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Ionizing radiation affects protein composition of exosomes secreted *in vitro* from head and neck squamous cell carcinoma

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Exosomes are membrane vesicles of endocytic origin that participate in inter-cellular communication. Environmental and physiological conditions affect composition of secreted exosomes, their abundance and potential influence on recipient cells. Here, we analyzed protein component of exosomes released *in vitro* from cells exposed to ionizing radiation (2Gy dose) and compared their content with composition of exosomes released from control not irradiated cells. Exosomes secreted from FaDu cells originating from human squamous head and neck cell carcinoma were analyzed using LC-MS/MS approach. We have found that exposure to ionizing radiation resulted in gross changes in exosomal cargo. There were 217 proteins identified in exosomes from control cells and 384 proteins identified in exosomes from irradiated cells, including 148 "common" proteins, 236 proteins detected specifically after irradiation and 69 proteins not detected after irradiation. Among proteins specifically overrepresented in exosomes from irradiated cells were those involved in transcription, translation, protein turnover, cell division and cell signaling. This indicated that exosomal cargo reflected radiation-induced changes in cellular processes like transient suppression of transcription and translation or stress-induced signaling.

Key words: exosomes, head and neck cancer, ionizing radiation, proteome, radiobiology

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INTRODUCTION

Exosomes are small (30–120 nm of diameter) membrane-derived vesicles secreted by many types of normal and tumor cells, both *in vitro* and *in vivo* (Mathivanan *et al*., 2012; Vlassov *et al*., 2012). These vesicles, covered by lipid membrane, contain different bioactive molecules such as proteins (e.g. cytoskeleton proteins, membrane trafficking proteins, transmembrane molecules, signaling molecules) (Thery *et al*., 2001), lipids (Subra *et al*., 2007), DNA (Balaj *et al*., 2011), mRNA (Gibbings *et al*., 2009) and microRNAs (Taylor & Gercel-Taylor, 2008). Exosomes are involved in communication between cells, and their cargo could be transported via circulation between different cells at diverse locations in the body. Exosomes and their cargo can influence phenotype of recipient cells. They can interact with receptors at a target cell surface, which initiates downstream intracellular signaling. Alternatively, exosomes are endocytosed by target cells, which is followed by the release of cargo proteins and RNA that directly affect functions of recipient cells (Stoorvogel *et al*., 2002).

Components of exosomes secreted from different cell types are currently systematized in database called ExoCarta (Mathivanan *et al*., 2012). Several different classes of exosomal proteins have been detected based on data from a wide variety of cells and body fluids, including: membrane adhesion (e.g., integrins) and membrane transport/trafficking proteins (e.g., annexins, Rab protein family), cytoskeletal components (e.g., actins, ERM proteins) and lysosomal markers (e.g., CD63, LAMP-1/2), antigen presentation factors (e.g., HLA class I and II/peptide complexes), death receptors (e.g., FasL, TRAIL), cytokines and cognate receptors (e.g., TNFα, TNFR1, TGFβ), enzymes (e.g., pyruvate kinase, enolase), drug transporters (e.g., ATP7A, ATP7B, MRP2) and iron transporters (e.g., TfR), heat shock proteins and tumor antigens (e.g., MelanA/Mart-1, gp100, CEA, HER2) (Mathivanan *et al*., 2012). The presence of specific proteins in exosomes secreted from different cell types, e.g. tumor-derived exosomes, suggests the existence of a selective protein-sorting mechanism during its formation (Zitvogel *et al*., 1998). Furthermore, changes in composition of exosomes secreted from cells exposed to different physiological and environmental conditions were detected (Villarroya-Beltri *et al*., 2014). Of note, in addition to "constitutive" exosome secretion through Trans-Golgi network, the release of exosomes via alternative stress-induced pathways was observed, which included Rab27 proteins as key regulators (Ostrowski *et al*., 2010).

Among the major environmental factors inducing cellular stress is ionizing radiation. Albeit damage to genetic material is generally considered the most critical lesion induced by radiation, other cellular effects are also ob-

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Abbreviations: AGC, Automatic Gain Control; BRUB, Britton & Robinson universal buffer; DMEM, Dulbecco's modified Eagle's medium; DTT, ditiotreitol ; FASP, filter aided sample preparation; FBS, fetal bovine serum; GO, gene ontology; HCD, higher-energy collisional dissociation; HD, hypergeometric distribution; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; IR, ionizing radiation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NO, nitric oxide; PBS, phosphate-buffered saline; PEP, posterior error probability; ROS, reactive oxygen species; SCX, strong cation exchange; SDS, sodium dodecyl sulfate; TEM, transmission electron microscopy

served, including increased levels of reactive oxygen species (ROS), nitric oxide (NO), cytokines (interleukins, TGFβ, TNFα) and disturbed calcium transport. These factors influence function of a target cell itself but can also affect neighboring not irradiated cells (so called radiation-induced bystander effect) (Jella *et al.*, 2014). Moreover, factors induced upon exposure to radiation could be putatively packed within exosomes, and therefore potentially reach distant recipient cells. It was shown that radiation-induced p53 protein controls *TSAP6* and *CHMP4C* genes responsible for exosome production (Yu *et al.*, 2009). Increased level of HSP72 was detected in exosomes isolated from serum of prostate cancer patients treated with radiotherapy (Hurwitz *et al.*, 2010), while B7-H3 protein (regulator of antitumor immunity) was detected in exosomes secreted from irradiated 22RV1 prostate cancer cells (Lehmann *et al.*, 2008). In case of proton irradiation increased level of survivin was detected in exosomes secreted from HeLa cells, yet the rate of exosome secretion was not influenced (Khan *et al.*, 2011). Moreover, radiation-induced changes in protein composition of exosomes might hypothetically exert an influence on recipient cells. In fact, recent studies indicated involvement of exosomes in radiation-induced bystander effect in human keratinocytes (Jella *et al.*, 2014), as well as enhanced migration of cells exposed to exosomes secreted from irradiated glioblastoma cells (Arscott *et al.*, 2013).

