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Analysis of DNA damage using the comet assay method in metastatic castration resistant prostate cancer patients receiving ^{177}Lu-PSMA-617 radioligand therapy

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ABSTRACT

Aim: To evaluate possible DNA damage in metastatic castration-resistant prostate cancer patients receiving ^{177}Lu-PSMA-617 radioligand therapy using the comet assay method.

Methods: Prospectively included patients were divided into four groups: a control group and three treatment groups receiving 2, 4, and 6 cycles, respectively. Agarose-coated comet assay slides were prepared using samples obtained from routine blood tests before treatment, and electrophoresis was performed to detect DNA damage. Descriptive statistics, normality tests, and multiple comparison tests were performed.

Results: Forty-five patients with a mean age of 75.5, Gleason scores of 3+4, 4+3, 4+5, and 5+5, and ECOG performance scores of 0/1, 2, and 3 were included in the study. In a valid Comet assay analysis, a similar number of cells were examined in the four treatment groups without any statistical differences. Group analyses revealed statistically significant differences between DNA damage levels. Specifically, the number of stage 0 cells showed a significant difference between the 0-2 and 4-6 cycles groups, while the number of cells with moderate/high levels of damage showed similarities between the 4-6 cycles groups.

Conclusions: This study demonstrates an increase in DNA damage in peripheral blood lymphocytes with cumulative doses in prostate cancer patients treated with ^{177}Lu-PSMA. These findings contribute to the understanding of the relationship between DNA damage during the treatment process and treatment cycles.

Key words: Prostate cancer, ^{177}Lu-PSMA, comet assay, DNA damage.

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Introduction

Metastatic castration-resistant prostate cancer (mCRPC) is defined as the condition where the disease progresses despite medical or surgical castration and can be observed in approximately 10-20% of prostate cancer patients [1]. The clinical management of mCRPC primarily involves various systemic chemotherapies, next-generation androgen deprivation therapies, palliative treatments for metastatic bone pain, and radio-ligand therapies (RLT) targeting the prostate-specific membrane antigen (PSMA). Among the RLTs, the use of Lutetium-177 (^{177}Lu) labeled with PSMA-617 peptide in mCRPC is becoming increasingly common

worldwide, including in our country [2]. Studies have demonstrated a high treatment response rate with a minimum side effect profile in ^{177}Lu-PSMA-617 therapy [3-4]. Radio-nuclides generating medium-energy beta radiation like ^{177}Lu have been successful in selectively destroying malignant tumors. In addition to its medium-level beta energy (497 keV: 78.6%, 384 keV: 9.1%, 176 keV: 12.2%), ^{177}Lu also emits two low-energy gamma emissions (208 keV: 11%, 113 keV: 6.6%) [6].

In repeated applications of various peptides labeled with ^{177}Lu, normal tissues are exposed to cumulative radiation, increasing the risk of genetic instability and potential accumulation of DNA damage that could potentially lead to carcinogenesis. However, imaging-based dosimetry findings are not sufficient alone to predict the effects and limit the toxicities of such treatments, particularly concerning hematological disorders [7, 8]. In patients receiving ^{177}Lu-labeled RLT, all organs, including blood, are exposed to β-particles emitted by circulating ^{177}Lu radio-nuclides and penetrating γ-radiation from distributed activity in the body [9]. As a consequence of exposure to ionizing radiation, free oxygen radicals may be formed, leading to secondary DNA damage within cells. Furthermore, at the DNA level, ionizing radiation can cause single and double-strand breaks, resulting in genotoxic effects [10-15].

The comet assay is a biochemical method commonly used to measure DNA damage in blood cells. When DNA damage occurs, DNA segments with disrupted supercoiled structures form comet-like tails under electrophoresis. The comet assay reveals the frequency of DNA breakage through the relative intensity of these tails under fluorescence microscopy. Additionally, the modified comet assay allows the detection of oxidative stress and subsequent DNA damage [16]. Due to the potential of ^{177}Lu-PSMA-617 RLT to play a more significant role in the treatment sequence of prostate cancer and become a widespread treatment option for different cancer types in the future, it is essential to understand the possible side effects in clinical applications and the relationship between increased treatment cycles and toxicity. In our study, we aim to analyze DNA damage in peripheral blood lymphocytes of prostate cancer patients receiving repeated ^{177}Lu-PSMA-617 RLT and determine the potential relationship between this damage and repeated treatment cycles. As the first study in the literature examining the potential effects of RLT on DNA damage, our work aims to serve as a guiding reference for future research in this field.

