Received: April 6, 2022
Accepted: May 6, 2022

Acad Med J 2022;2(1):82-98
UDC:618.19-006.6-085.849:575.117
DOI: 10.53582/AMJ2221082t
Original article

ASSOCIATION OF CCNG1 AND FDXR GENE EXPRESSION DURING RADIOTHERAPY WITH BIOCHEMICAL- AND LIFESTYLE-RELATED CONFOUNDING FACTORS IN BREAST CANCER PATIENTS

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Abstract

Introduction: CCNG1 and FDXR are well-established gene expression biomarkers of IR exposure. Expression alterations during radiotherapy (RT) in patients with different types of cancer has seldom been investigated, along with their potential associations with biochemical- and lifestyle-related confounding factors, that would help elucidate specific changes in RT response and individualize RT in breast cancer patients.

Materials and methods: A non-randomized, controlled, open-trial clinical study was performed in 57 breast cancer patients (intervention group, IG) and 56 healthy individuals (control group, CG). Gene expression was analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR) in leukocytes of peripheral blood samples.

Results: A significant up-regulation of FDXR was observed up to 48 h after the first RT fraction, with no significant expression alterations of CCNG1 at 24 h and 48 h. Fold changes of CCNG1 were slightly lower (1.13-1.23) compared to FDXR (1.49-2.08). RT-induced FDXR and CCNG1 expression alterations could not be significantly associated with patient age, increased WBC count (> $9x10^9$/L), increased C-reactive protein (CRP) during RT (> 5 mg/L) and decrease of increased CRP values during RT. Patients with diabetes mellitus had lower CCNG1 fold changes 24 h post-RT (0.89 ± 0.3 *vs*. 1.23 ± 0.6); identical was the finding for smokers and non-smokers (1.06 ± 0.5 *vs*. 1.22 ± 0.7).

Conclusion: RT-induced CCNG1 and FDXR changes could not be significantly associated with the examined biochemical- and lifestyle confounding factors, except for diabetes mellitus and smoking.

Keywords: radiotherapy, confounding factors, breast cancer, FDXR, CCNG1, gene expression

Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide, with 2.26 million cases in 2020 (11.7%) and is the fifth cause of cancer-related death with 685 000 deaths (6.9%)[1]. In North Macedonia in 2020, there were 988 new cases of breast cancer (59.8 persons per 100,000) and 316 (16.7 per 100,000) breast cancer-caused deaths[2].

Breast cancer is commonly treated by various combinations of surgery, RT, chemotherapy and hormone therapy as the most common therapeutic strategies, depending on the menopausal status of patients, disease stage, primary tumor grade, histological cancer type, estrogen receptor (ER) and progesterone receptor (PR) status and human epidermal growth factor type 2 receptor (HER2) overexpression and/or amplification. According to the ESMO Clinical Practice Guidelines, whole-breast RT is strongly recommended after breast-conserving surgery [I, A][3], as it reduces the 10-year risk of recurrence by 15% and the 15-year risk of breast cancer-related mortality by 4%[4]. In lymph node-positive patients, post-mastectomy RT reduces the 10-year risk of any recurrence by 10% and the 20-year risk of breast cancer-related mortality by 8%[5]. Finally, comprehensive locoregional RT encompasses the chest wall and all lymph nodes. RT doses for local and/or regional adjuvant irradiation have traditionally been 45-50 Gy divided in 25-28 fractions of 1.8-2.0 Gy, with a typical boost dose of 10-16 Gy in 2 Gy single doses[3]. Shorter fractionation schemes (e.g. 15-16 fractions with 2.5-2.67 Gy single dose) have shown similar effectiveness and comparable side effects[6].

The purpose of RT is to eradicate residual disease and reduce local reoccurrence by causing cancer cell death mainly by ionizing radiation (IR)-induced DNA double strand breaks, which trigger cascade of cellular events termed DNA-damage response (DDR), including damage sensing, signal transduction to the effectors of DNA repair, cell cycle arrest and apoptosis induction. Response to IR-induced DNA damage is subject of interindividual variations, possibly related to mutations of key DDR pathway genes or to the individual capacity to modulate the expression of DDR genes after IR exposure[7]. It can be influenced by age, smoking, diabetes, collagen vascular disease and genotype[8].

Identification and quantification of molecular biomarkers of IR is crucial for the follow-up of the progress of RT, as well as in prediction of its outcome early in a treatment regimen[9]. First suggested 22 years ago[10], the relative expression levels of radio-responsive genes in peripheral blood lymphocytes for estimation of radiation exposures is nowadays widely employed as superior, time- and cost-effective approach in biodosimetry, able to predict absorbed radiation dose within hours to days after exposure[11-14]. For the purpose of relevant and accurate analysis, estimation of radiation exposure by identifying and quantifying a group of genes, usually up-regulated under IR, using qRT-PCR platforms, representing the "gene signatures" or "metagenes" of IR is performed[11,15-17]. Most of the identified genes are regulated by p53 (e.g. MDM2, DDB2, FDXR, PCNA, GADD45, RPS27L and SESN1) and are involved in p53-pathways such as cell cycle regulation, DNA damage repair and apoptosis[18]. A combination of FDXR, DDB2 and CCNG is used to determine low doses and FDXR, DDB2 and PHPT1 to determine high doses[19]. Importantly, using the latter three, they obtained a linear fit with a high significance, suggesting good sensitivity and reproducibility[14]. CDKN1A, DDB2, CCNG1 and GADD45A were also reported as the most IR responsive genes[9]. BBC3, FDXR, CDKN1A, PCNA, XPC, GADD45A, DDB2 and POLH were found up-regulated in total body irradiated (TBI) patients; expression was found consistently higher 6 h after irradiation than after 24 h for all dose tested, suggesting its time-dependent decrease[20]. FDXR, AEN, DDB, PHLDA3, GADD45A, ZMAT3 and PCNA were up-regulated and MYC, PFKP and PTGDS were down-regulated after *ex vivo* human whole blood irradiation with three different doses (0.56 Gy, 2.23 Gy and 4.45 Gy)[17].

It is only in the last two decades that IR-induced gene expression alterations have been thoroughly investigated. A great understanding of the transcriptional response in cells to

IR exposure revealed complex alterations in expression of specific genes depending on radiation dose, dose-rate, radiation quality and lapse between stress and analysis[18]. However, the influence of other confounding factors including age, blood parameters and lifestyle habits (food intake, supplements, smoking, exercise etc.) remain largely unknown, except for few studies[21-24]. Assessment of their effects on gene expression of well-established breast cancer biomarkers would reveal valuable knowledge of the sources of all possible gene expression alterations that are not result of IR, which will allow establishment and application of more group- or patient-specific approaches during RT, resulting in better therapy outcomes.

Ferredoxin reductase (FDXR) is recognized as the best stand-alone biomarker of ionizing radiation exposure for assessing universal response to DNA damage. It encodes FDXR, a mitochondrial flavoprotein that initiates electron transport from NADPH for cytochrome P450 (CYP-450) enzymes. Recently, it has been shown that FDXR regulates several iron homeostasis-regulating components, among which iron regulatory protein 2 (IPR2) negatively regulates p53 expression[25]. The gene is regulated by p53 and sensitizes cells to oxidative stress-induced apoptosis. This gene has high sensitivity to radiation exposure, relatively low level of endogenous expression and variability among individuals[13] and very high levels of fold changes in peripheral blood[19]. FDXR has linear, dose-dependent transcriptional up-regulation, both *ex vivo* and *in vivo*[7,13,18,19,23], in one study in ranges 0.15 Gy-3.5 Gy[23]. It is the most accurate genes for providing dose estimates, when compared to GADD45A, CDKN1A, BBC3, BAX, DOB2, GDF15 and TNFSF4[13,26] and even to 247 genes[13].