Ionizing radiation, widely applied in cancer treatment, is known to provoke deleterious effects both in directly exposed cells and also in neighboring cells/tissues through different signal transduction systems (Jella *et al.*, 2014). Hypothetical involvement of exosome-based mechanisms in transduction of signal between exposed and unexposed cells appears an attractive idea, which validation still requires new lines of experimental evidence. Head and neck squamous cell carcinomas (HNSCC) belong to a group of malignancies with high radiosensitivity, and thus radiotherapy is the major treatment modality in this type of cancer (Sheridan *et al.*, 1997). On the other hand, acute radiation toxicity is frequently observed in normal tissues adjacent to irradiated tumor, hence HNSCC is an attractive model in studies of radiation-induced signal transduction mechanisms. Here, we characterized for the first time complete proteome of exosomes secreted *in vitro* from irradiated HNSCC cells using a shotgun proteomics approach aiming to identify components of protein network potentially involved in response to radiation.

MATERIALS AND METHODS

Experimental model. FaDu cell line derived from human head and neck squamous cell carcinoma (HNSCC) was used as an experimental model. These cell line is human papillomavirus (HPV) negative. Although this model is HPV-negative, the association of HPV infections is a serious issue and it was already shown that exosomes released from HPV-positive cells differ in composition and amount depending significantly on the level of HPV E6/E7 oncogene expression (Honegger *et al.*, 2013). Cells were grown in 20 ml of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 15% FBS at 37°C and 5% CO_2. Cells grown at 70–80% confluence were washed three times with 30 ml of phosphate-buffered saline (PBS) each time and replenished with 20 ml of DMEM with 5% exosome-depleted FBS and grown for additional 72h, then the culture flask was treated with 2 Gy radiation dose (6 MeV photons) using the linear accelerator (Clinac 600) and incubated for 18 hours before exosome isolation.

Isolation of exosomes

A. Exosome-precipitation approach using Total Exosome Isolation Kit. This method of exosome purification is based on the patented reagent designed to precipitate specifically exosomes, which are subsequently pelleted by centrifugation (at $10\,000 \times g$). Details of this procedure are described elsewhere (Schageman *et al.*, 2013). Briefly, medium from cell culture (20 ml) was centrifuged at $2000 \times g$ for 30 min to remove cell debris. The supernatant containing the cell-free medium was transferred to a fresh container and combined with 1/2 volume of the Total Exosome Isolation reagent (Invitrogen by life technologies, Austin, USA), then mixed well by vortexing. Samples were incubated at 4°C overnight and then centrifuged at 4°C at $10\,000 \times g$ for 1 h. The supernatant was aspirated and discarded and the pellet containing exosomes was resuspended in PBS buffer.

B. Purification based on a series of differential centrifugation steps. This procedure of exosome purification was based on multiple centrifugation steps aimed to eliminate cell debris and fragments of dead cells before sedimentation of the exosomal fraction as described in details elsewhere (Thery *et al.*, 2006). Briefly, the medium from cell culture was centrifuged at $2000 \times g$ for 20 min, and then the supernatant transferred to a fresh tube was centrifuged at $10\,000 \times g$ for 30 min. Before the final step of ultracentrifugation the volume of resulting supernatant was decreased using exosome concentrators Centricon Plus-70 Centrifugal Filter Units (Merck Millipore, Massachusetts, USA), which allowed ~100-fold volume reduction after 15 min centrifugation at $3500 \times g$, and then the sample was ultracentifuged at $100\,000 \times g$ for 70 min. The pellet was re-suspended in PBS and ultracentrifuged at $100\,000 \times g$ for another 60 min; all above steps were performed at 4°C. The resulting pellet consisted of exosomal fraction. The protein concentration was measured directly (i.e. without membrane lysis) using the Bradford assay; similar protein concentration was obtained in preparation of exosomes from both control and irradiated cells (3.8 and 4.2 μg/μl, respectively).

Western blot detection of exosome-associated protein CD63. Level of CD63 protein in whole cell lysate and exosomes from FaDu cells was assessed by Western blotting using anti-CD63 antibody (Santa Cruz Biotechnology, Inc; SC-15363). Protein samples (7 μg of proteins) were separated using PAGE/SDS and electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked, incubated with primary anti-CD63 antibody followed by secondary peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, USA), and then immune-complexes were detected using ECL plus Western Blot detection system (Amersham Biosciences, GE Healthcare Europe, Freiburg, Germany).

Transmission Electron Microscopy (TEM). Isolated exosomes were fixed with 2% paraformaldehyde in 0.1 cacodylic buffer (pH 7.4) containing 0.1M sucrose. Suspension of exosomes (5 μl) was placed on the Formvar/carbon-coated copper grids (300-mesh) for 1 hour. After rinse in 100 μl PBS the specimen was contrasted using 4% uranyl acetate (pH 7.0) for 5 min, and then washed with 100 μl drops of PBS. The excess of solution was removed using filter paper, the grid was air dried and analyzed using transmission electron micro-

scope TESLA BS500 with Frame-Transfer- CCD Camera, (TRS, Germany) at the 10 000× magnification; three grids were examined for each exosome preparation.

