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Communication

Ionizing radiation affects profile of serum metabolites: increased level of 3-hydroxybutyric acid in serum of cancer patients treated with radiotherapy

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Radiotherapy causes molecular changes observed at the level of body fluids, which are potential biomarker candidates for assessment of radiation exposure. Here we analyzed radiotherapy-induced changes in a profile of small metabolites detected in sera of head and neck cancer patients using the gas chromatography coupled with mass spectrometry approach. There were about 20 compounds, including carboxylic acids, sugars, amines and amino acids, whose levels significantly differed between pre-treatment and post-treatment samples. Among metabolites upregulated by radiotherapy there was 3-hydroxybutyric acid, whose level increased about three times in post-treatment samples. Moreover, compounds affected by irradiation were associated with several metabolic pathways, including protein biosynthesis and amino acid metabolism.

Key words: head and neck cancer; mass spectrometry; metabolomics; radiation response; serum metabolome

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Abbreviations: βHB, β-hydroxybutyrate; GC/MS, gas chromatography coupled with mass spectrometry; HNC, head and neck cancer; NMR, nuclear magnetic resonance; RT, radiotherapy

INTRODUCTION

Radiotherapy is an effective treatment of different types of cancer, which offers the possibility to preserve the structure and function of a target organ. However, successful radiotherapy of patients with advanced cancer usually requires aggressive methods of treatment that include accelerated schemes of fractionation or a combination of radiotherapy with different types of chemotherapy. Irradiation affects not only tumor but also damages normal tissue adjacent to the cancer target, which leads to different types of acute and/or late injury reactions. In extreme cases radiation injury reactions might significantly affect patient's comfort and effectiveness of the treatment. Hence, planning and monitoring of radiotherapy would be greatly facilitated if molecular markers of individual response to radiation were available in the clinical practice (Bentzen, 2008; Alsner *et al*., 2008). Moreover, molecular markers of exposure to ionizing radiation would have great applicability to exposure assessment after radiation accidents and for estimation of occupational or environmental exposure in epidemiological studies (Horneck, 1998; Amundson *et al*., 2003).

The overall response of a patient's organism to local body irradiation and other "pathological" conditions is mirrored in molecular composition of body fluids (e.g., blood or urine). There are several reports evidencing RT-related changes in serum proteome of cancer patients (Marchetti *et al*., 2006; Menard *et al*., 2006), including patients treated because of head and neck cancer (Widłak *et al*., 2013; Widłak *et al*., 2015). Metabolome is another molecular component of blood that is potentially affected upon exposure to radiation. In recent years monitoring of cancer-related metabolites in blood is an emerging approach to detection and diagnosis of different malignancies (Spratlin *et al*., 2009). However, there are relatively few studies addressing radiation-related changes in metabolome of body fluids. Among them there are works reporting changes in a profile of metabolites in urine of irradiated mice (Goudarzi *et al*., 2014) and rats (Mak *et al*., 2015). Moreover, changes in abundance of different phospholipids were detected in blood of irradiated rodents (Wang *et al*., 2009; Laiakis *et al*., 2014a). However, there have been only a few studies addressing the metabolome of humans exposed to radiation. These include reports showing radiation-induced changes in profile of metabolites in red blood cells (Patel *et al*., 2015) and in urine (Laiakis *et al*., 2014b). Moreover, changes induced by RT in fraction of serum phospholipids in patients treated because of HNC was reported (Jelonek *et al*., 2014). Most recently, RT-induced changes in small molecule metabolites were analyzed in serum by NMR spectroscopy in a similar group of patients, and differences associated with radiation toxicity were observed (Boguszewicz *et al*., 2016). Nevertheless, radiation-related changes in small metabolite component of blood is a relatively under-researched topic, despite the fact that this molecular component of a human body is a valuable resource of potential biomarkers. Here we aim to characterize the influence of local body irradiation on a profile of metabolites in serum of cancer patients, where the metabolome is analyzed using GC/MS approach in samples collected before and directly after the end of RT.

MATERIALS AND METHODS

Characteristics of the patient group. Twenty patients with head and neck squamous cell cancer were enrolled into this study; all participants were Caucasians

(14 men), 44–76 years old (median 59 years), mostly current smokers (85%) and alcohol consumers (80%). Cancer was located mostly in larynx (6), oropharynx (5) and hypopharynx (4); the primary tumor stage was: T2 (45%), T3 (50%) and T4 (5%), lymph node status: N0 (40%), N1 (25%), N2 (35%), all patients without distant metastases (M0). All patients were treated with radical radiotherapy alone (no surgery or chemotherapy was applied before RT) using continuous accelerated irradiation scheme (Skladowski *et al*., 2006) with 1.8 Gy daily dose fractions in the 7-day-a-week scheme to the total dose of 68.4–72 Gy (median 72 Gy); the overall treatment time was 36-42 days (median 39 days). The study was approved by the appropriate Ethics Committee and all participants provided informed consent indicating their conscious and voluntary participation.

