squamous cell carcinoma cell line (LU-HNSCC-26).

**Methods** The LU-HNSCC-26 cells were treated with proteasome inhibitor (bortezomib, carfilzomib or ixazomib) or MDM2 inhibitor (RG7112) in combination with cisplatin. Combinatorial effects were analyzed by isobolograms. Protein expression was investigated by Western blotting and cell cycle phase distribution by flow cytometry.

**Results** There was no synergy between the substances and cisplatin. All proteasome inhibitors displayed antagonistic effects while the MDM2 inhibitor was additive in combination with cisplatin. The expression of p53 was only marginally affected and apoptosis was not detected. The cell cycle progression was halted in G0/G1 with all inhibitors and in S phase with cisplatin. The expression of p21 increased by bortezomib or carfilzomib, ixazomib increased p21 in combination with cisplatin while RG7112 did not affect p21. There was no effect on ERCC1 with any of the substances.

**Conclusions** In the investigated HPV16-positive OPSCC cell line, proteasome inhibition decreased the effect of cisplatin. A possible mechanism for this includes low effects on p53 expression with concomitant increase in p21 expression and blocking of cell cycle progression in G0/G1 with preserved DNA damage repair. The combination of proteasome inhibition with ordinary cytotoxic treatment for HPV-positive OPSCC patients is thus questionable, and clinical trials should be preceded by thorough testing in adequate models.

## Background

Permanent prostate brachytherapy (PPB) using I-125 or Pd-103 is an established radical treatment for localised prostate cancer, yielding excellent local control and long-term biochemical control [1–4]. Like external beam radiation therapy (EBRT), including three-dimensional conformal radiation therapy and intensity-modulated radiation therapy, dose escalation improves the clinical outcomes of PPB. Stock et al. [5] reported that freedom from prostate-specific antigen failure at 10 years was closely associated with the biologically effective dose, which was the most significant predictor of positive post-treatment biopsy results. However, toxicity also increased as the total delivered dose increased. The incidence of acute genitourinary (GU) toxicity of grade  $\geq 2$  after PPB monotherapy ranged from 10–40% [6–9], and acute urinary retention (AUR) occurred in 5–15% of patients [10–13]. Kittel et al. [4] studied the long-term toxicity of PPB for prostate cancer and reported that the overall rates of late GU and gastrointestinal toxicities of grade  $\geq 3$  were 7.6% and 0.8%, respectively, and on multivariable analysis, age  $\geq 70$  years and prostate length  $\geq 5$  cm were predictive of grade  $\geq 3$  toxicity. As described above, the incidence and

severity of toxicity after PPB differed greatly among reports partly due to the difference in techniques, including the prescribed dose, seed placement, or treatment quality. However, it is speculated that the profiles of acute GU toxicity caused by PPB might not be the same as those caused by EBRT because the incidence of AUR after PPB is considered to be higher than that after EBRT.

Treatment-related toxicity has been assessed based on toxicity grading using standardised toxicity criteria such as the Radiation Therapy Oncology Group and the European Organization for Research and Treatment of Cancer (RTOG/EORTC) toxicity criteria or Common Terminology Criteria for Adverse Events. The severity of toxicity is graded based on the worst symptoms after treatment and is classified as acute or late toxicity according to the interval between treatment completion and occurrence of the evaluated toxicity. In other words, toxicity grading does not include factors regarding the duration of and recovery from toxicity. Furthermore, it is difficult to evaluate adverse events related to urinary toxicities, because patients with prostate cancer often have dysuria with benign prostatic hyperplasia. Another approach for assessing the severity of symptoms is to evaluate quality of life (QOL). The severity of treatment toxicities can be well assessed by evaluating QOL using standardised self-administered questionnaires. Among the existing methods of QOL evaluation, longitudinal assessments of QOL before and after treatment have the potential to provide important information regarding the duration of and recovery from toxicities as well as the toxicity severity. To our knowledge, few studies have reported a relationship between the grading of toxicities based on toxicity criteria and the longitudinal changes in the QOL score after PPB for localised prostate cancer.

In this study, we used the Expanded Prostate Cancer Index Composite (EPIC) as a proxy for disease-specific QOL. The EPIC comprises four separate domains (urinary, bowel, sexual, and hormonal domains) [14]; however, we mainly focused on the relationship between changes in the urinary domain, including its subscales, and grading of GU toxicities after PPB. We previously reported on the relationship between changes in the disease-specific QOL score, as assessed with the EPIC, and the grading of GU toxicity after high-dose rate brachytherapy with EBRT [15] and intermediate hypofractionated intensity-modulated radiation therapy (66 Gy in 22 fractions, 3 fractions per week) [16] for localised prostate cancer. Herein, we evaluated the relationship between changes in disease-specific QOL scores and grading of GU toxicities after PPB using I-125 alone for localised prostate cancer to clarify the usefulness of the disease-specific QOL in assessing treatment-related toxicity.

## Methods

### Patients

Between May 2007 and April 2010, among patients with localised prostate cancer (T1-3N0M0) treated with PPB alone using I-125 at our institution, we assessed 107 consecutive patients whose disease-specific QOL was evaluated before PPB and at 1, 3, 6, 12, and 24 months after PPB and who had been followed up for  $\geq 12$  months. The clinical risk group was defined using the D'Amico risk classification [17]. In general, androgen-deprivation therapy (ADT) was administered according to the risk classification,

as follows: no ADT to patients in the low-risk group, 4–6 months neoadjuvant ADT to those in the intermediate-risk group, and 4–6 months neoadjuvant ADT and 6 months adjuvant ADT to those in the high-risk group. ADT mainly comprised administration of a luteinising hormone-releasing hormone agonist plus a nonsteroidal or steroidal antiandrogen. Patients with gland sizes of  $\geq 50 \text{ cm}^3$  (even those with low-risk disease) received short-term (3–4 months) neoadjuvant ADT to achieve prostate volume reduction before PPB. Patients with a large transurethral prostatectomy cavity were excluded from this study.

