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Radiotherapy-Induced Changes in Serum Lipid Profile of Patients with Prostate or Head and Neck Cancer

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Abstract

Purpose

Local body irradiation induces molecular changes that could be detected at the level of blood metabolome. We hypothesized that changes in a mass profile of a lipid fraction of serum observed in response to cancer treatment are primarily affected by acute toxicity of radiotherapy.

Methods

129 patients with prostate cancer (PC) and 66 patients with head and neck cancer (HNSCC) treated with intensity-modulated RT were enrolled, for whom different treatment tolerance was observed. Blood samples were collected before, within and one month after the end of RT. Lipophilic fraction of serum was profiled in individual samples by MALDI-ToF mass spectrometry.

Results

RT affected abundances of several lipid components of serum. Majority of changes observed during the treatment was reversed during follow-up, yet some of them could be detected one month after completion of RT. The number and extent of changes observed in serum of HNSCC patients was generally higher than that in PC patients, even though doses and volumes of irradiated tissues were comparable in both groups. The frequency of RT-induced changes in serum lipid profiles was associated with intensity of acute radiation toxicity, which was apparently higher in HNSCC patients.

Conclusions

Response of patient's organism to local body irradiation during cancer treatment could be monitored at the level of serum lipidome, which changes are primarily associated with type and degree of radiation toxicity.

Keywords: Acute Toxicity; IMRT; Mass Spectrometry; Radiation Response; Serum Lipidomics

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Introduction

Radiotherapy (RT), either alone or in combination with other treatment modalities, is an effective treatment of patients suffering from different types of cancer. Intensity modulated RT (IMRT) helps to deliver precisely a higher dose of radiation to the tumor, and to reduce potentially dose and toxicity of radiation to the surrounding normal tissues. However, a potential drawback of IMRT is an exposure of large volume of normal tissues to low/medium doses, which could affect whole body response to the treatment [1-3]. IMRT is currently among the main treatment options for patients with prostate adenocarcinoma (PC) and patients with head and neck squamous cell cancer (HNSCC) because of its potential to preserve structure and function of a target organ [4-7]. However, aggressive treatment (e.g., dose-escalated hypofractionation, accelerated fractionation or chemoradiotherapy) is usually required in advanced cancer cases, which might be associated with increased risk of treatment toxicity [8,9]. Mucositis, frequently associated with inflammation and acute phase response, is the major symptom of acute radiation toxicity in HNSCC patients [10,11]. High risk of acute mucosal reaction (AMR) is caused by damage of epithelial cells in oral mucosa, which large volume is frequently irradiated with high doses (50-60 Gy). Most importantly, severe AMR can significantly affects the quality of life of patients, causing treatment discontinuation in extreme cases [12-14]. In contrast to rather frequent acute toxicity in HNSCC patients, properly planned image-guided IMRT is well tolerated in majority of PC patients. Improved outcome of contemporary RT in PC patients results among others from enhanced protection of major organs at risk: bladder and rectum [6,7,15]. Local body irradiation during cancer RT apparently induces a systemic response and affects different molecular components of blood, which reflects pleiotropic effects of the treatment associated with either regression of tumor and/or toxicity of normal tissues. There are several reports documenting effects of RT at the level of human serum proteome, which indicate important role of factors associated with acute phase and inflammation reactions [16-21]. However, the influence of RT on the composition of human serum lipidome is much less known. There is more than 500 different lipid species reported in human serum/plasma specimen. The most numerous category among them are phospholipids (PLs), which are both key components of biological membranes and signaling molecules involved in different cellular mechanisms [22]. Several reports showed that composition of PLs present in body fluids could be specifically changed in different disease states (e.g. in cancer) and reflect response of patient's organism to the treatment [23]. Animal studies revealed that metabolism and blood levels of PLs changed upon exposure to ionizing radiation [24,25]. However, there is only one report addressing radiation-induced changes in profile of serum lipidome, where MALDI-MS approach was applied to characterize IMRT-related effects in group of HNSCC patients. The study showed that abundances of several choline-containing PLs decreased significantly during RT, and then increased during followup. Moreover, several RT-induced effects correlated with intensity of AMR and volume of normal tissue irradiated with low-to-medium doses, which indicated association of lipidome changes with acute radiation toxicity [26]. Here we aimed to further characterize RT-induced changes in profiles of human serum lipidome. Two groups of cancer patients with different types of IMRT-mediated acute toxicite were compared – patients with PC and HNSCC. The study revealed that type and intensity of radiation toxicity induced in normal tissue might be the key factor determining extent of RT-related changes in serum lipidome.

