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Unique proteomic signature for radiation sensitive patients; a comparative study between normo-sensitive and radiation sensitive breast cancer patients

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ABSTRACT

Radiation therapy is a cornerstone of modern cancer treatment. Understanding the mechanisms behind normal tissue sensitivity is essential in order to minimize adverse side effects and yet to prevent local cancer reoccurrence. The aim of this study was to identify biomarkers of radiation sensitivity to enable personalized cancer treatment.

To investigate the mechanisms behind radiation sensitivity a pilot study was made where eight radiation-sensitive and nine normo-sensitive patients were selected from a cohort of 2914 breast cancer patients, based on acute tissue reactions after radiation therapy. Whole blood was sampled and irradiated *in vitro* with 0, 1, or 150 mGy followed by 3 h incubation at 37 °C. The leukocytes of the two groups were isolated, pooled and protein expression profiles were investigated using isotope-coded protein labeling method (ICPL). First, leukocytes from the *in vitro* irradiated whole blood from normo-sensitive and extremely sensitive patients were compared to the non-irradiated controls. To validate this first study a second ICPL analysis comparing only the non-irradiated samples was conducted. Both approaches showed unique proteomic signatures separating the two groups at the basal level and after doses of 1 and 150 mGy.

Pathway analyses of both proteomic approaches suggest that oxidative stress response, coagulation properties and acute phase response are hallmarks of radiation sensitivity supporting our previous study on oxidative stress response. This investigation provides unique characteristics of radiation sensitivity essential for individualized radiation therapy.

1. Introduction

The probability to be diagnosed with cancer before the age of 75 years is 22% for women in Europe [1]. The most common form of cancer for women is breast cancer [1]. In total, around 50% of all cancers are treated with radiation therapy [2]. The therapy is adjusted to the most sensitive patients where 5% severe acute adverse healthy tissue effects are accepted. The patients with no signs of adverse effects have a higher probability of local reoccurrence of cancer within 5 years, indicating that they would have benefited from a higher dose of ionizing radiation (IR) [3,4]. Finding biomarkers of radiation sensitivity would not only benefit the individual but also reduce healthcare costs.

Mechanisms behind individual radiation sensitivity have been challenging scientists for decades. We have previously shown that sensitive patients show different stress response compared to normo-sensitive patients using serum levels of 8-hydroxy-2′-deoxyguanosine (8-oxo-dG) as a biomarker of oxidative stress after

Abbreviations: ICPL, isotope-coded protein label; IR, ionizing radiation.

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low doses of *in vitro* irradiation of whole blood [5,6]. *In vitro* irradiation of whole blood increased 8-oxo-dG levels in the serum of normo-sensitive patients but not in those from sensitive patients. It was hypothesized that this difference was due to different capability to respond to oxidative stress [5].

This hypothesis was further investigated in the present study by analyzing the differences in proteomic profiles of leukocytes isolated from normo-sensitive patients and extremely sensitive patients using two independent labeling approaches. Here we show unique proteomic signatures separating the two groups both at the basal level (non-irradiated samples) and after gamma doses of 1 and 150 mGy. These low doses were chosen in order to visualize more subtle differences in response pathways between the two groups of patients [7] and to avoid drastic and immediate high dose effects such as DNA repair and apoptosis [8]. In order to reduce the individual variation within the two groups and facilitate the search for common responses associated with radiation sensitivity, the samples from the donors in each group were pooled. This approach has been used previously [9,10] in order to reduce the factors originating from the variability between individuals [11] not related to their radiation response. Pathway analyses of both proteomic approaches suggest that oxidative stress response, coagulation properties and acute phase response are hallmarks of radiation sensitivity. This is in good agreement with previous studies [12,13] also indicating a difference in radiation induced oxidative stress response between these two extreme groups.

2. Materials and methods

2.1. Patient material

As previously described in Skiöld et al. [5], a cohort of 2914 patients, including photographs of healthy tissue effects of each patient and assessment of the sensitivity according to the radiotherapy oncology group (RTOG), has been established at Karolinska University Hospital, Stockholm, Sweden. From this cohort nine normo-sensitive patients (RTOG 0) and eight sensitive patients (RTOG 4) were selected. Dose per fractions of 2 Gy (accumulated tumor doses ~50 Gy) or 2.66 Gy (accumulated tumor dose 42.6 Gy), were delivered during 5 days per week. The average age for RTOG 0 group was 52 years and for RTOG 4 group was 51 years. Five patients in the RTOG 0 group and six in the RTOG 4 group received chemotherapy prior to the radiation therapy. The patients were treated for their tumors 1–6 years before they participated in this study. This retrospective study was performed in accordance with the ethical standards of the Swedish Ethical Committee at the Karolinska University Hospital (Dnr 03-621). All the subjects that participated had given their approved consensus.