## **PPB**

PPB comprised transperineal implantation with I-125 seeds as monotherapy in all patients. A planned prescribed dose of 144 Gy was used according to the TG-43 protocol of the American Association of Physicists in Medicine guidelines [18]. The criteria for intraoperative planning were as follows: the percentage of prostate volume receiving the prescribed dose of 144 Gy was  $> 95\%$  and that receiving 150% of the prescribed dose was  $< 65\%$ , and the dose delivered to 90% of the prostate (D90) was  $> 160 \text{ Gy}$ .

## **Follow-up And Evaluation Of Toxicities**

Toxicities were evaluated at every visit, and all patients were followed up at 1–3-month intervals during the first 2 years, and at 3-month intervals thereafter. Toxicities caused by PPB were scored among all patients based on the severity of symptoms during the follow-up period, and the toxicities were graded based on the RTOG/EORTC toxicity criteria [19]. Each symptom was given a grade from 0 (no symptoms) to 5 (death directly related to radiation effects). Acute toxicities were those evaluated within 6 months after PPB completion and late toxicities were those evaluated thereafter.

## **Longitudinal Qol Evaluation**

Longitudinal disease-specific health-related QOL was prospectively evaluated just before PPB and at 1, 3, 6, 12, and 24 months after PPB using the EPIC to assess the time-course changes and recovery patterns of toxicities. The EPIC comprised a 50-item questionnaire that quantified the patient's prostate cancer-specific QOL in four separate domains (urinary, bowel, sexual, and hormonal domains) [14]. The urinary domain consisted of four subscales (urinary function, urinary bother, urinary irritation, and urinary incontinence) and the bowel, sexual, and hormonal domains each comprised two subscales (function and bother).

## **Statistical analysis**

The difference in the average value of the EPIC QOL score at each observation time point was tested using an analysis of variance. Comparative analyses between two groups were performed with unpaired two-tailed *t* tests. All statistical analyses were performed using Microsoft® Excel for Mac version 16.26

(Microsoft Corporation, Redmond, WA, USA). In all statistics analysed,  $P < 0.05$  was considered to indicate statistical significance.

## Results

### Patients

The median follow-up duration after completing PPB was 116 (range, 18–148) months. The characteristics of the patients/tumours are shown in Table 1. The risk groups were distributed as follows: 52 patients in the low-risk group (49%), 47 patients in the intermediate-risk group (44%), and 8 patients in the high-risk group (7%). Among all the patients, 61 (57%) received neoadjuvant therapy and/or ADT.

### Acute And Late Toxicity Based On The Rtog/eortc Criteria

Acute GU toxicity scores were grade 0–1 for 73 patients, grade 2 for 27 patients, and grade 3 for 7 patients. Late GU toxicity scores were grade 0–1 for 101 patients and grade 2 for 6 patients. Regarding grade 3 acute GU toxicity, five patients experienced nocturia hourly or less frequently after PPB, but these symptoms resolved gradually after completing PPB with transient administration of an  $\alpha 1$  blocker. Two patients developed urinary retention soon after PPB but recovered within 1 week after transient placement of a urinary balloon catheter. None of the patients experienced grade 4 acute GU toxicity.

Regarding late GU toxicity, none of the patients experienced grade  $\geq 3$  toxicity during the entire observation period. Regarding acute and late gastrointestinal toxicities, none of the patients experienced grade  $\geq 2$  toxicity during the entire observation period.

#### Clinical and dosimetric factors according to acute GU toxicity grade (grade 0–1 vs grade 2–3)

We investigated clinical and dosimetric factors, including the number of inserted seeds, prostate volume at post-implant dosimetry, and dose-volume histogram parameters, such as prostatic D90, V100, and V150; D5 and D30 of the prostatic urethra to clarify factors associated with the occurrence of grade 2–3 acute GU toxicities. Table 2 summarises the average and standard deviation values of these factors among all patients, those with grade 0–1 acute GU toxicity, and those with grade 2–3 acute GU toxicity. As shown in Table 2, there were no significant differences in these values between patients with grade 0–1 acute GU toxicity and patients with grade 2–3 acute GU toxicity.

#### Changes in the EPIC QOL scores of the general urinary domain and its subscales

EPIC QOL scores of all domains were linearly transformed to a scale of 0 (lowest) to 100 (highest), whereby higher domain scores (range, 0–100) represented better functioning and QOL. EPIC QOL scores were evaluated as average values with standard deviations at each point. Figure 1 shows the results of the changes in all domains among all patients. The urinary (Fig. 1a) and bowel domains (Fig. 1b) exhibited significant differences (both  $P < 0.01$ ) among the observation time points. The sexual (Fig. 1c; P

= 0.08) and hormonal (Fig. 1d;  $P = 0.38$ ) domains did not show significant differences among the observation time points.