Materials and methods

Study plan: Our study has been supported by the Scientific Research Projects Unit under the Rapid Support Project with the code number TF.HZP.21.42. Patients were prospectively enrolled in the study. This prospective study has been approved by our university's Clinical Research Ethics Committee and conducted in accordance with the ethical standards of the 1964 Helsinki Declaration (15.09.2021-2021/308). In our clinic, routine blood samples were taken from mCRPC patients who received ^{177}Lu-PSMA-617 treatment, and lymphocytes were isolated for Comet assay to analyze DNA damage. Blood samples were taken from the treated groups at least 6 weeks after treatment for analysis.

Patients with pathologically confirmed prostate adenocarcinoma, clinically diagnosed with mCRPC based on evaluations and ^{68}Ga-PSMA-11 PET/BT imaging, aged 18 or older, and willing to participate in the study were included if they were referred for potential eligibility for prostate cancer-specific radio-

ligand therapy or seeking to receive the relevant radio-ligand treatment. Patients with known secondary malignancies, previous radio-ligand therapy other than ^{177}Lu-PSMA-617, or known radiation exposure were excluded from the study. The enrolled patients were analyzed in four groups: control (untreated), 2 cycles treated, 4 cycles treated, and 6 cycles treated. DNA damage scores obtained from patients who received repeated radio-ligand therapy were compared with the DNA damage scores of the control group, which was deemed eligible for the same treatment but had not received it yet.

Comet assay: For the Comet assay analysis, samples were obtained from routine blood samples of patients before treatment. To prepare agarose-coated Comet assay slides, 25 µL of cell suspension was embedded in 75 µL of low-melting-point agarose (0.5%; diluted in phosphate-buffered saline without calcium and magnesium) and then covered with a layer of 480 µL of average 0.75% agarose (diluted in phosphate-buffered saline without calcium and magnesium) on a microscope slide. An additional top layer of 100 µL of 0.5% low-melting-point agarose was added. The prepared slides were immersed in a container containing cold lysis solution (1% Triton X-100, 10% dimethyl sulfoxide, and 89% 10 mmol/L Tris/1% Na-lauryl-sarcosine/2.5 mol/L NaCl/100 mmol/L Na_2EDTA, pH 10) at 4°C for 1 hour. Subsequently, the slides underwent pre-electrophoresis treatment for 20 minutes in electrophoresis buffer (300 mmol/L NaOH and 1 mmol/L Na_2EDTA, pH 10) and were then exposed to 1.14 V/cm and 300 mA for 20 minutes. Pre-incubation and electrophoresis were performed in an ice bath. After pre-treatment, the slides were neutralized three times for 5 minutes each with 0.4 mol/L Tris, pH 7.5, and DNA was stained by adding 50 µL of ethidium bromide (50 µg/mL) to each slide. The cells were analyzed using a fluorescent microscope at 500x magnification and computer-assisted image analysis. Images of at least 50 cells (25 cells from each of the two slides) were evaluated using NIH Image 1.54 software program (National Institutes of Health, Bethesda, MD). Two areas were selected for each image: one including all cellular DNA, including the tail region of the comet, and the other including only the tail region of the comet. Integrated densities (the sum of the grey values of all pixels in the selection) were measured for each selection, and the percentage of DNA in the tail region of the comet was calculated. This number represents the amount of DNA in the tail and is expressed as the percentage of DNA in the tail in the figures. Additionally, DNA damage was classified based on tail length. Stage 0 represents undamaged cells, stage 1 indicates mild/minimal damage, stage 2 indicates moderate damage, and stages 3-4 indicate high damage.