LC-MS/MS analysis. Isolated exosomes (40 μg of proteins) were suspended in 40 μL of 0.1M Tris, pH 8.0, 0.1M DTT and 4% SDS and incubated for 5 min at 95°C. Proteins were then subjected to trypsin digestion using filter aided sample preparation (FASP) approach (Wisniewski & Mann, 2012; Wisniewski *et al*., 2011; Wisniewski *et al*., 2009). Briefly, the sample was mixed (1:5; v/v) with 8M urea solution in 0.1 M Tris/HCl, pH 8.5, in the ultrafiltration units (cut off 30 kDa) and centrifuged for 10 min at 14 000 × *g*. Filter-bound proteins were treated with 5 mM iodoacetamide (at room temperature in the dark for 30 min) and digested for 16 h with trypsin (Promega, enzyme to protein ratio 1:100). Resulting peptides were released from ultrafiltration units using 0.05M Tris/HCl pH 8.5 then deionized water. The peptide solutions were diluted with Britton & Robinson Universal Buffer (BRUB) at pH 5.0 then loaded on SCX column. Peptides were eluted from SCX column to C18 column first with BRUB pH 5.0 then with BRUB pH 2.0, and then eluted from C18 columns with 60% ACN. All peptide fractions were dried in SpeedVac and resuspended in 20 μL of deionized water prior to LC-MS analysis performed on Dionex UltiMate 3000 RSLC nanoLC System (Thermo Fisher Scientific, Bremen, Germany) connected to Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides (5 μl of injected sample) were separated on reverse phase Acclaim PepMap RSLC nanoViper C18 column (75 μm × 25 cm, granulation of 2 μm, Thermo Fisher Scientific) using 240 min gradient from 4% to 60% of acetonitrile (in 0.1% formic acid) at a flow rate of 250 nL/min and constant temperature of 30°C. The spectrometer was operating in the data-dependent MS^2 mode with survey scans acquired at a resolution of 70 000 at m/z 200 in MS mode and 17 500 at m/z 200 in MS^2 mode, respectively. The spectra were recorded in the scan range of 300–2000 m/z in the positive ion mode. The maximum ion injection times for the survey scan and the MS/MS scans were 10 and 100 ms, respectively, and the Automatic Gain Control (AGC) target value for both scan modes were set to 10^6. Higher-energy collisional dissociation (HCD) ion fragmentation was performed with normalized collision energies set to 25. The MS data were analyzed using MaxQuant 1.4.1.1 software. Exosome protein identification was performed using the Mascot engine for searching against Swiss-Prot human database. The precision tolerance was 10 ppm for peptide masses and 0.05 Da for fragment ion masses. Protein hit was considered significant only when based on at least 2 unique peptide hits or unique peptide covering at least 2% of protein sequence, and the value of posterior error probability (PEP) was lower than 0.05.

Annotation of identified proteins into functional groups. All protein names were converted into gene identifiers (GI numbers), which were annotated at gene ontology (GO) terms using NucleoAnnot software (Polanski *et al*., 2014). Statistical overrepresentation of GO terms was assessed in two compared groups (control and IR-exposed) using the hypergeometric distribution (HD) test. For each GO term the ratio of HD *p*-value for control and IR-exposed samples was used as a measure of significance of differences between preparations (only GO terms for which HD ratios are higher than 1 000 are showed). In order to visualize differences in functional protein networks between exosomes isolated from control and IR-exposed cells two lists of proteins were compared: proteins identified exclusively in exosomes from control cells (so called IR-downregulated proteins) and proteins identified exclusively in exosomes from irradiated cells (IR-upregulated proteins). Each of these list of proteins was annotated at the functional protein association networks database STRING 9.1 (Jensen *et al*., 2009).

RESULTS AND DISCUSSION

The most critical problem in characterization of the content of exosomes is purity and homogeneity of preparation for further proteomics or genomics analyses. We have tested two different techniques for isolation of exosomes: (1) exosome-precipitation approach using Total Exosome Isolation kit (Schageman *et al*., 2013) and (2) purification based on series of differential centrifugation steps (Thery *et al*., 2006). Both types of exosome preparations were analyzed by TEM to compare their purity (i.e., proportion of exosomes and other structures) and homogeneity (i.e., size distribution of resulting vesicles). The first method based on reagents designed to precipitate specifically exosomes is relatively simple and quick. However, we observed lower purity and homogeneity in this type of material (not shown). Furthermore, some FBS peptides were detected in this preparation. Finally, we decided to use method of exosome purification based on differentiating centrifugation, which approach was successfully implemented in some previous proteomics studies (Jella *et al*., 2014; Raimondo *et al*., 2011). Exosomal preparation obtained in our study consisted of microvesicles with the average diameter of 70–80 nm (in the 40–120 nm range), which was verified by the TEM ultrastructure analysis (Fig. 1A). To further verify quality of exosome preparation the level of exosome-associated marker CD63 was analyzed by Western blotting in exosomal fraction and in whole cell lysates. CD63 protein is a member of tetraspanin family and it is usually found in a glycosylated form (Maecker *et al*., 1997). This form of CD63 (~60 kDa), commonly used for exosome labeling (Berditchevski & Odintsova, 2007), was detected only in preparation of these vesicles, while minor amounts of non-glycosylated CD63 (~43 kDa) was detected only in whole cell lysates (Fig. 1B). We concluded that centrifugation/ultracentrifugation protocol allowed on purification of exosomes, which could be used for further proteomics characterization.