Regarding the urinary domain, the general urinary domain score dropped significantly at 1 month ( $77.1 \pm 14.1$ ) after PPB completion as compared to the baseline score ( $92.2 \pm 8.2$ ) ( $P < 0.01$ ), and then returned gradually to the baseline value by 12 months ( $92.0 \pm 9.6$ ) after PPB completion (Fig. 1a). The baseline general urinary domain score and the scores at 3 and 6 months after PPB were significantly different, indicating that significant reductions in the EPIC QOL general urinary domain score continued until 6 months after PPB. Regarding the subscales of the urinary domain, the changes in the scores of all subscales, including function, bother, irritation, and incontinence, showed similar trends as those observed in the general urinary domain scores, indicating that the baseline subscale scores and those obtained at 1, 3, and 6 months after PPB were significantly different (all  $P < 0.01$ ) (Fig. 2).

### **Relationship between changes in the EPIC QOL scores of the general urinary domain and its subscales and GU toxicity grade**

To evaluate the effects of the GU toxicity severity on the longitudinal changes in EPIC QOL scores, we investigated the relationship between the changes in the scores of the general urinary domain and its subscales and the GU toxicity grade by stratifying patients according to GU toxicity grade, i.e. patients with grade 0–1 toxicities and those with grade 2–3 toxicities.

Figures 3 and 4 show changes in the general urinary domain and its subscale scores according to the acute and late GU toxicity grades, respectively. Reduction in the general urinary domain score after PPB was observed in both patients with grade 0–1 and grade 2–3 acute GU toxicities. However, the reduction was more prominent among patients with grade 2–3 acute GU toxicity than among those with grade 0–1 acute GU toxicity (Fig. 3a). The differences in the general urinary domain scores at 1 and 3 months after PPB between patients with grade 0–1 and grade 2–3 acute toxicity were significant (all  $P < 0.01$ ). Regarding the scores of the general urinary domain subscales, all subscale scores exhibited trends similar to those for the general urinary domain score (Fig. 3b–e). The differences in the scores of all subscales at 1 month after PPB between patients with grade 0–1 and grade 2–3 acute toxicity were significant (all  $P < 0.01$ ); however, the duration of the reduction in QOL scores differed according to the subscale. The significant reduction in the urinary irritation QOL score recovered faster than did the reductions in the other subscale scores, and the reductions in the urinary bother and function subscale scores continued until 6 months after PPB. Among the subscales, the reductions in the urinary incontinence score among patients with grade 2–3 acute toxicity at 1 and 3 months after PPB were remarkable, indicating that the urinary incontinence score was the most susceptible to PPB among the subscale scores investigated and that the urinary incontinence score persisted for a long time compared to that on other subscale scores. There were no significant differences in QOL scores between patients with grade 0–1 late GU toxicities and those with grade 2 late GU toxicities (Fig. 4).

## **Discussion**

The EPIC QOL scores of 107 consecutive patients treated with PPB alone showed that the general urinary domain score significantly decreased at 1 month after PPB completion as compared to the baseline score ( $P < 0.01$ ), and then returned gradually to the baseline level. Concerning the QOL survey using the EPIC, a prospective study by Ash et al. [20] examining long-term QOL after PPB using I-125 for localised prostate cancer demonstrated that the general urinary domain score fell to 69.3 at 6 weeks after PPB and returned to the pre-treatment level by 1 year post-treatment. In that study, the change in the general urinary domain score with time mirrored the change in the International Prostate Symptom Score. The pattern of change in the urinary scores in the prior study was almost the same as the pattern observed in the present study. Among the subscales, Ash et al. [20] also demonstrated that urinary bother and irritation scores were mostly affected by PPB. Changes in the subscale scores of the current study showed similar trends, with the reductions in the urinary bother and irritation subscale scores being greater than the reductions in the other subscale scores. Besides longitudinal changes among all patients, the results of this study demonstrated that the reductions in the scores for the general urinary domain and its subscales closely demonstrated a relationship with the acute GU toxicity grade. Regarding the subscales, the reductions in the urinary irritation scores recovered faster than did the reductions in the other subscale scores, the urinary continence score was the most susceptible to PPB among the subscale scores evaluated, and the influence of PPB on urinary continence persisted for a longer time period than it did for the other subscale scores. To the best of our knowledge, this study is the first report to demonstrate a closely relationship between the EPIC scores for the general urinary domain and its subscales and the GU toxicity grade.

Herein, the toxicity severity could be evaluated using QOL assessments because the QOL scores of all domains were linearly transformed to a scale of 0 to 100. Moreover, longitudinal QOL assessments before and after treatment provided valuable information regarding the persistence of and recovery from treatment-related symptoms. Especially, the EPIC was useful for performing detailed evaluations of symptoms that were susceptible to treatment because the urinary domain comprised four subscales (function, bother, irritation, and incontinence) and the changes in treatment-related symptoms could be evaluated according to each subscale.

Urinary toxicity profiles due to treatment may differ between EBRT and PPB; hence, detailed analyses of the changes in QOL scores that occur with each treatment may be an effective tool for exploring specific treatment-related morbidity, and may provide information for improving treatment quality. Ávila et al. [21] reviewed patient-reported outcomes after treatment for clinically localised prostate cancer and mentioned that small deteriorations in urinary incontinence, irritative-obstructive symptoms, sexual function, and bowel bother were observed in meta-analyses of patients who underwent brachytherapy. Pinkawa et al. [22] compared EPIC QOL scores after PPB using I-125 and EBRT (70.2–72.0 Gy) for prostate cancer and demonstrated that the decreases in urinary function and bother scores were significantly greater after PPB than after EBRT at both 1 and 16 months, although bowel function/bother scores tended to be higher after PPB than after EBRT. Several studies proposed various clinical and dosimetric factors that may affect disease-specific QOL after PPB. Using the cancer-specific EORTC core questionnaire, Van Gellekom et al. [23] reported that D90 and prostate volume significantly affected the urinary symptom score. Concerning dosimetric factors, Vordermark et al. [24] analysed longitudinal changes in QOL after