The cyclin G1 gene (CCNG1) [27] is one of the first genes shown to be overexpressed in breast cancer tissue[28]. It encodes the mitochondrial protein cyclin G1 associated with G2/M phase arrest in response to DNA damage, and also plays a role in promotion of cell growth following damage recovery. Through CCNG1, p53 mediates its role as an inhibitor of cellular proliferation, apoptosis, DNA repair, cell differentiation and angiogenesis. It is also one of the downstream genes of DDR-activated transcription factors that is IR-responsive in whole human blood and peripheral blood lymphocytes irradiated *ex vivo*[18,19,24] and *in vivo*[29]. CCNG1 has linear dose response at low doses (25-100 mGy) *ex vivo* with low interindividual variability[19].

Hypoxanthine phosphoribosyl transferase 1 (HPRT1) encodes for hypoxanthine phosphoribosyl transferase 1, an enzyme catalyzing the conversion of hypoxanthine and guanine to their respective mononucleotides, thereby having an essential role in generation in purine salvage pathway[30]. HPRT1 is a well-established housekeeping gene used for normalization of gene expression data[23,24,29,31] and despite observed low level RNA transcription[31,32] and variability across different organ tissues in cancer and healthy individuals and between healthy individuals and cancer patients[32-34], it is still widely used as an endogenous control gene for transcriptional cancer-related studies.

In this study, we investigated the association between changes in gene expression of FDXR and CCNG1 and housekeeping gene HPRT1 in peripheral blood samples of 57 breast cancer patients and 8 potential confounding factors, including:

1. age,
2. diabetes mellitus as comorbidity,
3. smoking,
4. curcumin supplementation during RT,
5. coenzyme Q10 supplementation during RT,
6. CRP blood levels,
7. decrease of CRP blood levels during RT, and
8. WBC count**.**

Patients were exposed to high doses (2 Gy/fraction) of X-rays during RT at three time points: before beginning of RT (time 0), at 24 h and at 48 h after the first RT fraction. To

examine the cumulative effects, quantified changes in FDXR and CCNG1 expression were compared between the second and the first RT fraction, between the third and the second RT fraction and between the third and the first RT fraction.

Materials and methods

Patients, blood sampling and irradiation

In this non-randomized, controlled open-trial clinical study, the IG consisted of 57 patients (56 female and 1 male), aged 55.3 ± 9.8, diagnosed with breast cancer and referred to elective RT at the University Clinic for Radiotherapy and Oncology in Skopje (Table 1). Forty patients (71.43%) were previously subjected to radical mastectomy with partial or complete dissection of axillary lymph nodes (type 1), 11 (19.64%) to quadrantectomy (type 2), while in the other 5 (8.93%) excision of the breast tumor was performed (type 3). Between surgery and RT, 26 patients were treated with cyclophosphamide/doxorubicin hydrochloride/ paclitaxel according to AP protocol, 18 patients with cyclophosphamide / epirubicine hydrochloride/paclitaxel according to EP protocol and 1 patient with cyclophosphamide/ epirubicine hydrochloride/docetaxel according to ED protocol. Additionally, 53 patients were treated with herceptin (s.c. 600 mg), and 51 patients were under hormone therapy. During RT, 51 patients were supplemented with curcumin and coenzyme Q10. Patients aged under 25 years were excluded from the study; those who were exposed to IR in the last 10 days prior to the beginning of RT during a diagnostic procedure, if they received concomitant RT or chemotherapy and/or were with severe comorbidities. The CG consisted of 56 healthy individuals which were not irradiated. Peripheral blood samples were processed identically for the healthy controls. Average Ct HPRT1, Ct CCNG1 and Ct FDXR in the CG were measured at first (lysate preparation *ex tempore*) and second time point (lysate preparation 24 h after blood sampling).

Table 1. General data of patients (IG) in the clinical study

Variable	n (%)
gender	
male	1 (1.75)
female	56 (98.25)
age	
mean ± SD	55.3 ± 9.8
surgery type	
1	40 (71.43)
2	11 (19.64)
3	5 (8.93)

An informed consent was obtained from each individual and the Ethics Committee for Research with people at the Faculty of Medicine, Ss. Cyril and Methodius University in Skopje, R. North Macedonia, approved investigation with human subjects according to the Code of Ethics of the World Medical Association - Declaration of Helsinki.

During regular laboratory routine control, 2-3 mL of peripheral blood was taken and filled in EDTA-coated vacutainer at three time points:

1) before beginning of RT;

2) 24 h after the first, and before the second fraction of RT and

3) 48 h after the first, and before the third fraction of RT. At each time point, CRP and WBC count were measured for all IG patients.

The adjuvant radiation treatment was carried out on linear accelerator Varian Clinac 23EX (Varian, Palo Alto, CA, USA) with photon energy of 15 MV, which is at the disposal at the University Clinic for Radiotherapy and Oncology in Skopje. The prescribed total tumor

dose for every patient was 50 Gy, divided in 25 fractions (5 weeks x 5 doses). The daily dose of 2 Gy was applied at every 24 h with dose-rate of 400 MU/min. The elective RT after the performed computerized tomography (CT) simulation was conducted with 2 tangential and 1 supra/infraclavicular field of frontal thoracic wall and of regional lymphatic pool with 46 patients and with 2 tangential fields of frontal thoracic wall with 11 patients without supra/infraclavicular radiation.

Total RNA Analysis

Total RNA analysis was performed as described in the previously published paper by Trenceva *et al.*[35].

Processing of biological material for obtaining a sample (lysate of total leukocytes) for RNA isolation

From the obtained 2-3 mL of EDTA-blood samples, a sample (lysate of total leukocytes) for RNA isolation was obtained by selective erythrocyte osmotic lysis with *ex tempore* prepared solution of NH_4Cl and NH_4HCO_3 in deionized water. 300 μL EDTA-blood and 1 mL of this solution was mixed for 10 min at room temperature following a 10-minute centrifugation and discarding of lysed erythrocytes. The remaining liquid mass from total leukocytes was immediately dissolved in 300 μL RLT solution, which contained guanidine thiocyanate. Thus, lysate from total leukocytes was obtained, in which the present endogenous and, possibly exogenous ribonucleases, were inactivated from the strong chaotropic agent guanidine thiocyanate.

Automated isolation of total RNA from lysate of total leukocytes

The isolation procedure of total RNA was made through entirely automated process of Biorobot EZ1 and appropriate EZ1 RNA Tissue Mini Kits (Qiagen, Hilden, Germany) at the Institute for Immunobiology and Human Genetics at the Faculty of Medicine in Skopje. Separation of the total RNA from the remaining cell components of this device are based on the application of magnetic particles in presence of chaotropic salt. The obtained isolate, as well as the lysate from leukocytes, were kept at -80°C until the next step - determination of expression of radioresponsive genes with qRT - PCR method.