Statistical analysis: Descriptive statistics, including mean and standard deviation for numerical variables, and frequency and percentage analysis for categorical variables, were provided for the data obtained from the study. The normality assumption of the variables was examined using the Shapiro-Wilk test. For variables that satisfied the normality assumption, Analysis of Variance (ANOVA) was performed, while for variables that did not meet the normality assumption, the Kruskal-Wallis test was used. To determine which groups the differences came from after the analyses, the post hoc multiple comparison test of Tukey/Dunn's test was used. The analyses were conducted using SPSS 22.0 software. A significance level of $p<0.05$ was chosen.

Results

A total of 45 patients with castration resistant metastatic prostate cancer eligible for ^{177}Lu-

PSMA-617 treatment were included in the study. Blood samples were obtained from the patients and analyzed using the Comet assay to assess DNA damage in four different groups: patients who had not received treatment yet (0 cycles), those who received 2 cycles, 4 cycles, and 6 cycles of treatment. The number of patients in each group was recorded as follows: 0 cycles (n=13), 2 cycles (n=19), 4 cycles (n=19), and 6 cycles (n=14).

The mean age of the patients was calculated as 75.5 years (SD±9.3). The Gleason scores of the patients were as follows: 3+4 (n=11, 24.4%), 4+3 (n=17, 37.8%), 4+5 (n=11, 24.4%), and 5+5 (n=6, 13.4%). The ECOG performance scores of the patients were distributed as follows: ECOG 0/1 (n=8, 17.8%), ECOG 2 (n=29, 64.4%), and ECOG 3 (n=8, 17.8%) (Table 1).

Table 1. Clinical, pathological, and demographic data.

Parameters	
Age; mean (±SD)	75.5 ±9.3
Gleason Score, median (IQR)	7 (7-10)
ECOG PS (%)	
0/1	8 (%17.8)
2	29 (%64.4)
3	8 (%17.8)

SD: Standard Deviation, IQR: Interquartile Range, ECOG PS: Eastern Cooperative Oncology Group, Performance Status.

DNA damage analysis: In the Comet assay analyses, a similar number of cells were examined in all four treatment groups without any statistically significant difference (p=0.191). However, in the group analyses, there was a statistically significant difference in the DNA damage stages among the different treatment cycles (p=0.001) (Table 2).

In the 0 cycles group, the number of stage 0 cells was significantly higher compared to the 2, 4, and 6 cycles groups. In the 2 cycles group, the number of stage 0 cells was significantly higher than the 4 and 6 cycles groups. There was no statistically significant difference between the 4 and 6 cycles groups in this regard (Table 2).

The number of stage 1 cells showed similarity between the 0-2 and 4-6 cycles groups. However, in the 0-2 cycles group, the number of stage 1 cells was significantly higher compared to the other two groups (Table 2).

The number of stage 2 cells was significantly higher in the 2 and 4 cycles groups compared to the 0 cycles group, and the 2-4 cycles groups showed similarity in this regard. In the 6 cycles group, the number of stage 2 cells was lower than the 2 cycles group, and there was no statistically significant difference between the 0 and 4 cycles groups and the 6 cycles group (Table 2).

The number of stage 3 cells in the 4 and 6 cycles groups showed similarity to each other, and it was significantly higher compared to the 0 and 2 cycles groups. The lowest number of stage 3 cells was observed in the 0 cycles group, and in the 2 cycles group, a higher number of stage 3 cells were recorded compared to the 0 cycles group.

The number of stage 4 cells was similarly the lowest in the 0 and 2 cycles groups. In the 4 cycles group, it was significantly higher compared to the 0 and 2 cycles groups, and in the 6 cycles group, it was significantly higher than all other groups.

The total number of cells with DNA damage was significantly lower in the 0 cycles group compared to the other groups. There was no statistically significant difference between the 2-4 cycles and 4-6 cycles groups. However, the number of damaged cells was significantly higher in the 6 cycles group compared to the 0 cycles group and in the 4 cycles group compared to the 0 cycles group. Additionally, the number of cells with moderate/high levels of damage was lower in the 0 cycles group compared to the other groups. This number was similar between the 4-

6 cycles groups. However, this number significantly increased in the 4 and 6 cycles groups compared to the 2 cycles group (Table 2).