Materials and Methods

Characteristics of patient group

Patients with two types of cancer were enrolled into the study: 129 patients with prostate adenocarcinoma (PC) and 66 patients with head and neck squamous cell carcinoma (HNSCC); all of them were Caucasians. Detailed description of patients and the treatment is shown in Table 1. All patients were subjected to IMRT using 6MV or 20MV photons. PC patients were treated according to the conventional 5-time a week irradiation scheme with 2 Gy dose fractions; overall treatment time was in the 50-85 days range (median 54 days). There were two subgroups of PC patients with different radiation plans: RT_1 – total dose 76 Gy was delivered to the gross tumor volume (GTV), and RT_2 – the treatment additionally included irradiation to the pelvic lymph nodes with total dose 44 Gy (the high risk group). HNSCC patients were treated according to the continuous accelerated irradiation scheme (CAIR) with 1.8 Gy daily fraction doses (7-time a week); overall treatment time was in the 38-49 days range (median 41 days). Dose-volume histograms (DVH) were calculated for all patients based on treatment plans; curves corresponding to average DVH are depicted for all three groups in Figure 1.

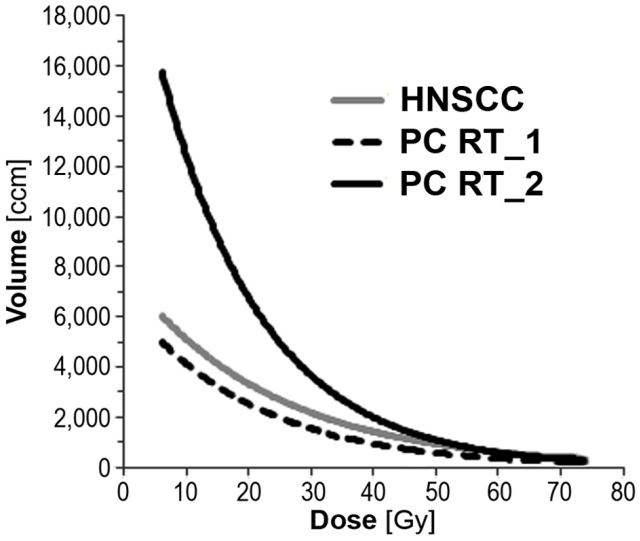

Figure 1. Curves corresponding to average dose-volume histograms in each patient group.

Treatment of PC patients was corrected on a daily basis using fiducial-based image-guided RT (IGRT). Androgen Deprivation Therapy was applied in about 80% of PC patients before and/or during RT (none of PC patients was subjected to prostatectomy). Neither surgery nor induction/concomitant chemotherapy was applied to HNSCC patients enrolled in the study. Acute mucosal reaction (AMR) in HNSCC patients, as well as gastrointestinal toxicity and genitourinary toxicity reaction in PC patients, was assessed during the RT according to the RTOG/EORTC protocol. The study was approved by the appropriate institutional Ethics Committee and all participants provided informed consent indicating their conscious and voluntary participation.

Parameter/group	PC patients / RT_1	PC patients / RT_2	HNSCC patients
number of patients	61 (100% men)	68 (100% men)	66 (71% men)
age (years)	49 - 78 (median 69)	54 - 84 (median 70)	45 - 82 (median 63)
type/location of cancer	prostate adenocarcinoma	prostate adenocarcinoma	squamous cell carcinoma: oropharynx (15), hypopharynx (6), larynx (45)
cancer stage	T1 - 54% T2 - 44% T3 - 0% T4 - 0%	T1 - 32% T2 - 44% T3 - 21% T4 - 3%	T1 - 21% T2 - 44% T3 - 26% T4 - 9%
	N0 - 100 %	N0 - 90%	N0 - 68%
	M0 - 100%	M0 - 100%	M0 - 100%
total GTV dose (Gy)	74-76 (median 76)	70-76 (median 76)	61.2-72 (median 66.6)
clinical target volume (ccm)	82-546 (median 254)	122-787 (median 293)	18-781 (median 327)
volume irradiated with 40 Gy dose (ccm)	256-1736 (median 790)	1340-5125 (median 2268)	551-2768 (median 1634)
maximal intensity of acute radiation toxicity (RTOG/EORTC grade)	gastrointestinal toxicity 0 – 77% 1 – 18% 2 – 5% 3 – 0% 4 – 0%	gastrointestinal toxicity 0 – 61% 1 – 21% 2 – 18% 3 – 0% 4 – 0%	acute mucosal reaction 0 – 0% 1 – 3% 2 – 22% 3 – 75% 4 – 0%
	genitourinary toxicity 0 – 41% 1 – 38% 2 – 15% 3 – 6% 4 – 0%	genitourinary toxicity 0 – 39% 1 – 40% 2 – 18% 3 – 3% 4 – 0%	

Table 1. Characteristics of analyzed groups of patients.