Proteins present in isolated exosomes were identified after trypsin digestion using nanoLC-ESI-MS/MS approach followed by analysis of registered peptides using the MASCOT engine search. Only proteins identified by at least 2 unique peptide hits or peptide covering at least 2% of protein sequence were considered positively identi-

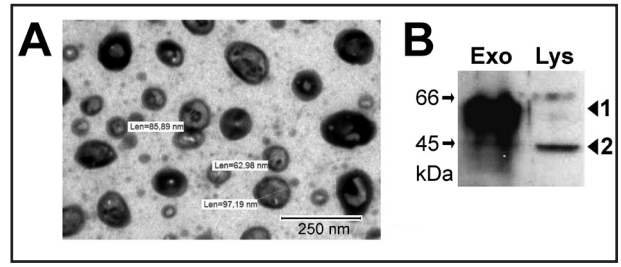

Figure 1. Characterization of exosomes secreted from FaDu cells.
(**Panel A**) Ultrastructure of isolated exosomes analyzed by TEM microscopy; depicted is a scale bar and diameters of selected vesicles. (**Panel B**) Western blot analysis of CD63 protein in exosome fraction (Exo) and whole cell lysate (Lys); marked are two forms of CD63: 1 — glycosylated and 2 — not glycosylated.

Table 1. Proteins identified in exosomes secreted from FaDu cells.
Common proteins were identified in exosomes from both control and irradiated cells, IR-upregulated proteins were identified exclusively in exosomes from irradiated cells, IR-downregulated proteins were identified exclusively in exosomes from control not irradiated cells.

common proteins	A1; A1M; A2; A2M; A3; A4; ACT7; ACTN4; AGRN; ALDOA; ANXA1; ANXA11; ANXA2; ANXA2P2; ATP1A1; BANF1; BETATUB85D; C3; CCT2; CCT4; CCT7; CCT8; CD9; CFL1; CFL2; CLTC; COL12A1; COL4A2; CPNE8; EEF1A; EF1ALPHA100E; EF1ALPHA48D; EFT-3; EHD1; EHD3; FASN; FAT2; FLNA; FN1; FTL1; H2AFV; H2AFZ; H2BFS; H2B-I; H2B-V; H2B-VIII; H3F3C; H4-I; H4-VIII; HIS-1; HIS2AV; HIS4; HIST1H1B; HIST1H1C; HIST1H1D; HIST1H1E; HIST1H2BB; HIST1H2BC; HIST1H2BD; HIST1H2BF; HIST1H2BH; HIST1H2BJ; HIST1H2BK; HIST1H2BL; HIST1H2BM; HIST1H2BN; HIST1H2BO; HIST1H4A; HIST1H4B; HIST2H2BB; HIST2H2BC; HIST2H2BD; HIST2H2BE; HIST2H2BF; HIST3H2BA; HIST3H2BB; HMGA1; HSP70-5; HSP90A.1; HSP90AA1; HSP90AB1; HSPA1A; HSPA1B; HSPA8; HSPG2; ITIH2; LAMA3; LAMA5; LAMB1; LAMB2; LAMB3; LAMC1; LDHB; LGALS3BP; M6PR; MEC-7; MMP1; MVP; MYH9; PDCD6IP; PKM; PLAU; PLOD3; POTEE; POTEF; POTEI; POTEJ; POTEKP; PSMA1; PSMA3; PSMA5; PSMA6; PSMA7; PSMA8; PSMB1; PSMB2; PSMB5; PSMB6; PTGFRN; RAB7A; RABG3E; RABG3F; RPS3; SERPINA1; SERPINA3; SLC16A1; SLC2A1; SLC3A2; TB10.70.5670; TBB-2; TBB-4; TEF1; TFRC; TGAS006M08.1; TOP1; TUBB; TUBB2; TUBB2B; TUBB4; TUBB4B; TUBB5; VCP; XRCC5; XRCC6; YBX1; YBX2; YBX3; YWHAZ;
IR-upregulated proteins	ABCE1; ACTR2; AHCY13; ALDH1A3; ALDOC; ANXA4; ANXA5; ANXA7; APC-1; APOB; APOC3; APP; ARF1; ARF2; ARF3; ARF79F; ARP14D; ARRDC1; ATP6V1A; BCAM; BROX; BSG; BUB3; CAF1; CCT3; CCT5; CD44; CD63; CES4A; CKMT1; CLSTN1; COL18A1; CTNNA1; CTSA; CTTN; DARS; DIRC2; DLST; DNPEP; DYNC1H1; EEF2; EFHD2; EHD4; EIF2S1; EIF4A1; EIF4A2; EPHA2; F37A4.5; FGFBP1; FKBP1A; FLOT1; GAPDH; GNB2L1; GPC1; GPNMB; GPR56; GSP1; GSP2; GSTP1; H2A.ZL; H2AFJ; H2AFX; H2AFY; H2A-IX; H3; HIS2A; HIS-74; HIST1H2AA; HIST1H2AB; HIST1H2AC; HIST1H2AD; HIST1H2AF; HIST1H2AG; HIST1H2AH; HIST1H2AJ; HIST1H2AK; HIST2H2AA1; HIST2H2AA3; HIST2H2AC; HIST3H2A; HLA-A; HLA-B; HLA-C; HLA-E; HLA-G; HLA-H; HNRNPAB; HNRNPC; HNRNPD; HRAS; HSPA5; HTZ1; IARS; IQGAP1; ISG15; KIAA1199; KRAS; KRT84; LAMC2; LET-60; LGALS3; LRP1; LSR; MAMU-F; MAT2A; METK; MFGE8; MTHFD1; MTS2; MYL12A; MYL12B; MYL6; MYL6B; MYOF; NACA; NHP2L1; NID1; NQO1; NRAS; PA2G4; PABPC1; PABPC1-A; PABPC1-B; PABPC3; PABPC4; PAICS; PARP1; PFKP; PHGDH; PHT1; PLP2; PP1-13C; PP1-87B; PP1ALPHA-96A; PPP1CB; PPP1CC; PRDX1; PRMT1; PRMT1-A; PRMT1-B; PRMT8; PROS26.4; PSMA2; PSMC2; PSMC5; PSMD14; PSMD6; QSOX1; RAB11A; RAB11B; RAB1A; RAB1B; RAB1C; RAB2A; RAB32; RAB5A; RAB5B; RAB5C; RABF2A; RABF2B; RAN-1; RAP1A; RAP1B; RASL2-9; RBBP4; RBBP7; RLC-A; RNH1; RPL11; RPL13; RPL15; RPL17; RPL18; RPL23A; RPL27; RPL3; RPL30; RPL5; RPL6; RPL7; RPL7A; RPL8; RPLP2; RPS11; RPS15A; RPS16; RPS18; RPS2; RPS20; RPS21; RPS4; RPS4X; RPS4Y1; RPS6; RPS8; RPSA; RPT2; RUVBL1; RUVBL2; S100A14; SAM1; SAM2; SCAMP3; SDC4; SDCBP; SEMA3C; SF3B3; SFN; SKP1; SLC16A3; SLC39A14; SMC3; SND1; SNRNP200; SPI1; SRSF5; SSBP1; ST13; ST13P4; TCP1; TKT; TLN1; TOPP6; TPP2; TUBA; TUBA4A; TXN; UBR4; UGP2; VAT1; VHA-13; VHA68-1; VHA68-2; WARS; YWHAG; YWHAQ;
IR-downregulated proteins	ACTN1; AHSG; APOA1; APOA4; APOH; C1R; C1S; C8A; CALM; CALM1; CALM1A; CAM; CAPNS1; CAV1; CD5L; CDC48B; CFH; CMD-1; EDIL3; EEF1G; FGB; HEXB; HNRNPH1; HNRNPH2; HP; HPR; HPX; IFITM1; IFITM2; IFITM3; IGHG1; IGHG2; IGHG3; IGKC; IGLC1; IGLC2; IGLC3; IGLC6; IGLC7; IGLL5; ITIH1; KATNAL2; LAMP1; LCN2; LDHA; NAP1L4; NME1; NME2; NME2P1; PAC1; PAS-3; PF14_0323; PLG; PRE9; PRMT5; PROS1; PROS29; PROSALPHA3T; PSMA4; RBP4; RPL13A; S100A9; SEC18; SERPINC1; SLC7A5; SPATA5; TF; TINAGL1; YWHAE;

