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Proteomic signatures of radioresistance: Alteration of inflammation, angiogenesis and metabolism-related factors in radioresistant oesophageal adenocarcinoma

Simone Marcone [a,*], Amy Buckley [a], Colm J. Ryan [b,c], Mark McCabe [a], Niamh Lynam-Lennon [a], David Matallanas [c], Jacintha O`Sullivan [a], Susan Kennedy [a]

[a] Department of Surgery, Trinity Translational Medicine Institute, Trinity St. James's Cancer Institute, St. James's Hospital, Trinity College Dublin, Dublin, Ireland
[b] School of Computer Science, University College Dublin, Dublin 4, Ireland
[c] Systems Biology Ireland, School of Medicine, University College Dublin, Dublin 4, Ireland

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ABSTRACT

The clinical management of locally advanced oesophageal adenocarcinoma (OAC) involves neoadjuvant chemoradiotherapy (CRT), but as radioresistance remains a major clinical challenge, complete pathological response to CRT only occurs in 20–30% of patients. In this study we used an established isogenic cell line model of radioresistant OAC to detect proteomic signatures of radioresistance to identify novel molecular and cellular targets of radioresistance in OAC. A total of 5785 proteins were identified of which 251 were significantly modulated in OE33R cells, when compared to OE33P. Gene ontology and pathway analysis of these significantly modulated proteins demonstrated altered metabolism in radioresistant cells accompanied by an inhibition of apoptosis. In addition, inflammatory and angiogenic pathways were positively regulated in radioresistant cells compared to the radiosensitive cells. In this study, we demonstrate, for the first time, a comprehensive proteomic profile of the established isogenic cell line model of radioresistant OAC. This analysis provides insights into the molecular and cellular pathways which regulate radioresistance in OAC. Furthermore, it identifies pathway specific signatures of radioresistance that will direct studies on the development of targeted therapies and personalised approaches to radiotherapy.

1. Introduction

Oesophageal cancer (OAC) is the eighth most common cancer worldwide accounting for approximately 400,000 deaths annually [1]. OAC is primarily classified into two different histological types, squamous cell carcinoma and oesophageal adenocarcinoma, the latter is usually located in the distal oesophagus or gastro-oesophageal junction [2]. Treatment options for OAC tumours include local mucosal resection, esophagectomy, chemotherapy and chemoradiation therapy both pre- and post-operatively [3]. The existent standard of care for OAC is represented by neoadjuvant treatment with chemotherapy (neoCT) alone or in combination with radiation (neoadjuvant chemoradiation, neoCRT) for locally advanced tumours, in advance to surgery [4]. Radiotherapy is a essential treatment method for local tumour control in many solid cancers, including OAC [5]. Radiotherapy is based on the administration of ionising radiation (IR) to kill cancer cells through direct and indirect cellular mechanisms, such as single and double strand breaks in the DNA and the generation of reactive oxygen species (ROS), which leads to DNA damage, cell stress, alteration of cellular signalling pathways, and cell death [6]. Unfavourably, only a minority of OAC patients demonstrate a complete regression of the tumour after receiving neoCRT with the vast majority of patients subjected to a toxic treatment with no apparent therapeutic benefit and a considerable delay to surgery [7]. Resistance to radiotherapy involves a number of processes associated with the capability of cancer cells to adapt to ionising radiation damage through the alteration of biological processes such as cellular energetics [8], angiogenesis [9] and inflammation [10]. Inflammation plays a critical role in regulating the response of cancer to

Abbreviations: OAC, Oesophageal cancer; CRT, Chemoradiation therapy; IR, ionising radiation; OE33R, OE33 radioresistant cells; OE33P, OE33 radiosensitive cells.

* Corresponding author: Department of Surgery, Trinity Translational Medicine Institute, Trinity St James's Cancer Institute, St. James's Hospital, Dublin 8, Ireland
E-mail address: marcones@tcd.ie (S. Marcone).