In Figure 1, fluorescent microscope images of DNA damage levels before treatment, after the 2nd cycle, and after the 4th cycle of ^{177}Lutetium-PSMA treatments for the same patient are presented. It can be observed that as the treatment cycle increases, DNA damage levels also increase.

Table 2. DNA damage analysis by cycle numbers.

Variables	Cycle No	Mean ± SD	Median (Q1-Q3)	*p*
Stage 0	0 [a]	37,62 ± 4,52	39 (36 -41)	*0,001*‡*
	2 [ab]	17,63 ± 3,96	18 (16 -21)	
	4 [c]	9,89 ± 2,38	9 (8 -12)	
	6 [c]	7,43 ± 2,82	6,5 (5 -9)	
Stage 1	0 [a]	28,46 ± 3,84	28 (25 -31)	*0,001*‡*
	2 [a]	26,53 ± 5,32	28 (24 -30)	
	4 [b]	16 ± 4,11	15 (13 -19)	
	6 [b]	14,64 ± 3,91	14 (12 -1)	
Stage 2	0 [c]	16,85 ± 4,81	17 (14 -18)	*0,001*§*
	2 [a]	25,63 ± 4,26	27 (21 -29)	
	4 [ab]	22,11 ± 4,04	23 (19 -24)	
	6 [bc]	20,36 ± 4,4	20 (16 -23)	
Stage 3	0 [c]	12,08 ± 5,85	12 (8 -15)	*0,001*§*
	2 [b]	19,68 ± 4,63	20 (16 -23)	
	4 [a]	28,37 ± 5,09	29 (25 -31)	
	6 [a]	25,79 ± 3,87	25,5 (24 -26)	
Stage 4	0 [b]	5,77 ± 2,74	5 (4 -8)	*0,001*‡*
	2 [b]	13,68 ± 4,67	13 (11 -16)	
	4 [c]	23,47 ± 4,85	24 (21 -27)	
	6 [a]	34,14 ± 3,3	33,5 (32 -36)	
Total Counted Cell	0	100,77 ± 5,12	101 (99 -105)	*0,191§*
	2	103,16 ± 4,79	103 (100 -107)	
	4	99,84 ± 5,04	101 (96 -104)	
	6	102,36 ± 5,05	102,5 (98 -106)	
Total Number of Cells Containing DNA Damage[#]	0 [b]	63,15 ± 7,95	64 (58 -67)	*0,001*‡*
	2 [c]	85,53 ± 4,94	86 (82 -89)	
	4 [ac]	89,95 ± 4,62	89 (86 -94)	
	6 [a]	94,93 ± 4,73	95 (93 -98)	
Total Cell Count with Moderate/High DNA Damage [##]	0 [b]	34,69 ± 8,84	38 (28 -41)	*0,001*‡*
	2 [c]	59 ± 7,15	58 (52 -65)	
	4 [a]	73,95 ± 5,35	73 (71 -79)	
	6 [a]	80,29 ± 5,62	81 (75 -84)	

**p<0,05; ‡Kruskal Wallis test, § Analysis of variance; a,b,c: Different letters represent the difference between groups (Tukey test/Dunn's test), [#]Stage 1-2-3-4, [##]Stage 2-3-4.*

In Figure 1, fluorescent microscope images of DNA damage levels before treatment, after the 2nd cycle, and after the 4th cycle of ^{177}Lutetium-PSMA treatments for the same patient are presented. In Figures 2 and 3, fluorescent microscope images of DNA damage levels before treatment, after the 2nd cycle of 177Lutetium-PSMA treatments for the same patient are presented. In Figure 4, fluorescent microscope images of DNA damage levels before treatment, after the 4nd cycle of 177Lutetium-PSMA treatments for the same patient are presented.

As seen in these examples, it is seen that the DNA damage levels increase as the treatment cycle increases.