fied (detailed information in the Supplementary File Table S1 at www.actabp.pl). We identified 217 proteins in exosomes secreted from control not irradiated cells and 384 proteins in exosomes secreted from cells irradiated with 2 Gy. After comparison of both protein lists we found 148 protein present in exosomes from both control and irradiated cells ("common proteins"), 69 proteins identified only in exosomes from control cells ("IR-downregulated proteins") and 236 proteins identified only in exosomes from irradiated cells ("IR-upregulated proteins). Complete lists of the common, IR-downregulated and IR-upregulated exosomal proteins are given in Table 1. We concluded that exposure to ionizing radiation affected the protein content of exosomes secreted by HNSCC cells *in vitro*. In fact, in addition to "common" set of exosomal proteins (e.g. different HSPs), large number of proteins putatively specific for radiation-mediated processes was detected. In consequence, much more protein species was identified in exosomes secreted from irradiated cells as compared to not irradiated controls. In the next step we compared identified proteins from control samples with previously known exosomal proteins. The most comprehensive catalogue of exosomal proteins is available in the ExoCarta database (Mathivanan *et al.*, 2012), which contains information about all molecules identified in exosomes from various sources. The latest version 4.1 of the database contains 4563 unique proteins summarizing 146 studies on multiple organisms and cell types, including 2583 human proteins. However, there is no data available from experiments performed on HNSCC cells. We found 68 proteins identified in exosomes from control FaDu cells, which were absent in the ExoCarta database yet, namely: A1, A2, A3, ACT7, APOH, BANF1, BETATUB85D, C8A, CALM, CALM1A, CAM, CDC48B, CMD-1, EF1ALPHA100E, EF1ALPHA48D, EFT-3, H2BFS, H2B-I, H2B-V, H2B-VIII, H3F3C, H4-I, H4-VIII, HEXB, HIS-1, HIS2AV, HIS4, HIST1H1C, HIST1H1D, HIST1H1E, HIST2H2BB, HIST2H2BD, HIST3H2BA, HMGA1, HNRNPH1, HSP70-5, HSP90A.1, IGLC6, IGLC7, IGLL5, ITIH1, KATNAL2, M6PR, MEC-7, NME2P1, PAC1, PAS-3, PF14_0323, POTEJ, PRE9, PRMT5, PROS29, PROSALPHA3T, RABG3E, RABG3F, RPL13A, SEC18, SERPINC1, SPATA5, TB10.70.5670, TBB-2, TBB-4, TEF1, TGAS006M08.1, TOP1, TUBB5, YBX2, YBX3. These proteins could be detected for the first time in exosomes due to a very high sensitivity of implemented LC-MS/MS technique. Alternatively, some of them could be specific for HNSCC cells, which cell type was not analyzed in studies summarized in the ExoCarta database.

Finally, we searched for biological processes associated with identified exosomal proteins. For this purpose names of proteins detected in exosomes were converted into corresponding gene's identifiers, which were annotated at gene ontology terms; there were 183 annotated genes/proteins in exosomes from control cells and 340 annotated genes/proteins in exosomes from irradiated cells. In the next step statistical overrepresentation of GO terms associated with exosomal proteins was assessed using the hypergeometric distribution test (assuming total number of annotated genes/proteins as 21804). Then, we searched for GO terms which showed significantly different representation in samples obtained from control cells and irradiated cells; complete information on GO terms associated with identified exosomal proteins is presented in the Supplementary File

Table 2. Biological processes associated with proteins, which presence in exosomes secreted from FaDu cells was affected by ionizing radiation.
Showed are GO terms, numbers of components (total and present in control or IR-treated samples) and ratios of p-values obtained with the hypergeometric distribution test for both type of samples (Ctr/IR HD ratio). Ctr/IR_HD_ratio >> 1 indicates relative overrepresentation of components associated with a given GO terms in exosomes from irradiated cells, while Ctr/IR_HD_ratio << 1 relative underrepresentation of such components.