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radiation and it has been demonstrated that inflammation is a negative condition decreasing treatment response in OAC [11]. Sustained angiogenesis is a well-recognised hallmark of cancer, which refers to vessel sprouting and development from pre-existing blood vessels in cancer tissues [12]. In fact, it has been demonstrated that anti-angiogenic factors increased tumour response to therapy when in combination with single dose radiotherapy in oesophageal SCC mouse models, and in pancreatic and colorectal cancer patients [13,14]. Anti-angiogenic factors can also increase the tumour response to radiotherapy by normalising the vasculature in the tumour and enhancing the reaction of endothelial cell to damage [15]. Angiogenesis is tightly interconnected with other biological processes including tumour metabolism, in fact, blood vessels supply oxygen and nutrients required to sustain cellular metabolism and, in turn, endothelial cells can secrete mediators which promote mitochondrial biogenesis [16]. It has been previously demonstrated that the dysfunction of mitochondria and energy metabolism is implicated in the radioresistance of OAC [8]. Through the generation of an isogenic cell line model of radioresistant OAC, the radioresistant cells (OE33R) showed altered mitochondrial function and morphology, altered gene expression and bioenergetics, and enhanced clonogenic survival following radiation [8]. Despite increasing evidence describing the mechanisms regulating resistance to radiotherapy, there are no validated clinicopathological markers which can be used to identify patients who will benefit from neoCRT, and there are no radiosensitizers approved for clinical use. Moreover, while mitochondrial metabolism, angiogenesis and inflammation play key roles in mediating ionizing radiation effects, the intrinsic radioresistance of OAC remains poorly understood.

In this study, we identify potential proteins responsible for the radioresistance of OAC by characterising the intracellular protein profiles of our established isogenic cell line model of radioresistant OAC using a quantitative mass-spectrometry based proteomics approach. Comparative whole proteome analysis between radiosensitive OE33 cells (OE33P) and radioresistant OE33 cells (OE33R) identified 251 proteins regulating vital cellular processes that were differentially modulated in OE33R. OE33R cells showed altered bioenergetics accompanied by an inhibition of apoptosis. In addition, radioresistant cells were predicted to have an activation of inflammatory and angiogenic pathways, when compared to the radiosensitive cells. Importantly, in this study we identify proteomic signatures of radioresistance, which provide insights into the molecular mechanisms that regulate radioresistance in OAC. Additionally, we describe potential biomarker signatures for future investigations, which may assist the development of effective radiosensitisers to aid treatment decisions, and allow a more tailored cancer patient management.

2. Materials and methods

2.1. Cells lines and cell culture

The human OE33 oesophageal adenocarcinoma cell line was purchased from the European Collection of Authenticated Cell Cultures. The isogenic model of radioresistant OE33 oesophageal adenocarcinoma OE33P (radiosensitive) and OE33R (radioresistant) was generated, characterised and cultured in our institute as previously described [17]. For experiments, cells were seeded in 100 mm dishes (n=4 biological replicates). Once confluent, cell culture medium was removed and cells were rinsed once in PBS. Subsequently, 300 μl of PBS was added to the plates, cells were disrupted using a cell scraper and collected in labelled eppendorfs. Cells were centrifuged (14,000 rcf, 10 min), and supernatant discarded. Cell pellets were snap frozen in liquid nitrogen and transported on ice to Systems Biology Ireland for mass spectrometry (MS) preparation.

2.2. Sample preparation for mass spectrometry

Cell pellets were resuspended in a lysis buffer (2% SDS (Fisher Scientific), 0.1 M Tris–HCl pH 8, cOmplete Cocktail Tablets (Roche) and phosSTOP (Roche). Lysates were sonicated (Syclon ultrasonic cell disrupter), boiled (95 °C, 5 min) and centrifuged at 14,000 rcf for 10 min at 4 °C). Pierce BCA protein assay kit (Thermo Scientific) and a SpectraMax M3 (Molecular Devices) were used to obtain protein concentration. 0.1 M DTT was added to the protein samples, and the samples were heated at 95 °C for 5 min. Protein digestion and peptides purification prior to MS analysis was performed using the Filter Aided Sample Preparation (FASP) procedure with 30 kDa Vivacon spin ultracentrifugation units (Sartorius) as previously detailed [18]. A multi-step protein digestion method was used as described by Wisniewski and Mann [19]. 10 μg of digest for each sample was loaded in handmade C18 StageTips as described previously [20]. Digested and purified peptides were then resuspended in15 μl of 0.1% acetic acid, and analysed by mass spectrometry. Full details of sample preparation for mass spectrometry is reported in the Supplementary Methods and Materials.