Gene expression analysis with qRT-PCR

The level of expression of the radio-responsive genes in peripheral blood from patients ionized with RT was determined by ΔΔCt method, in the Laboratory for molecular pathology, Institute of Pathology, Faculty of Medicine in Skopje. Reverse transcription was performed using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The temperature conditions for the process of reverse transcription were 25°C for 10 min, then 37°C for 120 min and 85°C for 5 min. After that, qPCR was performed on 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers used according to Kabacik *et al.*[18] (Table 2). The temperature conditions for the PCR were as follows: 10 min initial denaturation at 95°C, followed by 40 cycles of 15s denaturation at 95°C and 1 min annealing at 60°C. The following parameters were determined:

1. ΔΔCt (FDXR-HPRT1) and ΔΔCt (CCNG1-HPRT1), i.e., normalized expression of the target gene (FDXR, CCNG1) in relation to the endogen control, HPRT1,
2. $2^{-\Delta\Delta Ct}$ (FDXR-HPRT1) - fold change of the level of expression of the target gene FDXR in relation to HPRT1, and
3. $2^{-\Delta\Delta Ct}$ (CCNG1-HPRT1) - fold change of the level of expression of the target gene CCNG1 in relation to HPRT1.

Table 2. Oligonucleotide primers and probes used for qRT-PCR

Primer	Oligonucleotide sequence 5' to 3'
HPRT1 F	TCAGGCAGTATAATCCAAAGATGGT
HPRT1 R	AGTCTGGCTTATATCCAACACTTCG
HPRT1 Probe	CGCAAGCTTGCTGGTGAAAAGGACCC
FDXR F	GTACAACGGGCTTCCTGAGA
FDXR R	CTCAGGTGGGGTCAGTAGGA
FDXR Probe	CGGGCCACGTCCAGAGCCA
CCNG1 F	GGAGCTGCAGTCTCTGTCAAG
CCNG1 R	TGACATCTAGACTCCTGTTCCAA
CCNG1 Probe	ACTGCTACACCAGCTGAATGCCC

Statistical analysis

The statistical analysis was performed with the statistical program SPSS Statistics software 23.0 (IBM Corporation, Armonk, N.Y., USA). Kolmogorov-Smirnov test was used for testing of normality and distribution of data. Pearson product moment correlation and Spearman rank-order correlation were used to investigate the correlation of the fold change of the level of expression of FDXR and CCNG1 to HPRT1 and each of the investigated biochemical- and lifestyle-related parameters. The statistical significance for all tests was defined at the level of $p<0.05$.

Results

Gene expression profile of HPRT1, FDXR and CCNG1 during RT

The gene expression profile of HRPT1, FDXR and CCNG1 during RT are presented in Table 3[35]. The average expression of HPRT1, CCNG1 and FDXR at the three time points are comparable, with FDXR being slightly higher at all time points. No statistically significant differences were found in expression of the control gene, HPRT1, as expected, but also of CCNG1. Post-hoc comparison in pairs showed no significant differences for HPRT1, but significantly lower values for Ct CCNG1 at the 2nd time point in relation to the 1st ($p=0.009$) and at the 3rd time point in relation to the 1st ($p=0.028$). Finally, statistically significant difference in the threshold cycle for FDXR was observed ($p<0.0001$) in the analyzed period, among all compared time points, therefore, FDXR expression decreased. Min-max ranges were comparable for normalized CCNG1, which was not the case with normalized FDXR, where much wider ranges were observed. The average ΔCt (FDXR-HPRT1) values were significantly different between the defined time points. The fold change of the level of expression of CCNG1 and FDXR to HPRT1 had comparable mean ± SD values for the three variables for both genes, except for $2^{-\Delta\Delta Ct,\ B}$ (FDXR - HPRT1), with higher mean and SD values (2.08 ± 1.7).

Comparison of changes in gene expression levels of FDXR and CCNG1 among the defined time points revealed that for CCNG1, in all three cases, increase in CCNG1 expression levels was registered in ≥61% of patients, while only one third (≥33%) of patients exhibited decreased CCNG1 expression when each time point was compared to the previous one, but without a statistical significance. Similar, but more intense and statistically significant trend was observed for FDXR, where for each compared pair, only 17.54% and 12.28% of patients exhibited decrease in FDXR expression 24 h and 48 h after the first radiation when compared to pre-irradiation expression, respectively. The increase was less observable when comparing FDXR expression 48 h and 24 h after the first radiation, when 28.07% of patients had decreased FDXR expression, and in 71.93% of patients FDXR levels increased 48 h after the first radiation compared to 24 h.

Control group

The average Ct HPRT1, Ct CCNG1 and Ct FDXR at the first and the second time point measured after preparing the lysate either *ex tempore* or 24 h after blood sampling held on 2-8^{0}C had comparably similar mean, minimum and maximum values (Table 3). The average Ct HPRT1, Ct CCNG1 and Ct FDXR at the second time point were significantly different than the average Ct HPRT1, Ct CCNG1 and Ct FDXR at the first time point (Table 4). The mean, median, lower, upper quartile and standard deviation had comparable values for the 1st and the 2nd time point for ΔCt (CCNG1-HPRT1) and ΔCt (FDXR-HPRT1) (Table 5).

Table 3. Descriptive statistics of average Ct HPRT1, Ct CCNG1 and Ct FDXR at 1st and 2nd time point

Variable	Valid N	Mean	Minimum	Maximum	Std. dev.	Std. errror
Average Ct HPRT1 1st time point (*ex tempore* lysate preparation)	56	31.789	30.381	34.698	1.079	0.144
Average Ct HPRT1 2nd time point (lysate preparation 24 h after blood sampling)	56	31.481	30.332	32.568	0.671	0.090
Average Ct CCNG1 1st time point (*ex tempore* lysate preparation)	56	32.155	29.695	35.040	1.178	0.158
Average Ct CCNG1 2nd time point (lysate preparation 24 h after blood sampling)	56	31.825	29.194	33.137	0.938	0.125
Average Ct FDXR 1st time point *(ex tempore* lysate preparation)	56	35.398	32.316	38.973	1.154	0.202
Average Ct FDXR 2nd time point (lysate preparation 24 h after blood sampling)	56	34.885	32.515	37.097	1.194	0.160

Table 4. T-test for dependent samples; results for dependent samples at 2nd time point. df- degree of freedom

Variable	t value	df	p value
Average Ct HPRT1 (lysate preparation 24 h after blood sampling)	2.696	55	0.0093
Average Ct CCNG1 (lysate preparation 24 h after blood sampling)	2.858	55	0.006
Average Ct FDXR (lysate preparation 24 h after blood sampling)	3.603	55	0.0007

Table 5. Descriptive statistics of ΔCt (CCNG1-HPRT1) and ΔCt (FDXR-HPRT1) at 1st and 2nd time point for different lysate preparation methods

Variable	valid N	mean	median	lower quartile	upper quartile	st. dev.
ΔCt (CCNG1 - HPRT1) 1st time point (lysate preparation *ex tempore*)	56	0.367	0.506	0.262	0.732	0.811
ΔCt (CCNG1 - HPRT1) 2nd time point (lysate preparation 24 h after blood sampling)	56	0.344	0.592	0.262	0.856	0.887
ΔCt (FDXR - HPRT1) 1st time point (lysate preparation *ex tempore*)	56	3.609	3.684	3.216	4.005	1.067
ΔCt (FDXR - HPRT1) 2nd time point (lysate preparation 24 h after blood sampling)	56	3.404	3.628	3.024	3.879	0.964

Comparison of ΔCt (CCNG1-HPRT1) and ΔCt (CCNG1-FDXR) at the 1st and the 2nd time point using Wilcoxon matched pairs test (Table 6) revealed no statistically significant differences between normalized expression at the 1st and the 2nd time points for both genes ($p>0.05$).