PPB and reported that prostatic V150 was the only implant parameter significantly associated with both urinary and bowel symptoms at 4 weeks and 1 year post-treatment. In our analysis of all patients, we did not identify any significant dosimetric factors that influenced the reduction in QOL score and the occurrence of grade 2–3 acute GU toxicity. However, the general urinary domain score at baseline for all patients also differed. This implies that pre-treatment urinary symptoms may affect changes in treatment-related urinary symptoms and QOL, although the scores for the general urinary domain and its subscales at baseline did not differ, even after stratification according to acute GU toxicity grade or prostate volume. Roeloffzen et al. [25] evaluated the effects of AUR among patients treated with PPB using I-125 on short- and long-term QOL, as assessed by the EORTC QLQ-PR25. The authors reported that patients with AUR had a significantly worse urinary QOL at all time points than did patients without AUR [25]. They also demonstrated that the pre-treatment International Prostate Symptom Score and neoadjuvant ADT were predictors of AUR but that pre-treatment QOL did not have added predictive value for changes in QOL.

Assessing disease-specific and health-related QOL may also be useful for evaluating long-term changes in treatment-related symptoms. Roeloffzen et al. [26] reported patients' prospective health-related QOL for up to 6 years after PPB and concluded that the health-related QOL at 6 years after PPB did not significantly differ from that at baseline, although a statistically significant deterioration in health-related QOL at 6 years was observed for urinary symptoms, bowel symptoms, pain, physical functioning, and sexual activity. Long-term assessments of QOL, especially disease-specific QOL, might clarify time-course changes in late toxicities in addition to acute toxicities; hence, comparing baseline QOL scores to QOL scores at 5–6 years after treatment may provide valuable information regarding the long-term positive and negative effects of \_\_\_\_\_ on treatment-related symptoms. Further research is needed to ensure the validity of longitudinal evaluations of EPIC QOL scores for the precise assessment of treatment-related symptoms after PPB.

Limitations of our study include its retrospective nature. As such, despite the long follow-up period, data for EPIC QOL scores at > 24 months post-treatment were unavailable. Further, we were unable to draw a relationship between EPIC QOL scores and dose-volume histogram parameters of PPB and late toxicities.

## Conclusions

The present study revealed that the changes in the urinary domain EPIC QOL scores, including the scores for all subscales, closely demonstrated a relationship with the acute GU toxicity grade after PPB. Furthermore, longitudinal assessments of EPIC QOL scores provided additional information regarding time-course changes in acute toxicity after PPB. Our results suggest that longitudinal evaluations of EPIC QOL scores may be a useful tool for assessing the quality of prostate cancer treatment.

## Abbreviations

ADT

Androgen-deprivation therapy; AUR:Acute urinary retention; D90:Dose delivered to 90% of the prostate; EBRT:External beam radiation therapy; EPIC:Expanded Prostate Cancer Index Composite; GU:Genitourinary; PPB:Permanent prostate brachytherapy; QOL:Quality of life; RTOG/EORTC:Radiation Therapy Oncology Group and European Organization for Research and Treatment of Cancer

## **Declarations**

### **Ethics approval and consent to participate**

The cell line was established earlier and used after approval by Lund University regional ethical review board (LU 376-01).

### **Consent for publication**

Not applicable

### **Availability of data and material**

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study. Any raw data not included in the article is available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

NS designed, performed and analyzed the experiments, and drafted the manuscript.

GA took part in the cell cycle analyses and revised the manuscript.

SS conceived of the study and drafted the manuscript.

JW conceived of the study and revised the manuscript.

LE conceived of the study, designed and evaluated the experiments, and drafted the manuscript.

All authors have read and approved the manuscript.