2.3. Liquid chromatography and mass spectrometry

Mass spectrometry analysis was performed as previously described [21]. Briefly, 5μl of Lys-c/tryptic peptides from each sample was analysed by a Q-Exactive mass spectrometer (Thermo Scientific) operating in automatic data-dependant switching mode. Raw files were analysed and relative protein concentration and identification were determined by label-free quantification using the MaxQuant software suite [22]. MaxQuant software (version 1.5.0.25) was used to analyse the raw files with a human protein database (Uniprot HUMAN, release 2017_01) consisting of 20,242 entries. Default parameters were used in MaxQuant for label free quantification with the exception of digestion enzymes (LysC and Trypsin digests were separated between parameter groups). FDR=0.01 was used for peptide and protein identifications. Reverse and contaminant hits were excluded from the statistical analysis. Full details of liquid chromatography and mass spectrometry method is reported in the Supplementary Methods and Materials. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [23] partner repository with the dataset identifier PXD020303.

2.4. Mass spectrometry data analysis

Log2 transformed LFQ (label free quantification) was used for all analyses. Proteins were removed from the analyses if they were observed in fewer than 4 samples. Missing values for individual samples were replaced with the lowest observed value for that sample. Between-group comparisons were performed using a two-sided heteroscedastic *t*-test. The Benjamini and Hochberg) approach was used to calculate a false discovery rate (FDR). Proteins were considered differentially expressed if they had a p value< 0.05, FDR $< 10\%$ and an absolute difference in their median log2(LFQ) value greater than 1. Proteins found to be significantly altered between groups were classified for their GO term enrichment, specifically interrogating the database for cellular component, molecular function, and pathway analysis. To this aim, Ingenuity Pathway Analysis (IPA) (QIAGEN (Redwood City, CA)), STRING Database (v.11) and DAVID Bioinformatics Resources (v.6.8) were used. STRING was also run to obtain protein-protein interaction networks. The molecular activation prediction (MAP) algorithm in IPA was utilized to interrogate networks and pathways generated with up- and down-regulated proteins in our dataset, in order to simulate upstream and downstream effects of activation or inhibition of the associated network functions.

3. Results

3.1. LC-MS/MS analysis of OE33P and OE33R cell lines revealed 251 significantly modulated proteins

To determine mechanisms of radioresistance in oesophageal cancer cell lines we used an established mass spectrometry and bioinformatic analysis approach using n=4 biological replicates. A schematic representing the LC-MS/MS pipeline used for the analysis of OE33P and OE33R cell lines is shown in Supplementary Figure 1. In total, 5785 proteins were identified (Fig. 1A). Details of the entire protein list, including mass spectrometry data of the identified proteins in OE33P and OE33R cell lines can be found in Supplementary Table 1. To obtain a broad understanding of the proteome changes in the cells with a radioresistant phenotype, we performed a statistical analysis of the differences in protein levels between OE33P and OE33R cells, resulting in 251 significantly modulated proteins (p value<0.05, FDR<10%, difference greater than 1), of which 97 proteins were upregulated and 154 downregulated in OE33R cells, when compared to OE33P cells (Fig. 1B). Protein IDs and statistical details of the 251 significantly modulated proteins is reported in Supplementary Table 2. This data indicated that there are differences in the interactome of both cell lines which we hypothesised may contribute to radioresistance and relate to protein network rewiring.

3.2. Network and pathway analysis of the significantly modulated proteins showed that metabolism is increased in OE33R cells

Pathway reconstruction was performed using bioinformatic tools that allows us to identify protein network modules that are differentially regulated between the two cell lines. To this aim, network analysis was determined using the STRING database showing protein-protein interactions of the significantly modulated proteins. A network of proteins associated with "metabolic pathways" is highlighted in Fig. 2A, demonstrating a significant enrichment (FDR=0.001) of this pathway in OE33R, compared to OE33P. The initial whole proteome analysis identified differentially expressed proteins that can be related to physiological functions that are deregulated in radioresistant cells. Expanding on our initial findings, significantly modulated proteins were submitted to DAVID Bioinformatics Resources for Gene Ontology (GO) analysis. This analysis identified a number of significantly enriched pathways including metabolic pathways, proteoglycans in cancer, regulation of lipolysis in adipocytes, terpenoid backbone biosynthesis. TNF signalling pathway, VEGF signalling pathway, arachidonic acid metabolism, focal adhesion, microRNAs in cancer and Fc gamma R-mediated phagocytosis. amongst these, "metabolic pathways" is the most significant enriched pathway in our dataset (38/251 proteins, FDR=3.8E-05) (Fig. 2B). The Molecular Activation Prediction tool in IPA revealed many metabolic processes were modulated in the radioresistant OE33R (Fig. 2C). In particular, metabolic processes such as "metabolism of membrane and lipid derivative", "metabolism of prostaglandin", "metabolism of eicosanoids" and "metabolism of cholesterol" were predicted activated in OE33R; while "metabolism of nucleic acid components or derivatives" was predicted inhibited in OE33R. In addition, "cellular lipid metabolic process" was significantly altered in OE33R cell (Fig. 3A). These findings demonstrate that altered metabolism is a driver pathway altered in OE33 radioresistant subline. Furthermore, the observed altered expression levels of proteins such as PTGES, PTGE2, PLA2G4A, IDI1, CYP24A1, HMGCS1, KYNU, FDPS, GDA, MLYCD, and NT5E may represent a novel metabolic proteomic signature of radioresistance in OAC (Fig. 2C).