Table 6. Wilcoxon matched pairs test for ΔCt (CCNG1-HPRT1) and ΔCt (FDXR-HPRT1) at 1st and 2nd time points

Pair of variables	Valid N	T	Z	p-value
ΔCt (CCNG1 - HPRT1) 1st time point (lysate preparation *ex tempore*) and ΔCt (CCNG1 - HPRT1) 2nd time point (lysate preparation 24 h after blood sampling)	56	794.000	0.033	0.973
ΔCt (FDXR – HPRT1) 1st time point (lysate preparation *ex tempore*) and ΔCt (FDXR - HPRT1) 2nd time point (lysate preparation 24 h after blood sampling)	56	606.000	1.566	0.117

Comparison between CG and IG

Comparison of the average Ct HPRT1, Ct CCNG1 and Ct FDXR before RT and 24 h after the first RT fraction between CG and IG (Table 7) revealed statistically significant differences of the average Ct CCNG1 (p (1st point)=0.0012; p (2nd point)=0.002) and Ct FDXR (p (1st point)=0.0001; p(2nd point)=0.0001) between the irradiated breast cancer patients (IG) and the healthy individuals (CG), but not for Ct HPRT1. In all cases, the mean values for average Ct CCNG1 and Ct FDXR were higher in the control group and p variances were <0.000. Higher F ratio variances were observed for threshold cycles of the two genes at the 2nd time point compared to the 1st.

Table 7. Descriptive statistics of CG and IG (N=113) and t-tests

Variable	Patient group	N	mean	st. dev.	t-value	df	p-value	F-ratio variances	p-variances
Average Ct HPRT1 1st time point (lysate preparation *ex tempore*)	CG	56	31.789	1.079	1.675	111	0.097	-	-
	IG	57	31.227	2.269					
Average Ct HPRT1 2nd time point (lysate preparation 24 h after blood sampling)	CG	56	31.481	0.672	1.069	111	0.288	12.397	0.000
	IG	57	31.130	2.366					
Average Ct CCNG1 1st time point (lysate preparation *ex tempore*)	CG	56	32.155	1.178	3.333	111	0.0012	3.526	0.000006
	IG	57	31.041	2.213					
Average Ct CCNG1 2nd time point (lysate preparation 24 h after blood sampling)	CG	56	31.825	0.938	3.141	111	0.002	5.325	0.0000
	IG	57	30.836	2.165					
Average Ct FDXR 1st time point (lysate preparation *ex tempore*)	CG	56	35.398	1.514	4.061	111	0.0001	3.228	0.0000
	IG	57	33.711	2.721					
Average Ct FDXR 2nd time point (lysate preparation 24 h after blood sampling)	CG	56	34.885	1.194	4.142	111	0.0001	5.534	0.000
	IG	57	33.199	2.808					

Comparison of the normalized CCNG1 expression levels (Ct CCNG1-HPRT1) between CG and IG (Table 8) showed higher mean values for Ct (CCNG1-HPRT1) in IG in both, the 1st (mean =0.367) and the 2nd time point (mean =0.344) when compared to Ct (CCNG1-HPRT1) for CG (mean=-0.186 and -0.294, respectively). A Mann-Whitney U test indicated that these differences were statistically significant, $U_{Ct\ (CCNG1\text{-}HPRT1)}$ (N_{CG}=56, N_{IG}=57))= 686.000, z=5.223, p<0.05 and $U_{Ct\ (FDXR\text{-}HPRT1)}$ (N_{CG}=56, N_{IG}=57)=620.000, z=5.602, p<0.05. Identical findings were true for the normalized FDXR expression levels (ΔCt FDXR-HPRT1) between GC and IG, where the mean values were higher at the 1st and the 2nd time point in

Table 8. Descriptive statistics of CG and IG (N=113) for ΔCt (CCNG1-HPRT1) at 1st and 2nd time point in CG and IG and Mann-Whitney U test results (w/ continuity correction)

Variable	Patient group	N	mean	st. dev.	rank sum group 1	rank sum group 2	U	Z	p-value	Z adjusted	2*1 sided exact p
ΔCt (CCNG1-HPRT1) 1st time point (lysate preparation *ex tempore*)	CG	56	0.367	0.811	4102.000	2339.000	686.000	5.223	0.000	5.223	0.000
	IG	57	-0.186	0.611							
ΔCt (CCNG1-HPRT1) 2nd time point (lysate preparation 24 h after blood sampling)	CG	56	0.344	0.887	4168.000	2273.000	620.000	5.602	0.000	5.602	0.000
	IG	57	-0.294	0.611							

CG (mean=3.609 and 3.404, respectively), with comparable variances (Table 9). T-test indicated that such differences were not statistically significant (p=0.171 and p=0.932, respectively).

Table 9. Descriptive statistics of Ct (FDXR-HPRT1) at 1st and 2nd time point for CG and IG and t-test results

Variable	Patient group	N	mean	st. dev.	t-value	p-value	F-ratio variances	p variances
ΔCt (FDXR-HPRT1) 1st time point (lysate preparation *ex tempore*)	CG	56	3.609	1.067	6.106	0.000	1.448	0.171
	IG	57	2.484	0.887				
ΔCt (FDXR-HPRT1) 2nd time point (lysate preparation 24 h after blood sampling)	CG	56	3.404	0.964	7.321	0.000	1.024	0.932
	IG	57	2.069	0.975				

Association of gene expression changes with patient age

Pearson product moment correlation and Spearman rank-order correlation of the fold change of CCNG1 and FDXR expression in relation to HPRT1 to patient age did not reveal any statistically significant correlation (p>0.05) (Table 10). Additionally, no statistically significant difference between gene expression changes and the mean patient age were found (p>0.05) (Table 11). A statistically significant difference (p=0.048) was found between the mean age of patients that had decreased and increased CCNG1 expression levels at 48 h after the first RT fraction, compared to 24 h after the first RT fraction (51.95 ± 10.03 *versus* 57.25 ± 9.30).

Table 10. Correlation of fold changes of normalized CCNG1 and FDXR gene expression changes 24 h after first RT fraction (A), 48 h after first RT fraction (B) and difference between 48 h and 24 h after first RT fraction (BvsA) to patient age

Variable	Spearman R / Pearson R	p value
$2^{-\Delta\Delta Ct, A}$ (CCNG1 - HPRT1)	0.038	0.781
$2^{-\Delta\Delta Ct, B}$ (CCNG1 - HPRT1)	0.144	0.285
$2^{-\Delta\Delta Ct\ (BvsA)}$ (CCNG1-HPRT1)	0.216	0.107
$2^{-\Delta\Delta Ct, A}$ (FDXR - HPRT1)	-0.109	0.416
$2^{-\Delta\Delta Ct, B}$ (FDXR - HPRT1)	0.0201	0.882
$2^{\wedge-\Delta\Delta Ct\ (BvsA)}$ (FDXR-HPRT1)	0.195	0.146

Table 11. Correlation of gene-specific expression changes between two time points related to patient age (years)

Compared time points	Expression change	CCNG1		FDXR	
		patient age (mean ± SD, years)	p value	patient age (mean ± SD, years)	p value
24 h after first RT fraction/before RT	decreased	56.0 ± 8.20	t=0.42 0.673	59.10 ± 8.70	t=1.36 p=0.18
	increased	54.86 ± 10.80		54.49 ± 9.90	
48 h after first RT fraction/before RT	decreased	52.37 ± 8.90	t=1.61 0.11	59.0 ± 8.60	t=1.07 p=0.29
	increased	56.76 ± 10.02		54.78 ± 9.90	
48 h after first RT fraction/ 24 h after first RT fraction	decreased	51.95 ± 10.03	t=2.02	51.62 ± 11.10	t=1.79
	increased	57.25 ± 9.30	0.048	56.73 ± 9.00	p=0.08