Serum samples

Three consecutive blood samples were collected from each patient: pre-treatment sample A, within-treatment sample B (16-47 days after start of RT in PC group, median 25 days, and 10-22 days after start of RT in HNSCC group, median 15 days), and post-treatment sample C (21-75 days after end of RT in PC group, median 35 days, and 23-59 days after end of RT in HNSCC group, median 36 days). Blood samples (5 ml) collected into Vacutainer Tubes were incubated for 30 minutes at room temperature then centrifuged at 1000 x g for 10 minutes to remove clots; resulting sera were portioned and stored at -70°C. Total lipids were extracted according to modified Folch method [27]. In brief, 25 µL of serum was mixed with 350 µL of 1:1 methanol (Sigma-Aldrich, St. Louis, USA)/chloroform (Avantor Performance Materials, Gliwice, Poland) mixture (v/v) containing antioxidants: 0.01% (w/v) 2,6-di-tert-butyl-4-methylphenol (Sigma-Aldrich, St. Louis, USA) and 0.005% (w/v) retinol (Sigma-Aldrich, St. Louis, USA). The mixture was vortexed and incubated for 25 min at 4°C. Then 100 µL of water was added to the mixture, vortexed for another 10 seconds and centrifuged (5 min, 15,000 x g). Chloroform phase (the bottom one) was kept and stored at -70°C, until mass spectrometry analysis (within one week).

Registration of mass spectra

Samples were analyzed using Ultraflextreme MALDI-ToF mass spectrometer (Bruker Daltonics, Bremen, Germany). 0.6 µL of each sample was mixed directly on stainless steel target plate (Ground steel target, Bruker) with 0.6 µL of matrix solution - 20 µg/µL of 2,5-dihydroxybenzoic acid (DHB) (Bruker Daltonics, Bremen, Germany) dissolved in 30% ethanol (Sigma-Aldrich, St. Louis, USA) containing 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, USA). Positive ions were recorded in the 350-900 Da range using a reflectron mode of the analyzer. For each sample, four technical replicas were registered. Samples were spotted in a random sequence to avoid "batch effect".

Processing of MALDI spectra and statistical analyses

The spectra preprocessing included alignment of spectra, detection of outlier profiles (based on Dixon's Q test) and averaging of technical replicas, then additional alignment of averaged individual spectra, baseline removal and normalization of the total ion current (TIC) was performed according to standard procedures [28]. Preprocessed spectra were then analyzed with Spectrolyzer software suite (v.1.0, MedicWave AB, Halmstad, Sweden). The analysis included peak detection and binning (peak clustering) steps; SNR=6 threshold was applied to extract relevant peaks from MS spectra. Hypothetical identification of selected lipid species was performed based on annotation of masses of spectral components at the Human Plasma Standard Reference Material (SRM1950) lipid database [22], available at http://www.lipidmaps.org/data/results/nist/index.html; potential candidates were filtered based on their biological relevance (i.e., previous detection in human blood) and assuming mass tolerance below 0.5 Da. Estimation of statistical significance of differences between consecutive time points was performed for each spectral component using the R statistical software (http://www.R-project.org/). The Wilcoxon signed rank test was used for verification whether observed differences in abundances were significant (the null hypothesis was that the median value of intensities in the differential spectrum is equal to zero); p-values were corrected for multiple testing using the Storey's approach (q-value). Moreover, since the overall changes in lipidome profiles were under consideration the number of significantly changed components ($p<0.05$) was accompanied by the false discovery rate (FDR).

Results

Mass profiles of the lipophilic fraction of serum extracted with organic solvents were analyzed using Matrix-Assisted Laser-Desorption Ionization (MALDI) mass spectrometry in the positive ion mode. These conditions favor ionization and detection of "neutral" zwitterionic phospholipids, such as (lyso) phosphatidylcholines (PCs/LPCs), (lyso)phospatidylethanolamines (PE/LPE) or sphingomyelins (SM) [23].