Material collection. Two consecutive blood samples were collected from each patient: pre-treatment sample A (within one week before the start of RT) and post-treatment sample B (at the last day of the treatment). Samples (5 ml) collected into BD Vacutainer Tubes were incubated for 30 min. at room temperature then centrifuged at $1000 \times g$ for 10 min. to remove clots; the resulting sera were portioned and stored at –70°C.

Extraction of metabolites. 25 µl of serum was added to 200 µl of a mixture of MeOH:H_2O (1:1, v/v), vortexed for 20 min. and centrifuged for 10 min. at 14000 rpm, and then the supernatant 1 was collected in a new tube. The pellet was re-suspended in 200 µl of a mixture of CH_2Cl_2:MeOH (3:1 v/v). The mixture was placed in an ultrasonic bath for 5 min, then vortexed for 10 min and centrifuged for 10 min. at 14000 rpm, and finally supernatant 2 was collected in a new tube. Both supernatant fractions were combined and evaporated in a vacuum concentrator.

GC/MS analysis. Dried extracts were derivatized directly before GC/MS. Each sample was mixed with 25 µl of methoxyamine hydrochloride in pyridine (20 mg/ml) and vortexed (950 rpm) for 90 min. at 37°C, then 80 µl of N-methyl-N-trimethylsilyltrifluoroacetamide was added to the mixture and vortexed (950 rpm) for 30 min at 37°C. The GC/MS analysis was performed with Agilent 7890A gas chromatograph (Agilent Technologies) coupled with Pegasus 4D GCxGC-TOFMS mass spectrometer (Leco). Compounds were separated using DB-5 bonded-phase fused-silica capillary column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) (J&W Scientific Co); GC oven temperature program was as follows: 2 min at 70°C, raised by 8°C/min to 300°C and held for 16 min at 300°C (the total time of GC analysis was 46.75 min). Helium was used as the carrier gas at a flow rate of 1 ml/min. One microliter of each sample was injected in splitless mode. The initial injector temperature was 20°C for 0.1 min., then the temperature was raised to 350°C at 600°C/min. rate. The septum purge flow rate was 3 ml/min. and the purge was turned on after 60 s. The transfer line and ion source temperature was set to 250°C. In-source fragmentation was performed with 70 eV energy.

Analysis of spectra. Mass spectra were recorded in the mass range of 35-650 m/z. All spectra were subjected to automatic peak detection, deconvolution, retention index calculation and library search by Leco ChromaTOF-GC software (v4.51.6.0). The alkane series mixture (C-10 to C-36) was used to correct the retention time (Rt) and to determine the retention index (RI) for each compound. Automated identification of metabolites was based on the Replib, Mainlib and Fiehn libraries; the quality threshold was set for similarity index (SI) above 700 and retention index ± 10. The unique quantification masses were specified for each component and the samples were reprocessed in order to obtain accurate peak areas for the deconvoluted components. The obtained profiles were normalized against the sum of chromatographic peak area (using the TIC approach). All peaks that were identified as artifacts (column bleed, alkanes, plasticizer, derivatization reagents) and peaks corresponding to non-identified compounds were excluded from further analysis.

Statistical analysis. Quantitative analysis was performed following the log transformation of data and the missing data imputation using the k-nearest-neighbour approach with the standardized Euclidean metric on a per-group basis (only metabolites present in at least 60% of samples in each set were used in further analyses). To asses significance of differences between pre- and post-treatment samples the paired *t*-test was employed (the Benjamini-Hochberg approach was applied to the *p*-values for multiple testing correction).

Bioinformatic analysis. Metabolomic pathways were identified using the Metabolite Set Enrichment Analysis (available at http://www.msea.ca/MSEA/faces/Home.jsp); statistical significance of the resulting over-representation was estimated using the hypergeometric test.

RESULTS AND DISCUSSION

The GC/MS approach, a standard analytical tool in metabolomics study (Spratlin *et al*., 2009), was used to characterize the profile of metabolites in human serum. Patients irradiated locally because of HNC were selected, since independent RT could be proscribed in this group, thus confounding effects of a combined treatment with chemotherapy, (anti)hormone treatment or surgery could be excluded. Moreover, the enrolled group was very homogenous regarding the treatment plans, total doses of radiation and volumes of irradiated tissues. Furthermore, similar intensity of acute mucosal reaction was observed in the majority of patients (transient toxicity with grade 3 according to the RTOG/EORTC scale was observed in 80% of patients). Hence, in spite of relatively low number of patients statistically significant effects of the radiation exposure *per se* could be revealed.