GO Term	Total number of components	Number of components in control samples	Number of components in IR-treated samples	Ctr/IR HD ratio
mRNA metabolic process	224	16	51	5.1E+33
viral process	546	27	70	7.6E+29
RNA metabolic process	246	17	50	6.9E+29
gene expression	671	22	70	3.3E+29
translational initiation	118	2	32	2.5E+29
translational elongation	91	4	29	2.7E+27
viral transcription	82	2	27	1.3E+27
viral life cycle	93	2	28	9.5E+26
translational termination	85	2	27	4.6E+26
translation	250	4	38	5.7E+24
nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	118	2	28	7.9E+23
SRP-dependent cotranslational protein targeting to membrane	107	2	27	5.9E+23
cellular protein metabolic process	511	16	49	4.6E+18
nucleosome assembly	105	22	36	8.8E+13
antigen processing and presentation of peptide antigen *via* MHC class I	98	11	23	9.5E+10
antigen processing and presentation of exogenous peptide antigen *via* MHC class I, TAP-dependent	75	11	21	5.5E+09
antigen processing and presentation of exogenous peptide antigen *via* MHC class I	79	11	21	2.9E+09
small molecule metabolic process	1392	27	59	1.9E+07
small GTPase mediated signal transduction	320	3	21	7.7E+06
mitotic cell cycle	388	17	32	1.9E+06
regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	75	11	18	4.1E+05
cellular nitrogen compound metabolic process	185	11	21	3.3E+05
regulation of cellular amino acid metabolic process	51	11	17	3.2E+05
anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	80	11	18	2.4E+05
protein folding	164	10	19	1.1E+05
negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	65	10	16	5.8E+04
protein transport	390	5	20	4.3E+04
positive regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	71	11	17	2.9E+04
GTP catabolic process	108	1	9	8.8E+03
cell adhesion	429	12	24	3.5E+03
"de novo" posttranslational protein folding	38	8	12	2.6E+03
ATP catabolic process	202	7	15	2.2E+03
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	66	11	16	2.1E+03
protein polyubiquitination	113	11	17	1.4E+03
complement activation, alternative pathway	13	3	1	9.1E-04

acute-phase response	38	5	3	8.2E-04
UTP biosynthetic process	12	3	1	7.5E-04
fibrinolysis	18	4	2	4.5E-04
regulation of complement activation	24	4	1	1.7E-04
innate immune response	625	22	17	1.2E-06
Fc-gamma receptor signaling pathway involved in phagocytosis	91	12	4	3.6E-10
complement activation, classical pathway	51	13	1	6.5E-16
complement activation	48	14	1	5.4E-18