Association of gene expression change with diabetes mellitus as comorbidity

Statistically significant differences were observed only in the mean fold change values of CCNG1 expression at 24 h after the first RT fraction between patients with and without presence of diabetes mellitus as comorbidity (p=0.039) (Table 12). Here, higher mean values

Table 12. Association between gene expression change and diabetes mellitus as comorbidity *-Mann Whitney, **paired t-tests

diabetes mellitus	n	mean ± SD	median (IQR) / min - max	p value
$2^{-\Delta\Delta Ct, A}$ (CCNG1 - HPRT1)				
yes	10	0.89 ± 0.3	0.97 (0.79 – 1.09)	2.07*
no	47	1.23 ± 0.6	1.09 (0.94 – 1.36)	0.039
$2^{-\Delta\Delta Ct, B}$ (CCNG1 - HPRT1)				
yes	10	1.03 ± 0.3	0.19 (0.34 – 1.36)	1.48**
no	47	1.28 ± 0.5	0.33 (0.49 – 2.81)	0.14
$2^{-\Delta\Delta Ct\ (BvsA)}$ (CCNG1-HPRT1)				
yes	10	1.13 ± 0.2	1.18 (1.01 – 1.23)	0.64*
no	47	1.13 ± 0.5	1.06 (0.84 – 1.37)	0.52
$2^{-\Delta\Delta Ct, A}$ (FDXR - HPRT1)				
yes	10	1.34 ± 0.5	0.29 (0.54 – 2.02)	0.84**
no	47	1.52 ± 0.6	0.08 (0.61 – 2.91)	0.402
$2^{-\Delta\Delta Ct, B}$ (FDXR - HPRT1)				
yes	10	1.78 ± 0.7	1.74 (1.57 – 2.18)	0.03*
no	47	2.14 ± 1.8	1.63 (1.29 – 2.53)	0.97
$2^{-\Delta\Delta Ct\ (BvsA)}$ (FDXR-HPRT1)				
yes	10	1.36 ± 0.5	1.32 (1.09 – 1.54)	0.05*
no	47	1.59 ± 1.5	1.24 (0.99 – 1.37)	0.96

of $2^{-\Delta\Delta Ct, A}$ were observed in patients (n=47) that did not have diabetes mellitus (1.23 ± 0.6) compared to patients (n=10) with diabetes mellitus (0.89 ± 0.3). Also, a statistically significant difference was observed in CCNG1 expression level changes when comparing the 2nd and the 1st time point (24 h after the first RT fraction to before RT) among patients with and without diabetes mellitus (p=0.025) (Table 13). CCNG1 expression was decreased in 7 patients (70%) with and 15 patients (31.91%) without diabetes mellitus, while it was increased in 3 patients (30%) with and 32 patients (68.09%) without diabetes mellitus.

Table 13. Association of gene expression change with diabetes mellitus as comorbidity using Pearson Chi-squared test

Parameter	Parameter change	diabetes mellitus yes n (%)	diabetes mellitus no n (%)	p value
CCNG1				
Gene expression change 2nd *versus* 1st time point	decreased	7 (70)	15 (31.91)	X^2=5.05
	increased	3 (30)	32 (68.09)	p=0.025
Gene expression change 3rd *versus* 1st time point	decreased	4 (40)	15 (31.91)	X^2=0.24
	increased	6 (60)	32 (68.09)	p=0.62
Gene expression change 3rd *versus* 2nd time point	decreased	2 (20)	19 (40.43)	X^2=1.48
	increased	8 (80)	28 (59.57)	p=0.22
FDXR				
Gene expression change 2nd *versus* 1st time point	decreased	2 (20)	8 (17.02)	X^2=0.05
	increased	8 (80)	39 (82.98)	p=0.82
Gene expression change 3rd *versus* 1st time point	decreased	1 (10)	6 (12.77)	X^2=0.06
	increased	9 (90)	41 (87.23)	p=0.81
Gene expression change 3rd *versus* 2nd time point	decreased	2 (20)	14 (29.79)	X^2=0.39
	increased	8 (80)	33 (70.21)	p=0.53

Association of gene expression changes with smoking

Examination of the correlation of fold change in FDXR and CCNG1 expression 24 h after, 48 h after and between 48 h and 24 h after the first RT fraction with smoking in 51 patients from IG showed statistically significant differences in mean $2^{-\Delta\Delta Ct, A}$ values of CCNG1 between smokers and non-smokers (p=0.039) (Table 14). Higher mean values (1.22 ± 0.7) were observed in non-smoking breast cancer patients compared to smokers (1.06 ± 0.5).

Table 14. Association of gene expression changes with smoking *-Mann-Whitney test, **paired t-test

active smoker	n	mean ± SD	median (IQR) / min - max	p value
$2^{-\Delta\Delta Ct, A}$ (CCNG1 - HPRT1)				
yes	16	1.06 ± 0.5	1.03 (0.85 – 1.19)	2.07*
no	35	1.22 ± 0.7	1.07 (0.94 – 1.29)	0.039
$2^{-\Delta\Delta Ct, B}$ (CCNG1 - HPRT1)				
yes	16	1.14 ± 0.5	0.19 – 2.26	1.48**
no	35	1.28 ± 0.5	0.33 - 2.80	0.14
$2^{-\Delta\Delta Ct (BvsA)}$ (CCNG1-HPRT1)				
yes	16	1.08 ± 0.2	1.12 (0.88 – 1.25)	0.03*
no	35	1.16 ± 0.5	1.12 (0.84 – 1.31)	0.98
$2^{-\Delta\Delta Ct, A}$ (FDXR - HPRT1)				
yes	16	1.65 ± 0.6	0.29 – 2.66	1.34**
no	35	1.42 ± 0.6	0.08 – 2.91	0.19
$2^{-\Delta\Delta Ct, B}$ (FDXR - HPRT1)				
yes	16	2.11 ± 1.1	1.74 (1.48 – 2.67)	1.3*
no	35	2.08 ± 2.0	1.59 (1.13 – 2.18)	0.19
$2^{-\Delta\Delta Ct (BvsA)}$ (FDXR-HPRT1)				
yes	16	1.23 ± 0.3	1.20 (1.04 – 1.38)	0.49*
no	35	1.71 ± 1.7	1.26 (0.99 – 1.67)	0.62

However, the association of gene expression changes (decrease/increase) between time points with active smoking (data not shown) did not reveal any statistically significant differences between smokers (n=16) and non-smokers (n=35) of the examined breast cancer patients (p>0.05).

Association of gene expression changes with curcumin supplementation

According to our results, curcumin supplementation during RT in 51 breast cancer patients could not be significantly associated with fold changes of FDXR and CCNG1 expression 24 h, 48 h and between 48 h and 24 h after the first RT fraction (p>0.05, data not shown) and FDXR and CCNG1 expression changes at 24 h after compared to before RT, at 48 h after compared to 24 h after the first RT fraction and at 48 h after compared to before RT (p>0.05, data not shown).

Association of gene expression change with coenzyme Q10 supplementation

Similar to the curcumin supplementation, coenzyme Q10 supplementation during RT in 51 breast cancer patients could not be neither positively nor negatively associated with FDXR and CCNG1 fold changes (p>0.05, data not shown) and with change in their expression at the 2nd compared to the 1st, 3rd compared to the 1st and the 3rd compared to the 2nd time point (p>0.05, data not shown).