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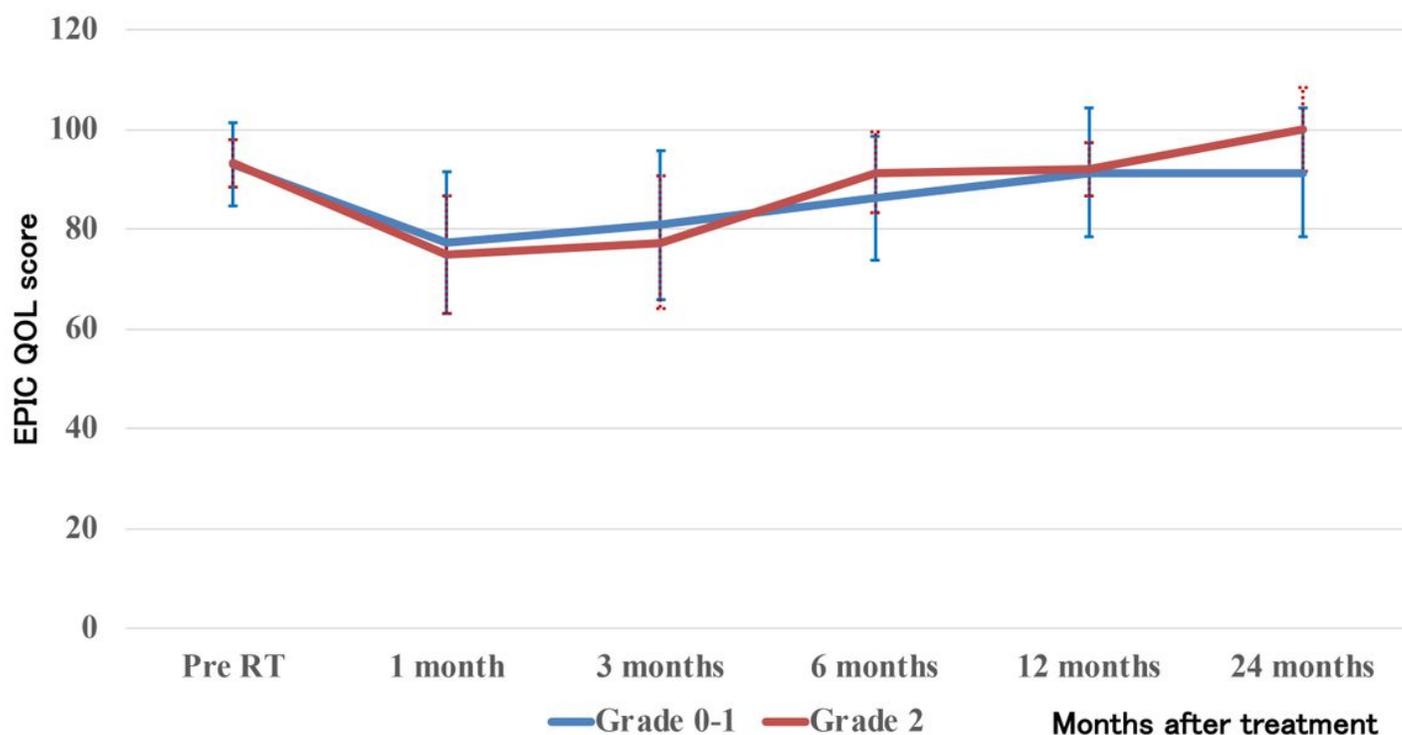
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## Figures

## (A) General Urinary



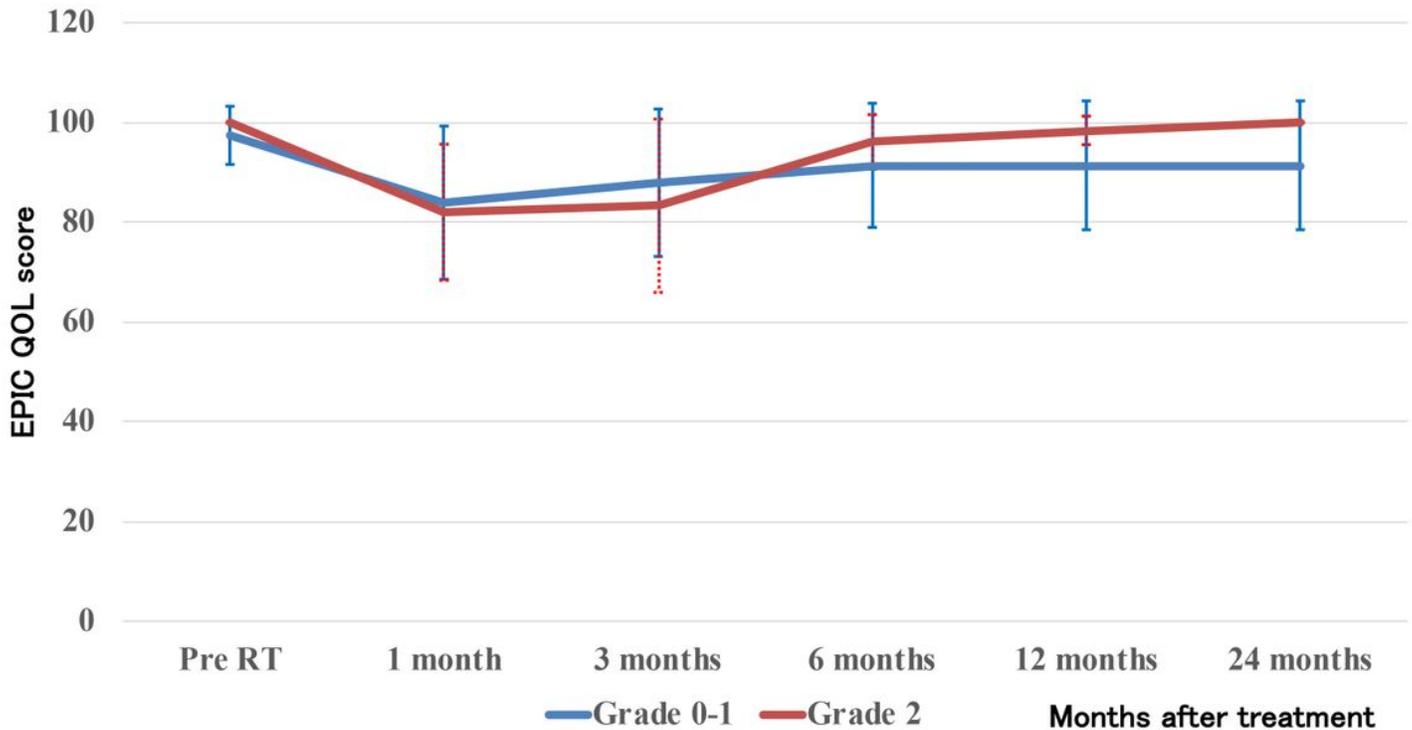
### Acute GU

<b>Grade 0-1</b>	<b>93.0 ± 8.3</b>	<b>77.3 ± 14.2</b>	<b>80.8 ± 14.9</b>	<b>86.2 ± 12.4</b>	<b>91.4 ± 12.9</b>	<b>91.4 ± 12.9</b>
<b>Grade 2-3</b>	<b>93.2 ± 4.7</b>	<b>74.9 ± 11.8</b>	<b>77.4 ± 13.3</b>	<b>91.4 ± 8.1</b>	<b>92.0 ± 5.3</b>	<b>100.0 ± 8.4</b>

**Figure 1**

Determination of IC50 for A. bortezomib, B. carfilzomib, C. ixazomib, and D. R7112 on HN26 cells. Cells were seeded in 96-well plates, incubated for five days and then analyzed by the SRB assay. Each experiment was repeated twice with six determination per drug concentration. Error bars show 95% confidence interval.