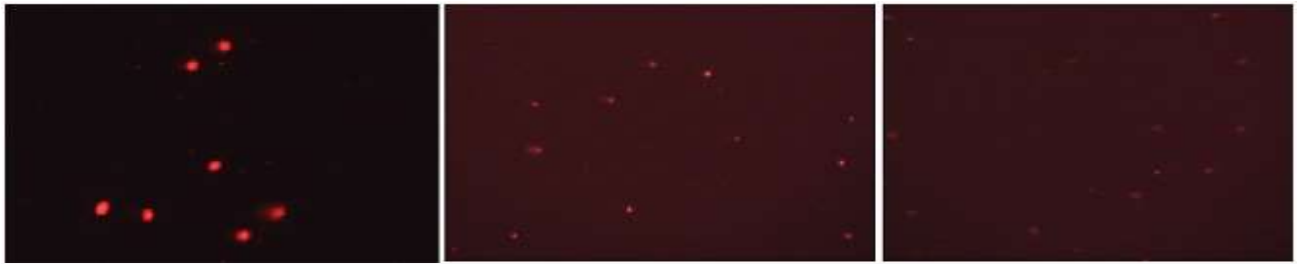

Figure1. Fluorescent microscope images of DNA damage levels after 177Lu-PSMA treatments before treatment (1a), 2nd cycle (1b), and 4th cycle (1c); As the treatment cycle increases, the level of DNA damage increases (1a: Stage 0-1 DNA damage; 1b: Stage 1-3 DNA damage; 1c: Stage 3-4 DNA damage, images belong to the same patient.).

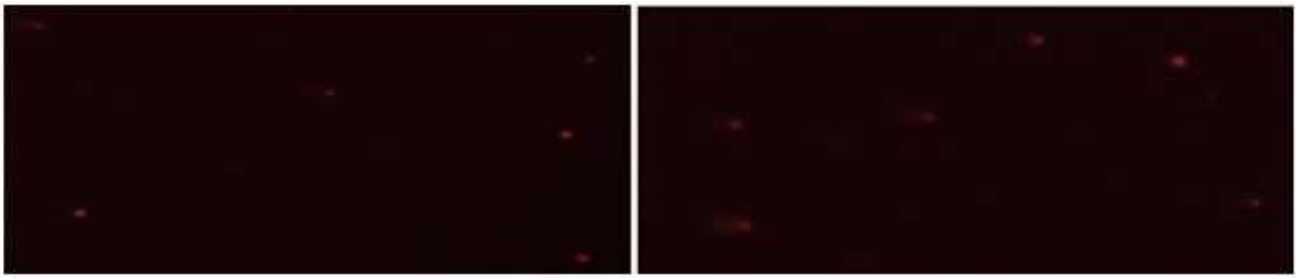

Figure 2. Fluorescent microscope images of DNA damage levels before treatment (2a) and after 2nd cycle (2b) 177Lu-PSMA treatments; (2a: Stage 0 and Stage1 DNA damage; 2b: Stage 0 and Stage 3 DNA damage, images are from the same patient).

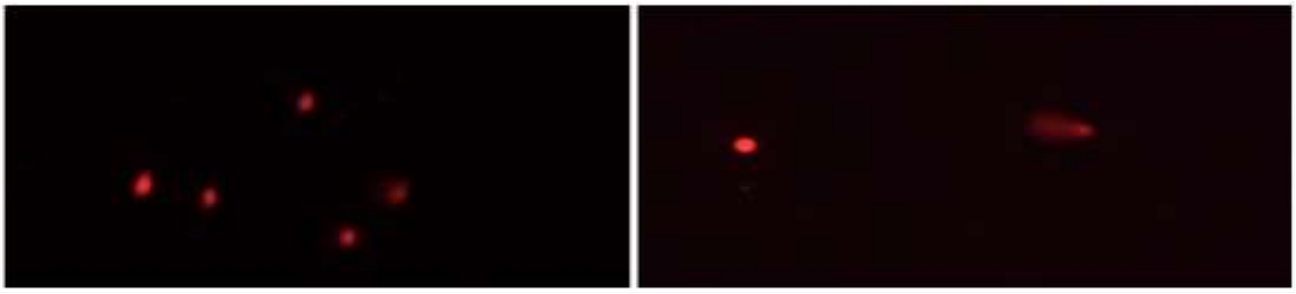

Figure 3. Fluorescent microscope images of DNA damage levels before treatment (3a) and after 2nd cycle (3b) 177Lu-PSMA treatments; (3a: Stage 0 and Stage 1 DNA damage; 3b: Stage 2 and Stage 3 DNA damage, images are from the same patient).