Association between gene expression changes and CRP values and its changes

During RT, CRP values were examined in 53 IG patients. We identified a lack of statistically significant difference between the fold change of FDXR and CCNG1 expression at 24 h, 48 h and comparing 48 h to 24 h after the first RT fraction between irradiated breast cancer patients that had peripheral blood CRP values > 5 mg/L (n=11) and <5 mg/L (n=42) during RT (p>0.05, data not shown), therefore the small observed differences were random. Also, no statistically significant difference was observed at FDXR and CCNG1 expression changes among different time points between patients with peripheral blood CRP values > 5 mg/L and <5 mg/L during RT (p>0.05, data not shown). Finally, no statistically significant difference between fold change of FDXR and CCNG1 expression 24 h, 48 h and between 48 h and 24 h after the first RT fraction among patients whose increased CRP decreased during RT (n=7) and patients whose increased CRP values remained unchanged during RT (n=4) was observed (p>0.05, data not shown).

***Association between gene expression change and WBC* count**

No statistically significant difference was found between the fold change of FDXR and CCNG1 expression at 24 h, 48 h and between 48 h and 24 h after first RT fraction of irradiated breast cancer patients which had WBC count >9 x 10^9/L during RT (n=4) and irradiated breast cancer patients with WBC count <9 X10^9/L during RT (n=53) (p>0.05, data not shown). Also, a lack of significant association was observed between the WBC levels and changes in FDXR and CCNG1 expression when comparing time points (2nd to 1st, 3rd to 2nd, 3rd to 1st) (p>0.05, data not shown), indicating that WBC count during RT in breast cancer patients cannot be associated with FDXR and CCNG1 expression changes.

Discussion

Analysis of gene expression signatures using qRT-PCR in prediction of IR dose and response is a promising method, as it is minimally invasive, fast, high throughput and requires minimal expertise[14,18,19,24,29]. Genes regulated through DDR have been identified as reliable gene expression biomarkers of IR[13]. FDXR and CCNG1 are shown to have strong responsiveness to IR *ex vivo*[19] and *in vivo* in previous studies[24]. Both CCNG1 and FDXR

were selected for this study as genes that can provide clear insight into the IR dose response and the associations between the gene expression levels (changes) and other disease-, treatment- and lifestyle-related confounding factors in breast cancer patients undergoing RT.

The gene expression profiles of CCNG1, FDXR and HPRT1 were analyzed in peripheral blood leukocytes of 57 breast cancer patients treated with RT (IG) and 56 healthy individuals (CG). Data analysis of all IG patients indicated slight up-regulation of CCNG1 and significant up-regulation of FDXR over time. FDXR expression was increased in most of the patients at 24 h and 48 h after the first RT when compared to before RT (82.46% and 87.72%), and for somewhat less patients when expression at 48 h after RT was compared to expression at 24 h after RT (71.93%). It is noteworthy that its expression was shown to be down-regulated in non-human primates (NHP) model[36] unlike in TBI patients, where it was strongly up-regulated[23]. FDXR is a ferredoxin reductase enzyme and its possible association with reactive oxygen species might imply questions regarding its specificity and further studies focused on this topic should be carried out[14].

A potential concern in our study was that the breast cancer patients may have alterations in pre-irradiation gene expression due to their clinical conditions and/or prior treatments that confound radiation signatures. The comparison between healthy individuals (CG) and breast cancer patients (IG) revealed no significant differences among threshold cycle of CCNG1 and FDXR at the 1st and the 2nd time point in terms of mean, median, lower and upper quartile. Both average Ct CCNG1 and average Ct FDXR at both time points were significantly higher in CG than in IG, with low p variances ($p<0.000$). The same was true for the normalized CCNG1 and FDXR values. Such findings of significantly lower basal expression levels for FDXR and CCNG1 in breast cancer patients compared to healthy controls were also confirmed previously, e.g. for CCNG1 in breast cancer patients[44] in a group of head and neck, prostate, breast, lung and endometrial cancer patients[24], while FDXR had similar pre-irradiation exposure levels in cancer (IG) and healthy (CG) patients[23]. Our findings have indicated the potential existence of novel, not thoroughly studied mechanisms of cancer development and DDR that influence basal FDXR and CCNG1 expression in breast cancer patients.

The age of breast cancer patients could not be recognized as an important confounding factor, despite observed significant decrease in CCNG1 expression at 48 h compared to 24 h post- RT in younger patients (51.95 ± 10.03 years) and increase in the older group (57.25 ± 9.30 years). Diabetes mellitus is known to increase breast cancer risk, also causing 17% increased risk of breast cancer mortality in women[38]. To date, there are no studies exploring its influence on gene expression alterations during RT in cancer, and even in breast cancer patients. In our study, there was a statistically significant difference ($p=0.039$) in CCNG1 fold change between patients with and without diabetes mellitus, such that patients with diabetes mellitus had lower CCNG1 fold changes 24 h after the first RT fraction (0.89 ± 0.3) compared to patients without diabetes (1.23 ± 0.6). More than two-thirds of patients without diabetes had an increased CCNG1 gene expression at 24 h after compared to beginning of RT (n=32, 68.09%), than decreased (n=15, 31.91%), while the results were opposite for patients with diabetes mellitus (n=3, 30% versus n=7, 70%).

Animal and *in vitro* human studies have found the polycyclic hydrocarbons, aromatic amines and N-nitrosamines from tobacco smoke to potentially induce breast tumors[39]. Additionally, tobacco smoking is shown to increase risk of recurrence and survival (behavior and progression) of breast cancer by altering the expression of other cancer-associated genes (APOC1, ARID1B, CTNNBL1, MSX1 etc.) but not of FDXR and CCNG1[40]. *Ex vivo* irradiation of peripheral WBC of smokers and non-smokers with 0.1, 0.5 and 2 Gy γ-rays identified 8 radio-responsive genes which expression was significantly affected by smoking status, among which FDXR and CCNG1 were not included[21]. In our study, breast cancer patients

that smoke had significantly lower CCNG1 fold change 24 h after the first RT fraction (1.06 ± 0.5) compared to non-smokers (1.22 ± 0.7), but no other significant associations were observed.

Curcumin is well-known for its anti-inflammatory, free radical scavenging, enzyme modulating, cardioprotective, hepatoprotective, antioxidant and radioprotective properties[41]. The radioprotective activity is exhibited through modulation of NF-kB and Nrf2, reduction of DNA damage and lipid peroxidation and induction of the enzymatic (e.g., catalase, superoxide dismutase, glutathione peroxidase, glutathione S-transferase) and non-enzymatic (glutathione, ascorbic acid) antioxidant defense systems. However, curcumin did not significantly affect the expression pattern of FDXR 2 h and 18 h after 2 Gy and that radioprotection is not a result of enhanced DNA repair[22]. Also, at 24 h after IR, curcumin did not have any regulatory effect on FDXR gene expression alone or combined with IR exposure[23]. However, curcumin counteracted the CCNG1 expression induced by IR 24 h after exposure[24].