In general, there were 166 unique metabolites identified in the analyzed samples. Among them there were 102 compounds detected and quantified in the vast majority of both pre-RT and post-RT samples (samples A and B, respectively). Individual AΔB differences in abun-

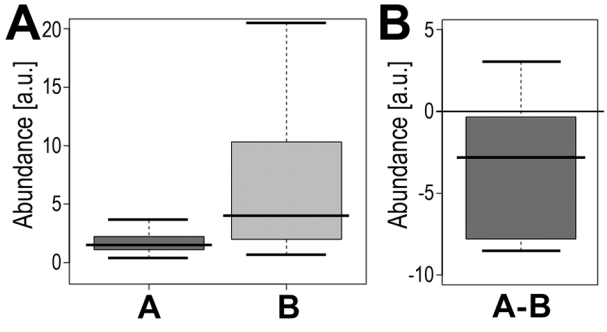

Figure 1. Serum level of 3-hydroxybutyric acid.
Panel A, Abundance in pre-RT and post-RT samples (A and B, respectively). **Panel B**, Individual differences of metabolite level in pre-RT and post-RT samples (A–B). Boxplots show minimum, lower quartile, median, upper quartile and maximum values.

Table 1. Compounds with increased abundance in post-RT samples

Metabolite	Type of compound	Fold change (median B/A)	A (pre-RT)		B (post-RT)		AΔB	
			mean value (a.u.)	C.V.	mean value (a.u.)	C.V.	Difference (median A-B)	*p*-value
3-Hydroxy-butyric acid	carboxylic acid	3.07	5.99	0.63	18.40	1.97	-2.78	0.050
Glycolic acid	carboxylic acid	2.99	4.61	0.46	1.05	0.84	-0.64	0.003
Cadaverine	(poly)amine	2.84	1.40	1.06	3.16	0.86	-1.74	0.020
Inositol	sugar	2.42	0.41	0.86	0.67	0.75	-0.38	0.021
Pyrimidine	heterocyclic compound	2.42	0.30	0.88	0.30	0.56	-0.13	0.014
gamma-Aminobutyric acid	amino acid	2.17	2.83	1.03	5.55	0.77	-2.63	0.031
Putrescine	(poly)amine	2.08	9.84	0.98	3.29	0.89	-1.62	0.036
D-Mannose	sugar	2.00	1.77	0.62	2.39	0.64	-1.00	0.012
Sebacic acid	carboxylic acid	1.79	0.60	0.73	0.83	0.85	-0.18	0.040
Pregn-4-ene-3.11.20--trione 3.20-bis (O-methyloxime)	polycyclic compound	1.66	0.63	0.67	0.66	0.78	-0.20	0.041
Phenylethylamine	amine	1.44	1.27	0.62	2.36	0.98	-0.59	0.050
Succinic acid	carboxylic acid	1.24	1.00	0.29	0.85	0.62	-0.15	0.039
Cystathionine	amino acid	a)	1/20*		12/20*			

a) compound quantified only in post-RT samples; showed is the number of A and B samples where the compound was detected (*)

dance of these metabolites were assessed, which revealed 18 compounds that showed statistically significant difference between individual pre-RT and post-RT samples ($p \leq 0.05$). We found 12 compounds with abundances increased in post-RT samples (fold change 1.24 to 3.07). On the other hand, there were 6 compounds with abundances decreased in post-RT samples (fold change 0.71 to 0.54). 3-Hydroxybutyric acid was the most upregulated metabolite; its level increased three times in post-RT samples (Fig. 1). Moreover, several compounds could be quantified only in one group of samples (i.e., their levels were below the detection limit in the majority of samples from the other group). There were 3 metabolites detected in the majority of pre-RT samples ($\geq 60\%$) and in very few post-RT samples ($\leq 20\%$); these metabolites could be considered as RT-downregulated. On the other hand, one metabolite (namely cystathionine) was detected in the majority of post-RT samples and in very few pre-RT samples, and could be considered as RT-upregulated. Furthermore, we analyzed inter-individual variability in the levels of the detected metabolites. In general, variation in abundances of the detected compounds was similar in both pre-RT and post-RT samples (the mean coefficient of variation was 0.60 and 0.78 in pre-RT and post-RT samples, respectively). It is noteworthy, however, that for the most radiation-upregulated compound (i.e., 3-hydroxybutiric acid) the treatment markedly increased the individual variability (coefficient of variation equal 0.63 and 1.97 in pre-RT and post-RT samples, respectively).