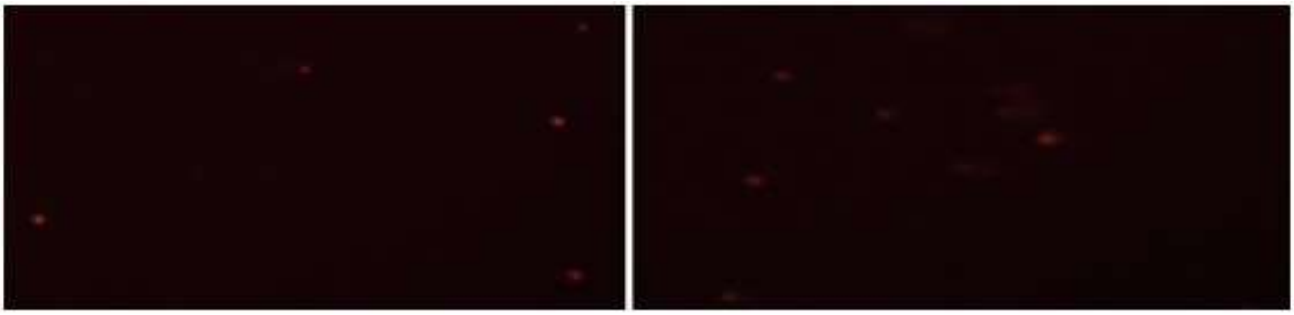

Figure 4. Fluorescent microscope images of DNA damage levels before treatment (4a) and after 4th cycle (4b) 177Lu-PSMA treatments; (4a: Stage 0 and Stage 1 DNA damage; 4b: Stage 3 and Stage 4 DNA damage, images are from the same patient).

Discussion

In the present study, the highest DNA damage was observed in the 6-cycle group, while there were similar levels of moderate/high damage in the 4 and 6-cycle groups. In the 2-cycle group, overall DNA damage was found to be higher compared to untreated patients, but lower than in the 4 and 6-cycle groups. Consequently, it was revealed that DNA damage in patients receiving ^{177}Lu-PSMA-617 therapy is parallel to the cumulative number of treatment cycles.

In a study involving 16 prostate cancer patients receiving ^{177}Lu-PSMA therapy, blood samples were collected immediately before treatment and at 1, 2, 3, 4, 24, 48, and 96 hours after treatment to assess DNA damage. Unlike the current study, the analysis of DNA damage in that study was performed using γH2AX+53BP1 focus assay instead of comet assay. Double-strand DNA damage was evaluated in peripheral blood lymphocytes over time and dose. It was noted that DNA damage increased in the first hours following treatment but decreased later due to repair processes [17]. In the present study, the aim was to demonstrate the potential DNA damage induced by cumulative doses over longer periods. Considering that blood samples were taken at least 6 weeks after treatment doses, it can be inferred that DNA damage persisted in peripheral blood lymphocytes despite repair processes. Additionally, in the study involving 16 patients, it was shown that the administered treatment dose did not significantly contribute to DNA damage in the initial hours, but as the treatment dose increased, DNA damage increased in the following hours [17]. In the current study, dose-dependent analysis was not performed, as patients were treated with similar doses as per the protocol. However, routine clinical practice of administering ^{177}Lu-PSMA therapy at 6-8 week intervals highlighted that cumulative dose increase was significantly associated with DNA damage.