## (B) Urinary Function



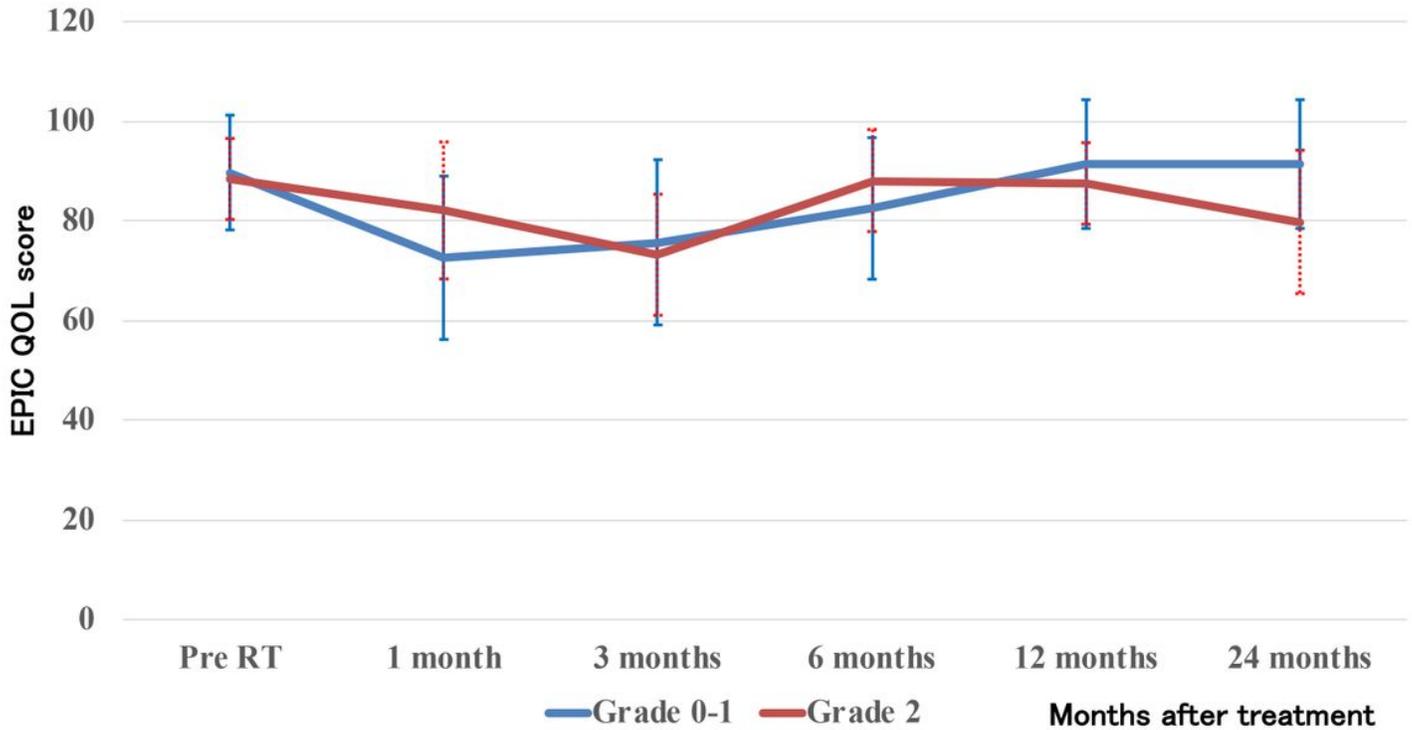
### Acute GU

<b>Grade 0-1</b>	<b>97.4 ± 5.8</b>	<b>83.9 ± 15.4</b>	<b>87.9 ± 14.8</b>	<b>91.4 ± 12.4</b>	<b>91.4 ± 12.9</b>	<b>91.4 ± 12.9</b>
<b>Grade 2-3</b>	<b>100.0 ± 0</b>	<b>82.0 ± 13.6</b>	<b>83.3 ± 17.4</b>	<b>96.1 ± 5.5</b>	<b>98.4 ± 2.9</b>	<b>100.0 ± 0</b>

**Figure 2**

Representative relative isobolograms for the interactions between cisplatin and the different substances on HN26 cells. All IC<sub>50</sub> values for each graph were determined in single experiments to avoid cell batch and dilution variations (as detailed in the Materials and Methods section) and plotted as fractions of the respective single substance values. The cells were incubated for one hour with varying concentrations of cisplatin and then for five days with varying concentrations of the other substances. A solid line was drawn between the IC<sub>50</sub> values of both substances in single treatment (filled circles) with the dotted lines indicating the 95% confidence interval (open circles). Squares indicate the IC<sub>50</sub> values for cisplatin at different concentrations of the other substance and triangles the IC<sub>50</sub> value of the other substance at different cisplatin concentrations. Error bars indicate 95% confidence interval. The analyses were repeated 3 times with similar results. All experiments were performed with 0–50 μmol/L cisplatin in combination with (A) bortezomib, 0–59.5 nmol/L, (B) carfilzomib, 0–200 nmol/L, (C) ixazomib, 0–400 nmol/L, and (D) RG7112, 0–100 μmol/L.