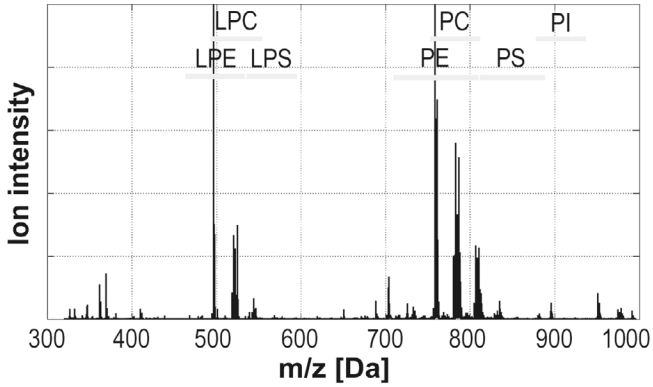

Figure 2. Typical MALDI-ToF mass profile of the lipid fraction of serum. Marked is position of spectral peaks corresponding to major classes of phospholipids along a representative spectrum: LPC/PC – (lyso)phosphatidylcholines, LPE/PE – (lyso)phosphatidylethanolamines, LPS/PS – (lyso)phosphatidylserines, PI – phosphatidylinositols

About 240 spectral components were detected in the 350-900 Da range of resulting spectrograms, including components of their isotope envelopes (representative mass profile is presented on Figure 2).

In order to find RT-related changes individual differential spectra were computed paired with respect to consecutive time points (i.e. changes AΔB, BΔC and AΔC), and then the statistical significance of differences in component's abundances was estimated. This is noteworthy that using this experimental design all RT-induced changes could be related directly to individual pre-RT reference samples, hence confounding effects associated with a type and stage of malignancy could be omitted.

change/patient group		PC / RT_1	PC / RT_2	HNSCC
AΔB	q < 0.05	0	0	13
	p < 0.05	6 [100%]	14 [74%]	46 [18%]
BΔC	q < 0.05	0	0	12
	p < 0.05	1 [100%]	14 [94%]	37 [31%]
AΔC	q < 0.05	0	0	31
	p < 0.05	1 [100%]	13 [94%]	57 [17%]
total	q < 0.05	0	0	48
	p < 0.05	7 [100%]	30 [39%]	85 [12%]

Table 2. Number of serum components, which levels changed significantly between analyzed time points in compared groups of patients. Showed are differences whose statistical significance reached the 0.05 threshold for q-value or p-value (with associated FDR value in square parentheses); total number of changes corresponded to components, where significant change was detected for at least one of three steps AΔB, BΔC or AΔC.

Several spectral components changed their abundances significantly between compared time points ($p<0.05$ and $q<0.05$ were selected as statistical significance thresholds), which numbers are shown in Table 2.

Different numbers of spectral components (i.e. lipid species), whose abundances were affected by RT, were detected in three groups of patients that were compared (i.e., PC/RT_1, PC/RT_2 and HNSCC). The highest number of RT-affected components was detected in serum of patients with HNSCC (only in this group the statistical significance of several differences reached the $q<0.05$ threshold). Moreover, when two groups of PC patients were compared a number of RT-affected components was higher in a group of patients, whose treatment plans included irradiation of the pelvic lymph nodes (i.e. group where larger volumes of normal tissue were irradiated with low and medium doses – PC/RT_2; see Figure 1); however, in neither group statistical significance of observed differences reached the $q<0.05$ threshold.

In the next step we looked for specific modality and time-dependence of RT-induced changes in abundance of detected spectral components (Table 3).

Abundances of corresponding components followed either downregulation/upregulation (d/u) or upregulation/downregulation (u/d) mode of changes, which modes were represented by 85%, 60% and 50% of RT-affected components in PC/RT_1, PC/RT_2 and HNSCC groups, respectively (when $p<0.05$ was selected as a significance threshold). Nevertheless, several RT-induced changes could be still detected about one month after completion of the treatment (i.e. the AΔC change). This was noted in the case of 1, 14 and 37 RT-affected components in PC/RT_1, PC/RT_2 and HNSCC groups, respectively ($p<0.05$). Importantly, even though the extent of RT-induced effects was apparently higher in group of HNSCC patients than in groups of PC patients, a mode of changes was similar for about 2/3 of particular components (i.e. a specific component upregulated in HNSCC patients was likely upregulated in PC patients). Moreover, 48 spectral components whose RT-induced changes reached the threshold of a high statistical significance ($q<0.05$) in the HNSCC patient group were listed in Table 3. These components were putatively annotated as sphingolipids (11 components), phosphatidylcholines (7 components),