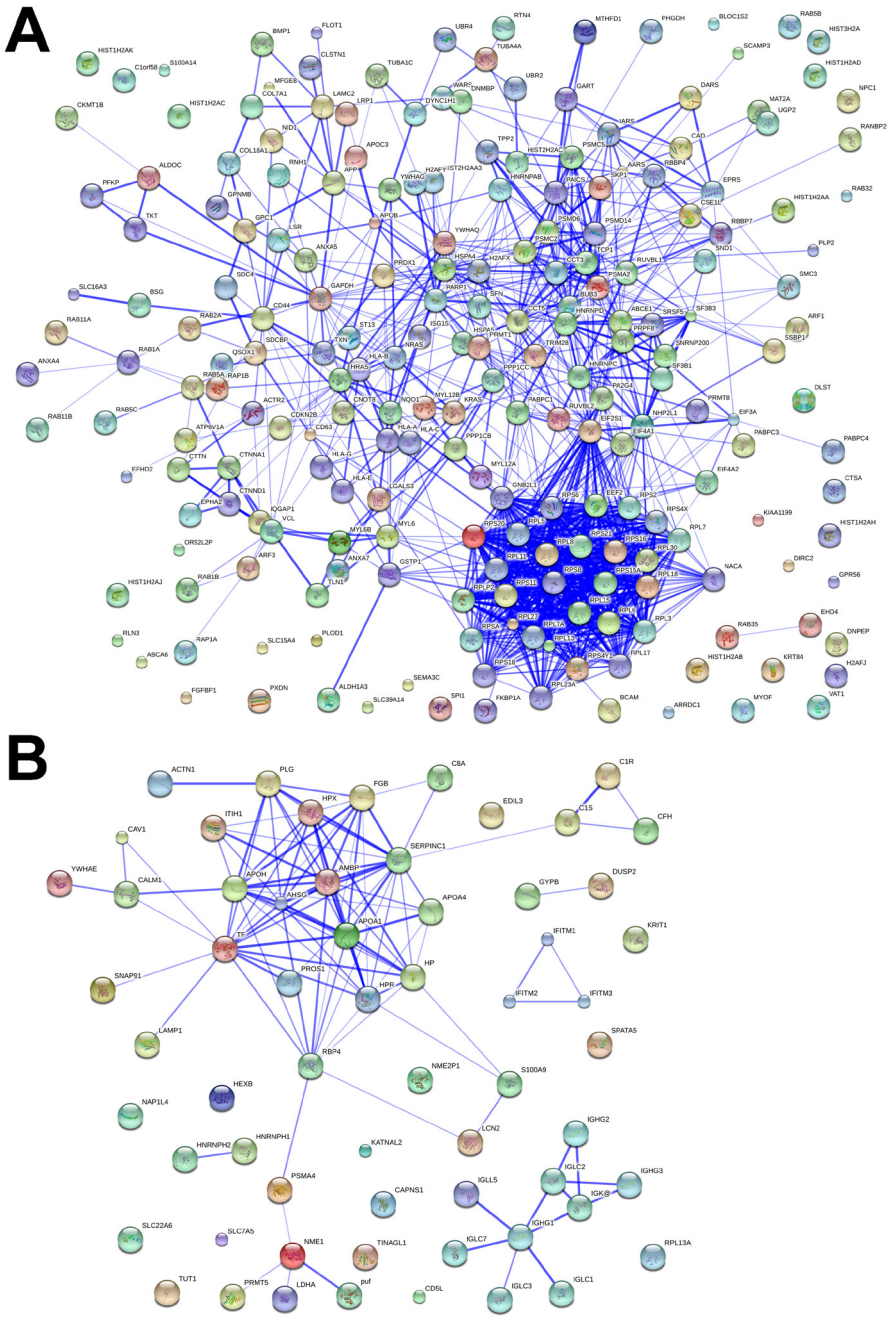

Figure 2. Network of potential interactions between exosomal proteins.
(**Panel A**) Proteins identified exclusively in exosomes from irradiated cells (217 annotated proteins. (**Panel B**) Proteins identified exclusively in exosomes from control cells (62 annotated proteins). Interactions between proteins are marked with blue lines; the thickness of the line correlates with the level of confidence.

Table S2 (at www.actabp.pl). Table 2 shows examples of GO terms that were differentially represented in material from control and irradiated cells – only GO terms for which p-values from the HD test obtained for both types of exosomal preparations (HD_ratio) differed at least 1000-fold were considered as differentially represented. Among the major biological processes associated with proteins present in exosomes secreted from irradiated cells (i.e. GO terms relatively overrepresented in IR-treated samples) were those involved in RNA metabolism, transcription, translation, protein folding and degradation, regulation of cell division and nucleosome assembly as well as viral infection and antigen presentation. On the other hand, among proteins associated with biological processes overrepresented in control samples were those involved in acute phase and immune response. We have also searched for potential functional interactions between proteins upregulated and downregulated in exosomes secreted from irradiated cells. Functional protein association networks were obtained after annotation of identified proteins at the STRING database (Jensen *et al.*, 2009). Networks containing proteins detected only in exosomes from control cells (IR-downregulated proteins) and only in exosomes from irradiated cells (IR-upregulated proteins) are depicted on Fig. 2. Among IR-upregulated proteins involved in the most numerous and confident interactions were those involved in transcription and translation (EIF proteins and ribosomal proteins) as well as (co)chaperones (e.g. CCT proteins) and proteasomal components (PSM proteins). (Fig. 2A). On the other hand, among IR-downregulated proteins involved in the most confident interactions were apolipoproteins and immunoglobulins (Fig. 2B).

Available information on radiation-induced changes in protein cargo of exosomes is limited to a few published works, which are based on different cellular models and modes of exposure to radiation. In general, both changes in levels of specific proteins and overall rates of exosome secretion have been reported (Arscott *et al.*, 2013; Hurwitz *et al.*, 2010; Jella *et al.*, 2014; Khan *et al.*, 2011; Lehmann *et al.*, 2008), yet generality of observed changes and their functional importance remains unclear. Many proteins identified here in exosomes secreted from FaDu HNSCC cells belong to classes previously described as a "typical" exosomal cargo. These groups included HSP proteins, metabolic enzymes, antigen presentation factors, viral assembly factors, annexins and other membrane proteins. Unexpectedly, however, we also detected large number of structural chromatin/nuclear proteins including histones and lamins. Histones were detected in exosome fraction purified from either irradiated cells and untreated controls. Hence, their presence could not be explained by hypothetical contamination of exosomes with apoptotic bodies, chromatin-containing vesicles derived from cells dying because of irradiation. Furthermore, only very low level (~0.2%) of cells in subG1 fraction (putatively corresponding to dying cells) was observed 18 hrs. after exposure of FaDu cells to 2 Gy radiation dose. This observation could suggest existence of a hypothetical exosome-directed chromatin-packing mechanism. Alternatively, chromatin fragments could interact with external surface of exosomes, which mechanism was suggested for exosomes present in urine (Miranda *et al.*, 2010). There were several functional groups of proteins detected in this work, which levels in exosomes secreted from HNSCC cells were affected by exposure to ionizing radiation. Major groups of proteins upregulated (or overrepresented) in exosomes released from irradiated cells were those involved in transcription and translation (e.g. EIFs, PSMs, RPLs and RPSs proteins), as well as regulation of cell cycle/division (including chaperones, ubiquitination-related factors and proteasome components) and cellular signaling (e.g. ARFs, RABs and RASs proteins). One should expect that arrest of the cell cycle and resulting blockade of transcription, translation and cell division would be the primary response of cells to radiation. Hence, the presence of transcription/translation-related factors in cargo of exosomes secreted from irradiated cells could reflect dynamic adaptation of cells to stress-induced conditions (e.g. removal of excessive/unnecessary components). Similarly, exosomal presence of GTPase-related signaling factors (ARFs, RABs and RASs proteins) could reflect and/or mediate stress-induced response triggered in cells exposed to radiation. However, elucidation of functional importance in radiation response of exosomal status of proteins identified in this work requires further protein- and pathway-oriented studies.

CONCLUSION

We have found that exposure to ionizing radiation significantly affect composition of proteins detected in exosomes secreted from human HNSCC cells. Several protein species were upregulated or downregulated in exosomes released *in vitro* from irradiated cells. Among proteins specifically overrepresented in exosomes from irradiated cells were those involved in transcription, translation, cell division and cell signaling. This indicated that exosomal cargo reflected changes induced by radiation in cellular processes (e.g. transient suppression of transcription/translation or stress-induced signaling).