### (C) Urinary Bother



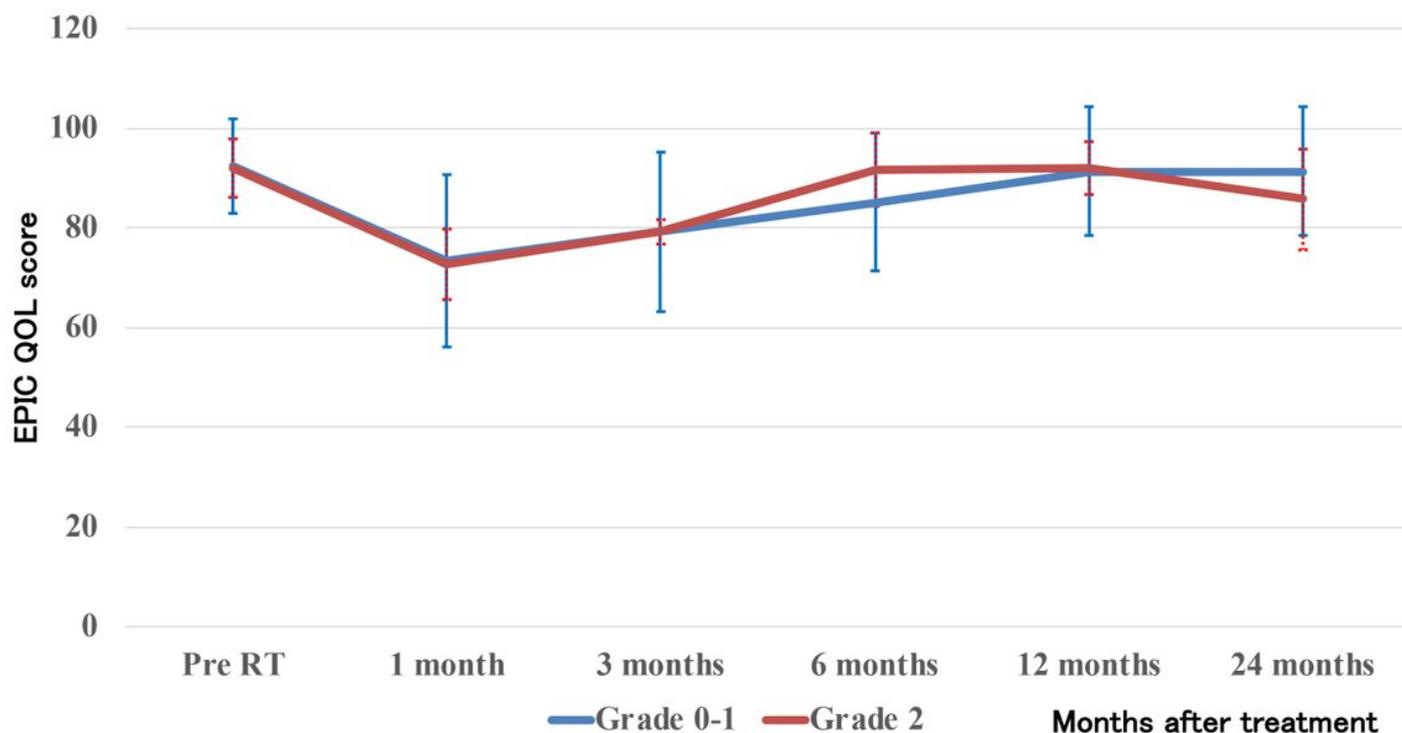
#### Acute GU

<b>Grade 0-1</b>	<b>89.7 ± 11.5</b>	<b>72.6 ± 16.4</b>	<b>75.7 ± 16.6</b>	<b>82.5 ± 14.2</b>	<b>91.4 ± 12.9</b>	<b>91.4 ± 12.9</b>
<b>Grade 2-3</b>	<b>88.4 ± 8.1</b>	<b>82.1 ± 13.7</b>	<b>73.2 ± 12.2</b>	<b>88.1 ± 10.2</b>	<b>87.5 ± 8.2</b>	<b>79.8 ± 14.4</b>

**Figure 3**

HN26 cells (50–80% confluent) were treated with the IC50 concentrations of bortezomib (8.9 nmol/L) and cisplatin (0.99 μmol/L [12]). For bortezomib alone the cells were treated with the inhibitor for the indicated times. For combination treatments with the two drugs, the cells were first treated with cisplatin for 1 h and then with bortezomib for the indicated times. For cisplatin alone, the cells were treated with cisplatin for 1 h and then with R10 medium for the indicated times. Each expression was normalized according to protein load and the relative expression, in comparison with control, calculated and indicated above the protein bands.