mode of changes /patient group	PC/RT_1	PC/RT_2	HNSCC	HNSCC* / m/z component [Da]	
d / d	0	5	16	11	481.7; 482.5; 483.4; 494,4; 496.5; 497.5; 498.5; 500.1; 524.5; 525.5; 679.6
d / u	1	12	13	7	526.5; 734.8; 744.7; 786.8; 787.8; 788.8; 789.8
u / d	5	6	29	16	446.3; 448.4; 675.7; 696.6; 702.7; 718.7; 721.7; 731.7; 806.7; 807.7; 834.8; 835.8; 836.8; 837.8; 874.8; 898.7
u / u	1	7	27	14	673.7; 677.9; 678.9; 717.7; 719.7; 720.7; 725.7; 726.7; 727.7; 729.7; 742.7; 743.7; 746.7; 747.7

Table 3. Number of spectral components representing different modes of RT-induced changes. Represented are components, whose changes in abundances reached the level of statistical significance ($p<0.05$ or $q<0.05$*) for at least one of three steps AΔB, BΔC or AΔC; d – downregulation, u – upregulation for subsequent changes AΔB and BΔC.

We found that the majority of RT-induced changes noted during the first stage of treatment, when about 30-50% of proscribed dose was delivered (i.e. the AΔB change), were somehow reversed or compensated by changes noted during one month follow-up after completion of the treatment (i.e. the BΔC change).

lysophosphatidylcholines (4 components) and phospatidylethanolamines (4 components). Examples of lipid components affected by RT in analyzed groups of patients are presented in Figure 3; which shows changes in abundances of two lipophilic species: m/z=788.7 Da (the u/d mode; putatively phosphatidylcholine PC[36:1]) and m/z=806.6 (the d/u mode; putatively phosphatidylcholine PC[38:6])

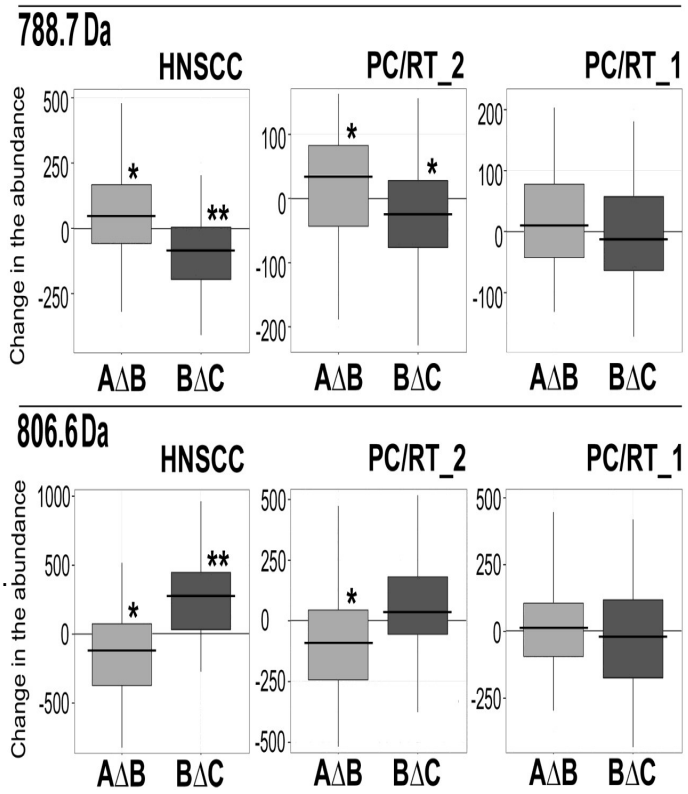

Figure 3. Examples of serum lipid components affected by RT in analyzed groups of patients. Presented are differences in abundances between consecutive time points (A-B and B-C) for components representing two modes of RT-induced changes: upregulation/downregulation and downregulation/upregulation (788.7 Da and 806.6 Da, respectively). Boxplots show: minimum, lower quartile, median, upper quartile and maximum values (outliers were removed from the plots); statistically significant changes are marked with asterisks ($p<0.05$) or doubled asterisks ($q<0.05$).