3.3. Gene Ontology and pathway analysis of the 251 significantly modulated proteins demonstrated that "angiogenesis" and "inflammatory response" were activated in the OE33R cell line compared to OE33P cells

DAVID Bioinformatics Resources was used to further characterise the 251 proteins significantly modulated between OE33P and OE33R cells in order to determine biological processes (Fig. 3A) and molecular functions (Fig. 3B) enriched in our dataset. Analysis of biological processes revealed proteins mapped to many cellular metabolic processes in the cells, as well as proteolytic and apoptotic processes (Fig. 3A). Additionally, IPA analysis revealed changes in other GO such as "angiogenesis" (Fig. 3C) and "inflammatory response" (Fig. 3D) showing that these pathways were activated in the radioresistant OE33R cell line compared to OE33P. Interestingly, angiogenesis and inflammation processes have been shown to regulate the response to ionising radiation in cancer cells and are associated with radioresistance. Therefore, analysis of the OE33R and OE33P cell line proteome has resulted in a potential pathway-specific proteomic signature of radioresistance in OAC; in particular, altered expression levels of ICAM1, LCN2, IFI16, MMP7, PIK3CA, PLA2G4A, PROCR, PTGES, RFN 213, DKK1, SEMA3A, SEMA3C, TGFBI, TGM2, TUBB3, CAV1, CAV2, CDKN2A, PTGS2 were associated with the activation of angiogenesis; while altered expression levels of ICAM1, LCN2, LGALS9, MGLL, MMP7, NFKBIA, NFRKB, PLA2G4A, PTGES, PTGS2, PYCARD, SIRPA, TGM2, TUBB3, TBC1D23, PROCR, CAV1, CYBA, PON2 and NT5E were associated with the inflammatory response pathway. These findings demonstrated that there is extensive rewiring of angiogenesis and inflammatory pathways in the process of acquiring resistance to ionising radiation.

3.4. Pathway analysis of the mitochondrial proteins significantly modulated in OE33R showed that "metabolic processes" are enriched and that "apoptosis" is inhibited in OE33R cells

The cytotoxic effects of ionising radiation are generated by direct and indirect cellular mechanisms, often altering the functionality of cellular organelles. Increasing evidence suggests that IR affects the biological processes on the endoplasmic reticulum, lysosome, plasma membrane, and profoundly alters mitochondrial function [24]. DAVID Bioinformatics Resource was used to obtain information regarding the cellular organelle localization of the 251 significantly modulated proteins (Fig. 4). This analysis revealed organelle specific signatures of proteins altered in OE33R and provided an overview of the proteome changes in radioresistant phenotype of OAC. One of the most prominent GO in this analysis was mitochondria with 31 significantly modulated mitochondrial proteins (p=0.031). Mitochondria are doubled membraned organelles that are at the junction of various important biological processes such as metabolism, gene expression, apoptosis, and ROS production [25-27]. Alteration of mitochondrial function in cancer have been associated with radioresistance [8]. To further investigate the role of the alteration of mitochondrial proteins in radioresistant OE33 subline, we performed pathway analysis of the 31 significantly modulated mitochondrial proteins in OE33R and OE33P cells (Fig. 5A and Supplementary Table 3). Noteworthy, in our proteomics analysis mitochondrial glutamate carrier 1 (SLC25A22) was increased 2.15 fold in OE33R, consistent with previously published data from our group in which mRNA levels of SLC25A22 was upregulated 2-fold in OE33R compared to OE33P [8]. STRING analysis of protein-protein interaction networks of the 31 mitochondrial proteins demonstrated these proteins were associated with "metabolic processes" (Fig. 5B). In addition, analysis of biological process (Fig. 5C) of the significantly modulated mitochondrial proteins obtained using DAVID confirmed that many of the metabolic processes regulated by mitochondria, are significantly