Coenzyme Q10 is antioxidant with a crucial role of central cofactor of mitochondrial respiratory electron transport chain to produce cellular ATP, a non-specific simulant for the immune system, in cellular biogenesis and oxidative balance. It protects mitochondrial proteins and membrane phospholipids from free-radicals induced oxidative stress as a powerful radical scavenger and suppresses inflammatory signaling pathways by regulating inflammatory transcription[42]. In breast cancer patients, coenzyme Q10 is associated with tumor regression and increased survival years, as it is responsible for down-regulation of pro-inflammatory cytokines (IL-6, IL-8, TNF-α), VEGF, CRP, impairs expression of matrix metalloproteinases (MMP-2 and MMP-9) and increases serum levels of tissue inhibitor of metalloproteinases (TIMPs)[43]. However, no studies were found that investigate the association of coenzyme Q10 supplementation with RT-induced alterations in gene expression to date. In our study, supplementation with curcumin and coenzyme Q10 could not be significantly associated with any alterations in FDXR and CCNG1 expression during RT. Finally, CRP levels in peripheral blood of our breast cancer patients (below or above 5 mg/L) during RT, the decrease of increased CRP values over the RT course and WBC count in peripheral blood (below or above $9x10^9$/L) could not be associated with observed FDXR and CCNG1 expression alterations during RT.

The findings of our study should be carefully observed, considering the extensive inter-individual variation in transcriptional response to IR exposure[45]. Additionally, although in many cases a statistical significance could not be reached, it should be considered that it may be a result of the limited number of patients involved in our study. Furthermore, recent studies have found a significant increase in HPRT1 expression in most tissues upon malignancy (with the highest average HPRT1 in breast tissue), this was not the case in our study. The increased target gene (CCNG1 and FDXR) expression observed may be more significant than the detected, as gene expression increase may be masked by the concomitant increase in cancer HPRT1 expression[34].

In conclusion, this is the first study investigating the association between two IR exposure biomarkers in human peripheral blood irradiated *in vivo* with 8 potential confounding factors in a single study. Further assessment found that some of the confounding factors can modify FDXR and CCNG1 expression and consequently could affect estimation of dose but to an extent that should not affect their use in monitoring IR exposure. To extend this research, employment of different housekeeping genes should be performed in order to examine result reliability and accuracy. Also, inclusion of time points closer to the beginning of RT, e.g. 2 h, 4 h, 6 h, 12 h after the first RT could provide more beneficial insight into the time- and dose-dependent gene expression.

Conflict of interest statement. None declared.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA *Cancer J Clin* 2021; 71(3): 209-249. doi: 10.3322/caac.21660.
2. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, *et al*. Global Cancer Observatory: Cancer Today. Lyon, France: *International Agency for Research on Cancer* 2020. [cited 2022 Feb 9]. Available from: https://gco.iarc.fr/today.
3. Cardoso F, Kyriakides S, Ohno S, Penault-Llorca F, Poortmans P, Rubio IT, *et al*. Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology* 2019; 30(8): 1194-1220. doi: 10.1093/annonc/mdz173.
4. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effect of radiotherapy after breast-conserving surgery on 10-year recurrence and 15-year breast cancer death: meta-analysis of individual patient data for 10 801 women in 17 randomised trials. *Lancet* 2011; 378(9804): 1707-1716. doi: 10.1016/S0140-6736(11)61629-2.
5. EBCTCG (Early Breast Cancer Trialists' Collaborative Group). Effect of radiotherapy after mastectomy and axillary surgery on 10-year recurrence and 20-year breast cancer mortality: meta-analysis of individual patient data for 8135 women in 22 randomised trials. *Lancet* 2014; 383(9935): 2127-2135. doi: 10.1016/S0140-6736(14)60488-8.
6. Whelan TJ, Pignol JP, Levine MN, Julian JA, MacKenzie R, Parpia S, *et al*. Long-Term Results of Hypofractionated Radiation Therapy for Breast Cancer. *N Engl J Med* 2010; 362: 513-520. doi: 10.1056/NEJMoa0906260.
7. Palumbo E, Piotto C, Calura E, Fasanaro E, Groff E, Busato F, *et al*. Individual Radiosensitivity in Oncological Patients: Linking Adverse Normal Tissue Reactions and Genetic Features. *Front Oncol* 2019; 9: 987. Available from: https://www.frontiersin.org/article/10.3389/fonc.2019.00987/full.
8. Human radiosensitivity: report of the independent advisory group on ionising radiation. London: Health Protection Agency; 2013.
9. Amundson SA, Grace MB, McLeland CB, Epperly MW, Yeager A, Zhan Q, *et al*. Human in vivo Radiation-Induced Biomarkers: Gene Expression Changes in Radiotherapy Patients. *Cancer Res* 2004; 64(18): 6368-6371. doi: 10.1158/0008-5472.CAN-04-1883.
10. Amundson SA, Do KT, Shahab S, Bittner M, Meltzer P, Trent J, *et al*. Identification of Potential mRNA Biomarkers in Peripheral Blood Lymphocytes for Human Exposure to Ionizing Radiation. *Radiat Res* 2000; 154(3): 342-346. doi: 10.1667/0033-7587(2000)154[0342:iopmbi]2.0.co;2.
11. Paul S, Barker CA, Turner HC, McLane A, Wolden SL, Amundson SA. Prediction of In Vivo Radiation Dose Status in Radiotherapy Patients using Ex Vivo and In Vivo Gene Expression Signatures. *Radiat Res* 2011; 175(3): 257-265. doi: 10.1667/RR2420.1.
12. Macaeva E, Mysara M, De Vos WH, Baatout S, Quintens R. Gene expression-based biodosimetry for radiological incidents: assessment of dose and time after radiation exposure. *Int J Radiat Biol* 2019; 95(1): 64-75. doi: 10.1080/09553002.2018.1511926.
13. Abend M, Badie C, Quintens R, Kriehuber R, Manning G, Macaeva E, *et al*. Examining Radiation-Induced In Vivo and In Vitro Gene Expression Changes of the Peripheral Blood in Different Laboratories for Biodosimetry Purposes: First RENEB Gene Expression Study. *Radiation Res* 2016; 185(2): 109-123. doi: 10.1667/RR14221.1.
14. Kultova G, Tichy A, Rehulkova H, Myslivcova-Fucikova A. The hunt for radiation biomarkers: current situation. *Int J Radiat Biol* 2020; 96(3): 370-382. doi: 10.1080/09553002.2020.1704909.