Discussion

In this study we observed that mass profiles of the lipophilic fraction of serum were affected in cancer patients exposed to local body irradiation during IMRT, yet the extent and frequency of detected changes was apparently different between groups of patients. Results of our previous studies focused on HNSCC patients revealed the correlation between intensity of acute radiation toxicity and RT-induced effects detected at the level of serum peptidome [19] or serum lipidome [26]. Here we hypothesized that differences observed at the level of serum lipidome between groups of patients undergoing IMRT mirrored differences in type and intensity of acute radiation toxicity induced by the treatment. In general, high grade of transient AMR resulting from radiation-mediated damage of oral mucosa is characteristic for HNSCC patients subjected to continuous accelerated RT [14,29]. It is also noteworthy that escalation of RT-induced AMR is frequently associated with inflammation and acute phase response [10,11], which apparently affected molecular composition of blood. In marked contrast, RT is well tolerated in PC patients and relatively low radiation toxicity in usually observed in patients undergoing properly planned and executed treatment [6,7,15]. Data on radiation toxicity assessed in analyzed groups of patient was coherent with literature data: 75% of HNSCC patients showed grade 3 of mucosal toxicity (which corresponded to a confluent mucositis), while only a few PC patients showed grade 3 of genitourinary toxicity (observed gastrointestinal toxicity was even lower in analyzed groups of PC patients; see Table 1). Although toxicity grade observed in HNSCC patients and PC patients cannot be compared directly, one should assume lower overall acute toxicity in the latter group. Moreover, higher radiation toxicity was observed in group of PC patients, whose treatment plans included irradiation of the pelvic lymph nodes (i.e. the PC/RT_2 group); see Table 1. Hence, we concluded that the extent of RT-related changes detected at the level of serum lipidome was associated with both type and intensity of acute radiation toxicity induced by the treatment. It is noteworthy that strong effect of acute radiation toxicity induced by RT in group of HNSCC patients were previously observed at the level of serum proteome, while comparable effects observed in group of patients treated because of PC were much weaker [21,30]. It should be emphasized that similar estimated doses of radiation (2.5-3.5 Gy) were putatively absorbed during the overall treatment by whole blood of patients in both HNSCC and PC/RT_2 groups. Therefore, differences observed between patients treated with RT because of head & neck cancer and prostate cancer could by attributed primarily to specific response of involved tissues and organs damaged by radiation. Moreover, RT-induced changes observed at the level of serum lipidome might be associated with a different tumor response to the treatment, yet data on efficacy of RT were not available for compared groups of patients.

The correlation between volume of tissue irradiated with low-to-medium doses and intensity of changes induced by RT in serum lipidome and peptidome was previously reported for group of HNSCC patients treated with IMRT [26,19]. Here we compared two groups of PC patients, whose treatment plans included large difference in volume of normal tissue irradiated with low and medium doses: volumes exposed to doses below 50 Gy were 2-3-fold larger in group with irradiated pelvic lymph nodes (PC/RT_2 group); see Figure 1. Interestingly, the extent of changes induced by RT in the serum lipidome profile was higher in the PC/RT_2 group than in the PC/RT_1 group (Table 2 and 3). However, intensity of acute radiation reactions, especially gastrointestinal toxicity, was also higher in the PC/RT_2 group than in the PC/RT_1 group (Table 1), which suggested that higher toxicity associated with larger volume of irradiated tissue might be the key factor. This possibility was strengthened when effects induced in HNSCC patients and PC patients were compared. Dose-volume histograms were rather comparable between group of HNSCC patients and group

of PC patients without irradiated pelvic lymph nodes (the PC/RT_1 group, see Figure 1), yet RT-induced changes in serum lipidome were apparently stronger in the former group of patients. Similarly, in spite of markedly larger volume of tissue irradiated with low/medium doses in the PC/RT_2 group, the extent of RT-induced changes in serum lipidome was smaller than in the HNSCC group, which further confirmed an essential role of RT-related toxicity.

Conclusion

One could assume that RT-induced changes observed at the level of serum lipidome reflected general response of patient's body to radiation, including inflammation and acute phase response. Here we concluded that type of acute radiation toxicity associated with specific features of irradiated tissue might be the primary factor determining the extent of such molecular response. On the other hand, volume of normal tissue irradiated with low-to-medium doses could have secondary effect, most likely associated with probability of acute radiation toxicity. Nevertheless, presented data indicated that risk and extent of acute radiation toxicity could be mirrored in a molecular profile of the lipid fraction of serum of cancer patients undergoing IMRT.

Acknowledgments

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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