Blood was collected by venipuncture in three heparin tubes and kept on ice until the start of the assay (<3 h). The blood samples were exposed to gamma radiation on ice with doses of 1 mGy (15 mGy/h) or 150 mGy (0.4 Gy/min). For exposing samples to 1 mGy a cell culture incubator equipped with low active ^{137}Cs source yielding a dose rate of 15 mGy/h and for 150 mGy Scanditronix (Sweden) ^{137}Cs source yielding a dose rate of 0.4 Gy/min were used. The control samples were kept on ice. The samples (irradiated and non-irradiated) were then incubated for 3 h at 37 °C, followed by isolation of leukocytes with red cell lysis buffer (RCLB). The dose response and time for protein expression as well as the other details about *in vitro* irradiation of whole blood have been discussed in our previous publication [6]. Isolated leukocytes were stored at −80 °C as a dry pellet.

The reasons for choosing *in vitro* irradiation of whole blood in the present study were that blood sample is easy to obtain from patients, it is a non-invasive method, low costs for collection and contains great numbers of cells. Additionally, our aim is to find biomarkers which can be used as a predictive test for determination of individual radiosensitivity prior radiotherapy and this makes *in vitro* exposure of whole blood a great candidate toward such a study.

2.2. ICPL analysis and LC–ESI-MS/MS analysis

2.2.1. Protein extraction

Proteins from the leukocytes were isolated with "Qproteome mammalian protein prep kit" (Qiagen). The protein concentration was measured using "2D-Quant kit" (Amersham Biosciences) and 75 μg of protein from each donor was used for the pooled sample of either RTOG 0 group or RTOG 4 group. RTOG 0 pool consisted of samples from nine patients and RTOG 4 pool of samples from eight patients. As we used non-irradiated and irradiated blood for pooling it resulted in altogether six pooled samples consisting of RTOG 0 samples irradiated with doses of 0, 1, or 150 mGy and RTOG 4 samples irradiated with similar doses (0, 1, or 150 mGy). The samples were further processed for the proteomics analysis using the ICPL (isotope-coded protein label) method (Fig. 1).

2.2.2. Protein ICPL labeling and separation

The protein lysate was precipitated by acetone and resuspended in a labeling buffer (SERVA) compatible for ICPL. Protein concentration was determined by Bradford assay following the manufacturer's instructions (Thermo Fisher). The duplex or triplex labeling was done as previously reported [14,15]. Schematic representation of labeling work flow from three biological replicates is shown in Fig. 1. A protein mixture with known ratios of heavy and light label containing bovine serum albumin, chicken ovalbumin and bovine carbonic anhydrase II was used as an internal standard for labeling efficiency and data acquisitions. The labeling was done using three technical replicates. The labeled samples were combined, and separated by 12% SDS gel electrophoresis before staining with colloidal Coomassie solution.

2.2.3. LC–ESI-MS/MS analysis

After Coomassie blue staining, each lane in the gel was cut into five equal slices and subjected to in-gel digestion with trypsin (Sigma–Aldrich) and mass spectrometry analysis on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific) equipped with a nanospray ionization source, as described previously [16]. Briefly, the peptides were accumulated on a nano trap column (Acclaim PepMap100, C18, 5 μm, 100 Å, 300 μm i.d. ×5 mm) and then separated by reversed phase chromatography (Acclaim PepMap100, C18, 3 μm, 100 Å, 75 μm i.d. ×15 cm) operated on a nano-HPLC (Ultimate 3000 HPLC; Dionex), with a nonlinear 170 min gradient of acetonitrile (ACN) in 0.1% formic acid (FA) at a flow rate of 300 nl/min. The gradient settings were subsequently: 0–140 min: 5–32% ACN, 141–145 min: 32–93% ACN, 146–150 min: stay at 93% ACN and then equilibrate for 20 min to starting conditions. Full scan MS spectra (from *m/z* 300 to 1500) were acquired in the Orbitrap with a resolution of 60,000. The up to 10 most intense ions were selected for fragmentation in the linear ion trap using collision-induced dissociation, depending on signal intensity. High resolution MS scans in the Orbitrap and MS/MS scans in the linear ion trap were acquired in parallel. Target peptides already selected for MS/MS were dynamically excluded for 60 s.

2.2.4. Quantification with ICPL

Data processing for protein identification and quantification of ICPL pairs was performed using Proteome Discoverer version 1.3 (Thermo Fisher) as described before [15,17]. The MS/MS spectra were searched against the Ensembl human database (version: 2.4, 96,580 sequences) using the Mascot search engine (version 2.3.02;