In another study, DNA damage in peripheral blood lymphocytes was analyzed using the comet assay in 10 patients receiving ^{225}Ac (Actinium)-PSMA therapy for the first time, and in 10 patients receiving ^{177}Lu-PSMA therapy. Blood samples were collected immediately before treatment and at 3, 21-24, and 42-48 hours after treatment. The comet assay analysis showed a mild and significant increase in DNA damage at 3 hours after treatment, but the number of damaged cells decreased in the following hours. Interestingly, in contrast to the current study, the analysis of DNA damage in patients receiving ^{225}Ac-PSMA therapy did not show a significant difference compared to the DNA damage levels before treatment. This observation raises the possibility that DNA damage may be closely related to the specific radionuclide used. However, it seems contradictory that ^{225}Ac radionuclide, which has a higher energy profile compared to ^{177}Lu, did not contribute significantly to DNA damage in peripheral blood. In this context, in vitro studies incubating blood samples from healthy volunteers with both ^{225}Ac-PSMA and ^{177}Lu-PSMA radiopharmaceuticals for 5 days under the same conditions showed that ^{225}Ac-PSMA caused significantly higher levels of DNA damage compared to ^{177}Lu-PSMA. The discrepancy observed in vivo is largely attributed to the rapid clearance of PSMA ligand from the blood, ultimately emphasizing the importance of considering the pharmacokinetic properties of the radiopharmaceutical used [18]. It should be noted that the current study used a single type of radiopharmaceutical, and the results obtained may not be generalized to other radiopharmaceuticals developed with ^{177}Lu.

In the conducted study, in contrast to the literature, it was shown for the first time that

cumulative ^{177}Lu-PSMA doses led to increased levels of DNA damage in peripheral blood lymphocytes even at least 6 weeks after treatment. The observed DNA damage was found to be dose-dependent, with higher levels seen in patients who received two treatment cycles compared to those who did not receive any treatment. However, this trend was much more pronounced in patients who received four and six treatment cycles compared to both untreated patients and those who had only received two treatment cycles. Therefore, it is recommended to evaluate treatment response after every two treatment cycles using up-to-date treatment response criteria such as RECIST 1.0. Furthermore, giving importance to prognostic analyses during pre-treatment or interim evaluations will contribute significantly to appropriate patient selection and decision-making regarding the continuation of treatment, thus facilitating the avoidance of high numbers of treatment cycles that have been shown to cause DNA damage in the current study [19,20]. In addition to the sample size, there are several limitations in the current study. Other parameters that may contribute to DNA damage in peripheral blood lymphocytes have not been excluded. The analysis of disease volume, which could potentially contribute to total tumor activity retention, was not included as a sub-parameter. Furthermore, the study only utilized single blood samples due to routine practices, instead of obtaining multiple samples over time.

As a result, the current study has demonstrated that the cumulative dose of ^{177}Lu-PSMA treatment in prostate cancer patients leads to increased levels of DNA damage in peripheral blood lymphocytes. By investigating the impact of ^{177}Lu-PSMA-617 treatment on DNA damage levels in castration-resistant metastatic prostate cancer, this study contributes to a unique field and enhances our understanding of the possible molecular-level side effects of targeted radioligand therapy in cancer treatment.

The findings related to DNA damage during treatment, especially in the context of high numbers of treatment cycles, can shed light on the potential long-term side effects of radioligand therapy and influence future treatment decisions. This can lead to optimizing treatment strategies, developing approaches to minimize DNA damage and long-term effects, and ultimately improve patient outcomes. Additionally, this study lays the groundwork for future research, exploring the impact of DNA damage on treatment outcomes and adverse events, as well as investigating strategies to minimize DNA damage while enhancing treatment efficacy and exploring the potential use of radioprotective agents.

Conclusions

In conclusion, focusing on a specific group of castration-resistant metastatic prostate cancer patients, this study fills a gap in the existing literature by providing valuable insights into the effects of targeted radioligand therapy in this patient population, particularly concerning the potential DNA damage caused by high numbers of treatment cycles. The lack of sufficient information about DNA damage related to the use of ^{177}Lu-PSMA-617 treatment in this patient group and the absence of guidelines specifying the maximum number of treatment cycles make this study unique and relevant, providing valuable information about the effects of the intended radioligand therapy in this specific patient population.

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