## (D) Urinary Irritation



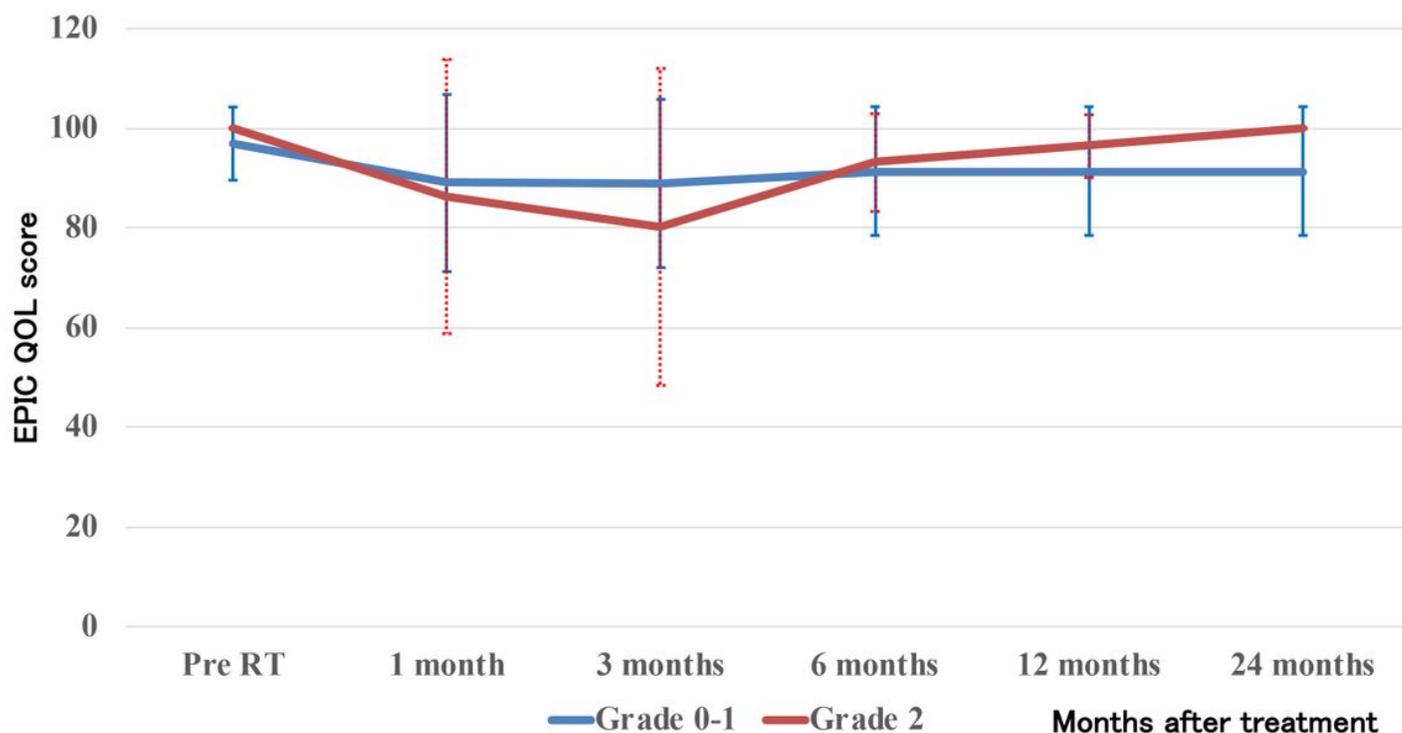
### Acute GU

<b>Grade 0-1</b>	<b>92.4 ± 9.5</b>	<b>73.4 ± 17.3</b>	<b>79.2 ± 16.0</b>	<b>85.2 ± 13.8</b>	<b>91.4 ± 12.9</b>	<b>91.4 ± 12.9</b>
<b>Grade 2-3</b>	<b>92.0 ± 5.9</b>	<b>72.7 ± 7.1</b>	<b>79.2 ± 2.5</b>	<b>91.7 ± 7.3</b>	<b>92.0 ± 5.3</b>	<b>85.7 ± 10.1</b>

**Figure 4**

HN26 cells (50–80 % confluency) were treated with the IC50 concentration of each inhibitor for 24 h: 8.9 nmol/L bortezomib, 25 nmol/L carfilzomib, 47 nmol/L ixazomib, 7.8 μmol/L RG7112 (Table 1). For cisplatin, the cells were treated with IC50 concentration (0.99 μmol/L [12]) for 1 h and then incubated with R10 medium for 24 h. For the combination treatments, the cells were first incubated with cisplatin for 1 h and then with the inhibitors for 24 h. Each expression was normalized according to protein load and the relative expression, in comparison with control, calculated and indicated above the protein bands. C33A2 cells were used as positive control for p53 (1/10 of the protein amount in the HN26 lanes was loaded to enable comparable detection intensities).

## (E) Urinary Incontinence



### Acute GU

<b>Grade 0-1</b>	<b>96.9 ± 7.3</b>	<b>89.0 ± 17.8</b>	<b>88.9 ± 16.9</b>	<b>91.4 ± 12.9</b>	<b>91.4 ± 12.9</b>	<b>91.4 ± 12.9</b>
<b>Grade 2-3</b>	<b>100.0 ± 0</b>	<b>86.3 ± 27.5</b>	<b>80.2 ± 31.8</b>	<b>93.1 ± 9.8</b>	<b>96.4 ± 6.3</b>	<b>100.0 ± 0</b>

Figure 5

HN26 cells (40–60% confluent) were treated with IC50 concentrations of each substance (8.9 nmol/L bortezomib, 25 nmol/L carfilzomib, 47 nmol/L ixazomib, 7.8 μmol/L RG7112 (Table 1) for 24 h after which the cell cycle distributions were determined by flow cytometry. For cisplatin, the cells were incubated with 0.99 μmol/L substance [12]) for 1 h and then with R10 medium or bortezomib for 24 h before flow cytometry measurement. The treatments were performed in triplicate.