15. Paul S, Amundson SA. Development of gene expression signatures for practical radiation biodosimetry. *Int J Radiat Oncol Biol Phys* 2008; 71(4): 1236-1244. doi: 10.1016/j.ijrobp.2008.03.043.
16. Knops K, Boldt S, Wolkenhauer O, Kriehuber R. Gene Expression in Low- and High-Dose-Irradiated Human Peripheral Blood Lymphocytes: Possible Applications for Biodosimetry. *Radiat Res* 2012; 178(4): 304-312. doi: 10.1667/rr2913.1.
17. Ghandhi SA, Smilenov LB, Elliston CD, Chowdhury M, Amundson SA. Radiation dose-rate effects on gene expression for human biodosimetry. *BMC Med Genomics* 2015; 8(1): 22. Available from: http://bmcmedgenomics.biomedcentral.com/articles/10.1186/s12920-015-0097-x.
18. Kabacik S, Mackay A, Tamber N, Manning G, Finnon P, Paillier F, *et al*. Gene expression following ionising radiation: Identification of biomarkers for dose estimation and prediction of individual response. *Int J Radiat Biol* 2011; 87(2): 115-129. doi: 10.3109/09553002.2010.519424.
19. Manning G, Kabacik S, Finnon P, Bouffler S, Badie C. High and low dose responses of transcriptional biomarkers in ex vivo X-irradiated human blood. *Int J Radiat Biol* 2013; 89(7): 512-522. doi: 10.3109/09553002.2013.769694.
20. Budworth H, Snijders AM, Marchetti F, Mannion B, Bhatnagar S, Kwoh E, *et al*. DNA Repair and Cell Cycle Biomarkers of Radiation Exposure and Inflammation Stress in Human Blood. *PLoS One* 2012; 7(11): e48619. doi: 10.1371/journal.pone.0048619.
21. Paul S, Amundson SA. Gene expression signatures of radiation exposure in peripheral white blood cells of smokers and non-smokers. *Int J Radiat Biol* 2011; 87(8): 791-801. doi: 10.3109/09553002.2011.568574.
22. Srinivasan M, Rajendra Prasad N, Menon VP. Protective effect of curcumin on γ-radiation induced DNA damage and lipid peroxidation in cultured human lymphocytes. *Mutat Res* 2006; 611(1-2): 96-103. doi: 10.1016/j.mrgentox.2006.07.002.
23. O'Brien G, Cruz-Garcia L, Majewski M, Grepl J, Abend M, Port M, *et al*. FDXR is a biomarker of radiation exposure in vivo. *Sci Rep* 2018; 8(1): 684. doi: 10.1038/s41598-017-19043-w.
24. Cruz-Garcia L, O'Brien G, Donovan E, Gothard L, Boyle S, Laval A, *et al*. Influence of confounding factors on radiation dose estimation in in vivo validated transcriptional biomarkers. *Health Phys* 2018; 115(1): 90-101. doi: 10.1097/HP.0000000000000844.
25. Zhang Y, Qian Y, Zhang J, Yan W, Jung Y-S, Chen M, *et al*. Ferredoxin reductase is critical for p53-dependent tumor suppression via iron regulatory protein 2. *Genes Dev* 2017; 31(12): 1243-1256. doi: 10.1101/gad.299388.117.
26. Brzóska K, Kruszewski M. Toward the development of transcriptional biodosimetry for the identification of irradiated individuals and assessment of absorbed radiation dose. *Radiat Environ Biophys* 2015; 54(3): 353-363. doi: 10.1007/s00411-015-0603-8.
27. Kimura SH, Ikawa M, Ito A, Okabe M, Nojima H. Cyclin G1 is involved in G2/M arrest in response to DNA damage and in growth control after damage recovery. *Oncogene* 2001; 20(25): 3290-3300. doi: 10.1038/sj.onc.1204270.
28. Reimer CL, Borras AM, Kurdistani SK, Garreau JR, Chung M, Aaronson SA, *et al*. Altered Regulation of Cyclin G in Human Breast Cancer and Its Specific Localization at Replication Foci in Response to DNA Damage in p53+/+ Cells. *J Biol Chem* 1999; 274(16): 11022-11029. doi: 10.1074/jbc.274.16.11022.
29. Tichy A, Kabacik S, O'Brien G, Pejchal J, Sinkorova Z, Kmochova A, *et al*. The first in vivo multiparametric comparison of different radiation exposure biomarkers in human blood. Woloschak GE, editor. *PLoS ONE* 2018; 13(2): e0193412. https://doi.org/10.1371/journal.pone.0193412.

30. Sculley DG, Dawson PA, Emmerson BT, Gordon RB. A review of the molecular basis of hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency. *Hum Genet* 1992; 90(3): 195-207. https://doi.org/10.1007/BF00220062.
31. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem Biophys Res Commun.* 2004 23;313(4):856-62. doi: 10.1016/j.bbrc.2003.11.177.
32. Townsend MH, Felsted AM, Ence ZE, Piccolo SR, Robison RA, O'Neill KL. Failling from grace: HPRT is not suitable as an endogenous control for cancer-related studies. *Mol Cell Oncol* 2019; 6(2): 1575691. doi: 10.1080/23723556.2019.1575691.
33. de Jonge HJM, Fehrmann RSN, de Bont ESJM, Hofstra RMW, Gerbens F, Kamps WA, *et al*. Evidence Based Selection of Housekeeping Genes. Lichten M, editor. *PLoS ONE* 2007; 2(9): e898. doi: 10.1371/journal.pone.0000898.
34. Sedano JM, Ramos EI, Choudhari R, Harrison AL, Subramani R, Lakshmanaswamy R, *et al*. Hypoxanthine Phosphoribosyl Transderase 1 is upregulated, predicts clinical outcome and controls gene expression in breast cancer. *Cancers* 2020; 12(6): 1522. doi: 10.3390/cancers12061522.
35. Trenceva K, Eftimov A, Petlichkovski A, Jakjovski Z, Topuzovska S. CCNG1 and FDXR expression levels after radiation therapy in breast cancer patients. Acad Med J 2021; 1(2): 78-88.
36. Port M, Majewski M, Herodin F, Valente M, Drouet M, Forcheron F, *et al*. Validating Baboon ex Vivo and in vivo Radiation-Related Gene Expression with Corresponding Human Data. *Radiat Res* 2018; 189(4): 389-398. doi: 10.1667/RR14958.1.
37. Li S, Lu X, Feng J-B, Tian M, Wang J, Chen H, *et al*. Developing Gender-Specific Gene Expression Biodosimetry Using a Panel of Radiation-Responsive Genes for Determining Radiation Dose in Human Peripheral Blood. *Radiat Res* 2019; 192(4): 399-409. doi: 10.1667/RR15355.1.
38. Wolf I, Sadetzki S, Catane R, Karasik A, Kaufman B. Diabetes mellitus and breast cancer. *Lancet Oncol* 2005 6(2): 103-111. doi: 10.1016/S1470-2045(05)01736-5.
39. Reynolds P. Smoking and Breast Cancer. *J Mammary Gland Biol Neoplasia* 2013; 18(1): 15-23. doi: 10.1007/s10911-012-9269-x.
40. Andres SA, Bickett KE, Alatoum MA, Kalbfleisch TS, Brock GN, Wittliff JL. Interaction between smoking history and gene expression levels impacts survival of breast cancer patients. *Breast Cancer Res Treat* 2015; 152(3): 545-556. doi: 10.1007/s10549-015-3507-z.
41. Soltani B, Ghaemi N, Sadeghizadeh M, Najafi F. Redox maintenance and concerted modulation of gene expression and signaling pathways by a nanoformulation of curcumin protects peripheral blood mononuclear cells against gamma radiation. *Chemico-Biol Interact* 2016; 257: 81-93. doi: 10.1016/j.cbi.2016.07.021.
42. Crane FL. Biochemical Functions of Coenzyme Q10. Journal of the American College of Nutrition 2001; 20(6): 591-598. https:// doi.org/10.1080/07315724.2001.10719063.
43. Alimohammadi M, Rahimi A, Faramarzi F, Golpur M, Jafari-Shakib R, Alizadeh-Navaei R, *et al*. Effects of coenzyme Q10 supplementation on inflammation, angiogenesis, and oxidative stress in breast cancer patients: a systematic review and meta-analysis of randomized controlled- trials. *Inflammopharmacology* 2021; 29(3): 579-593. doi: 10.1007/s10787-021-00817-8.
44. Wu D, Han B, Guo L, Fan Z. Molecular mechanisms associated with breast cancer based on integrated gene expression profiling by bioinformatics analysis. *J Obstet and Gynaecol* 2016; 36(5): 615-621. doi: 10.3109/01443615.2015.1127902.
45. Correa CR, Cheung VG. Genetic Variation in Radiation-Induced Expression Phenotypes. *Am J Hum Genet* 2004; 75(5): 885-890. doi: 10.1086/425221.