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Conflict of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the article.

REFERENCES

Arscott WT, Tandle AT, Zhao S, Shabason JE, Gordon IK, Schlaff CD, Zhang G, Tofilon PJ, Camphausen KA (2013) Ionizing radiation and glioblastoma exosomes: implications in tumor biology and cell migration. *Transl Oncol* **6**: 638–648.

Balaj L, Lessard R, Dai L, Cho YJ, Pomeroy SL, Breakefield XO, Skog J (2011) Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat Commun* **2**: 180.

Berditchevski F, Odintsova E (2007) Tetraspanins as regulators of protein trafficking. *Traffic* **8**: 89–96.

Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O (2009) Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol* **11**: 1143–1149.

Honegger A, Leitz J, Bulkescher J, Hoppe-Seyler K, Hoppe-Seyler F (2013) Silencing of human papillomavirus (HPV) E6/E7 oncogene expression affects both the contents and the amounts of extracellular microvesicles released from HPV-positive cancer cells. *Int J Cancer* **133**: 1631–1642.

Hurwitz MD, Kaur P, Nagaraja GM, Bausero MA, Manola J, Asea A (2010) Radiation therapy induces circulating serum Hsp72 in patients with prostate cancer. *Radiother Oncol* **95**: 350–358.

Jella KK, Rani S, O'Driscoll L, McClean B, Byrne HJ, Lyng FM (2014) Exosomes are involved in mediating radiation induced bystander signaling in human keratinocyte cells. *Radiat Res* **181**: 138–145.

Jensen LJ, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, Doerks T, Julien P, Roth A, Simonovic M, Bork P, von Mering C (2009) STRING 8 — a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res* **37**: D412–D416.

Khan S, Jutzy JM, Aspe JR, McGregor DW, Neidigh JW, Wall NR (2011) Survivin is released from cancer cells via exosomes. *Apoptosis* **16**: 1–12.

Lehmann BD, Paine MS, Brooks AM, McCubrey JA, Renegar RH, Wang R, Terrian DM (2008) Senescence-associated exosome release from human prostate cancer cells. *Cancer Res* **68**: 7864–7871.

Maecker HT, Todd SC, Levy S (1997) The tetraspanin superfamily: molecular facilitators. *Faseb j* **11**: 428–442.

Mathivanan S, Fahner CJ, Reid GE, Simpson RJ (2012) ExoCarta 2012: database of exosomal proteins, RNA and lipids. *Nucleic Acids Res* **40**: D1241–D1244.

Miranda KC, Bond DT, McKee M, Skog J, Paunescu TG, Da Silva N, Brown D, Russo LM (2010) Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. *Kidney Int* **78**: 191–199.

Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, Moita CF, Schauer K, Hume AN, Freitas RP, Goud B, Benaroch P, Hacohen N, Fukuda M, Desnos C, Seabra MC, Darchen F, Amigorena S, Moita LF, Thery C (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol* **12**: 19–30 (sup pp 11–13).

Polanski A, Polanska J, Jaksik R (2014) NucleoAnnot application was developed as a part of the GENEPI low-RT project (FP6-036452). Available online: http://cellab.polsl.pl/index.php/software/index.php?option=com_content&view=article&id=27.

Raimondo F, Morosi L, Chinello C, Magni F, Pitto M (2011) Advances in membranous vesicle and exosome proteomics improving biological understanding and biomarker discovery. *Proteomics* **11**: 709–720.

Schageman J, Zeringer E, Li M, Barta T, Lea K, Gu J, Magdaleno S, Setterquist R, Vlassov AV (2013) The complete exosome workflow solution: from isolation to characterization of RNA cargo. *Biomed Res Int* **2013**: 253957.

Sheridan MT, O'Dwyer T, Seymour CB, Mothersill CE (1997) Potential indicators of radiosensitivity in squamous cell carcinoma of the head and neck. *Radiat Oncol Investig* **5**: 180–186.

Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G (2002) The biogenesis and functions of exosomes. *Traffic* **3**: 321–330.

Subra C, Laulagnier K, Perret B, Record M (2007) Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. *Biochimie* **89**: 205–212.

Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol* **110**: 13–21.

Thery C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* **Chapter 3**: Unit 3.22.

Thery C, Boussac M, Veron P, Ricciardi-Castagnoli P, Raposo G, Garin J, Amigorena S (2001) Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol* **166**: 7309–7318.

Villarroya-Beltri C, Baixauli F, Gutierrez-Vazquez C, Sanchez-Madrid F, Mittelbrunn M (2014) Sorting it out: regulation of exosome loading. *Semin Cancer Biol* **28**: 3–13.

Vlassov AV, Magdaleno S, Setterquist R, Conrad R (2012) Exosomes: current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim Biophys Acta* **1820**: 940–948.

Wisniewski JR, Mann M (2012) Consecutive proteolytic digestion in an enzyme reactor increases depth of proteomic and phosphoproteomic analysis. *Anal Chem* **84**: 2631–2637.

Wisniewski JR, Zielinska DF, Mann M (2011) Comparison of ultrafiltration units for proteomic and N-glycoproteomic analysis by the filter-aided sample preparation method. *Anal Biochem* **410**: 307–309.

Wisniewski JR, Zougman A, Mann M (2009) Combination of FASP and StageTip-based fractionation allows in-depth analysis of the hippocampal membrane proteome. *J Proteome Res* **8**: 5674–5678.

Yu X, Riley T, Levine AJ (2009) The regulation of the endosomal compartment by p53 the tumor suppressor gene. *Febs j* **276**: 2201–2212.

Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G, Amigorena S (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* **4**: 594–600.