Table 2. Compounds with decreased abundance in post-RT samples

Metabolite	Type of compound	Fold change (median B/A)	A (pre-RT)		B (post-RT)		AΔB	
			mean value (a.u.)	C.V.	mean value (a.u.)	C.V.	Difference (median A-B)	*p*-value
Taurine	other	0.54	0.77	0.69	0.40	0.46	0.30	0.009
Creatinine	heterocyclic compound	0.59	1.01	0.52	0.61	0.51	0.36	0.009
D-Turanose	sugar	0.61	0.15	0.54	0.08	0.40	0.05	0.005
beta-Hydroxypyruvic acid	carboxylic acid	0.66	14.63	0.38	9.28	0.70	5.08	0.013
L-Alanine	amino acid	0.67	101.90	0.36	77.94	0.61	41.49	0.012
2-Deoxy-erythro-pentonic acid	sugar acid	0.71	5.07	0.42	3.84	0.62	1.59	0.025
Dimethylglycine	amino acid	b)	18/20*		3/20*			
Uridine	nucleoside	b)	14/20*		4/20*			
Glycerol-2-O-galacto-pyranoside	other	b)	13/20*		3/20*			

b) compound quantified only in pre-RT samples; showed is the number of A and B samples where the compound was detected (*)

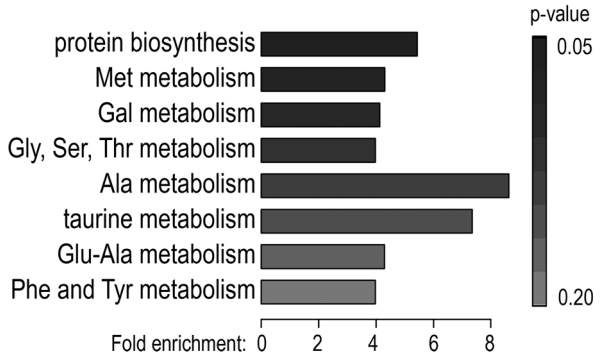

Figure 2. Metabolite sets enrichment overview of compounds affected in post-RT samples.
Showed are relative over-representation (fold enrichment) of pathways associated with RT-affected metabolites and its statistical significance (only pathways showing fold enrichment >4 are presented).

The complete lists of metabolites, whose serum levels were either increased or decreased in post-RT samples are presented in Table 1 and Table 2, respectively. There were differeSnt classes of metabolites represented among the compounds whose abundances in serum were affected after local body irradiation, which included carboxylic acids, sugars, biogenic amines and heterocyclic compounds. To reveal systemic information about the potential functional importance of these changes the identified compounds were annotated to metabolic pathways using the Metabolite Set Enrichment Analysis (Fig. 2). This type of analysis allowed to identify "over-represented" pathways associated with metabolites affected by RT (i.e., pathways associated with types of compounds that were more numerous than expected by chance). It is noteworthy that the major pathways associated with compounds upregulated in post-RT samples included methionine metabolism and galactose metabolism. On the other hand, the major pathways associated with compounds down-regulated in post-RT samples included glycine, serine, threonine and alanine metabolism as well as taurine metabolism. Hence, we concluded that among metabolic pathways primarily affected by radiotherapy were those related to biosynthesis of biogenic amines, amino acids and proteins.

We showed here that radiotherapy caused a significant increase in the serum level of 3-hydroxybutyric acid. The elevated level of this compound was observed in post-RT samples also in an NMR-based study (Boguszewicz *et al*., 2016; personal communication). 3-hydroxybutyric acid (also known as β-hydroxybutyric acid or β-hydroxybutyrate, βHB) is a ketone body generated during fasting. However, 3-hydroxybutyric acid is also involved in the extracellular signaling via specific receptors and is an endogenous inhibitor of histone deacetylases (Newman & Verdin, 2014). It has been recently showed that 3-hydroxybutyric acid could increase resistance to oxidative stress via upregulation of antioxidant enzymes mediated by histone acetylation (Shimazu *et al*., 2013). Moreover, 3-hydoxybutyrate dehydrogenase, which catabolizes 3-hydroxybutyric acid to acetoacetate, can be damaged and inactivated by ionizing radiation (McIntyre *et al*., 1983). Hence, RT-induced upregulation of 3-hydroxybutyric acid seems to be an important element of response to oxidative stress related to irradiation. It is noteworthy, however, that large interpersonal differences were observed in post-treatment levels of this compound. Thus, even though 3-hydroxybutyric acid seems to be an important component of molecular signature of radiation response, its potential applicability in assessment of radiation exposure has to be verified in further studies.

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