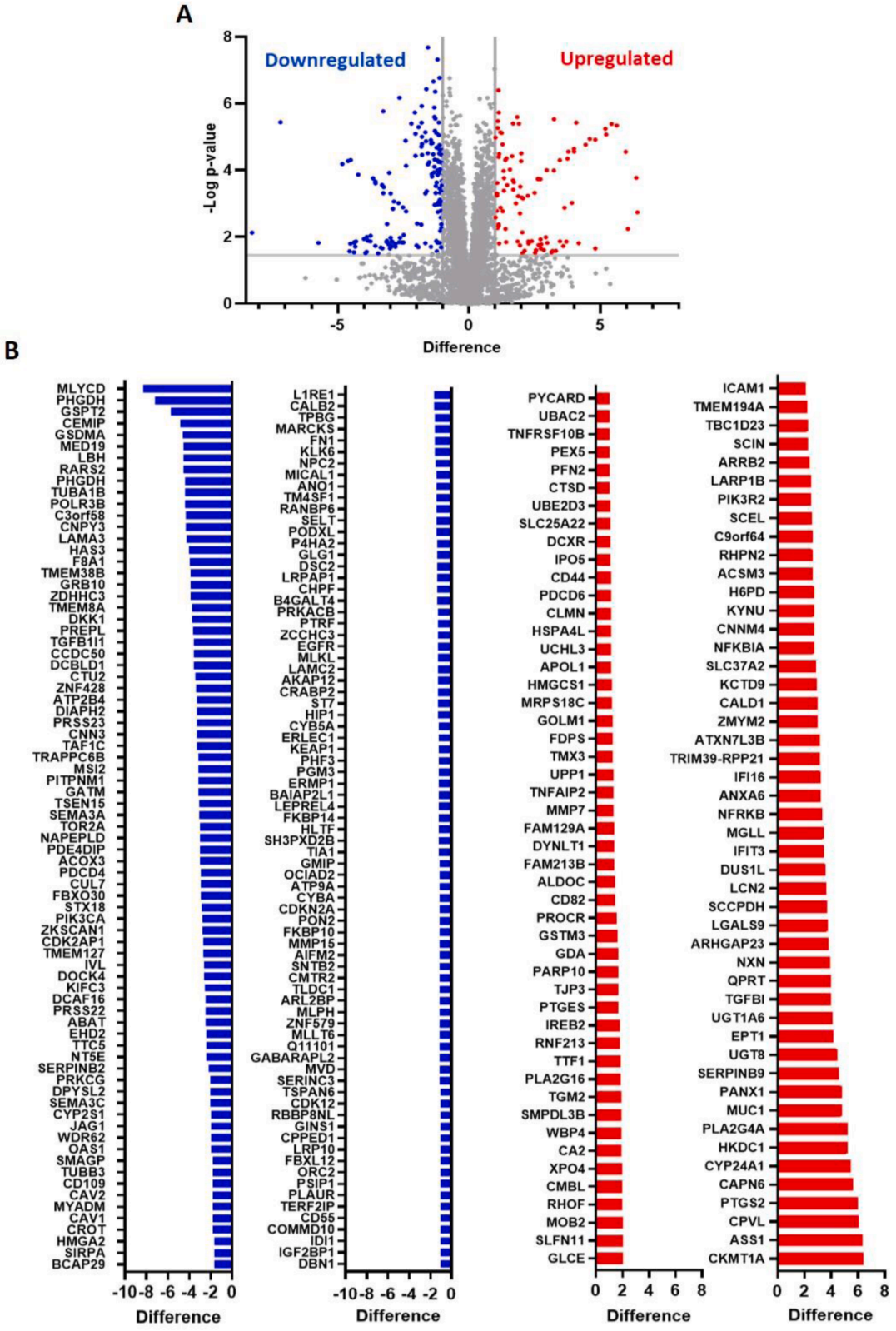
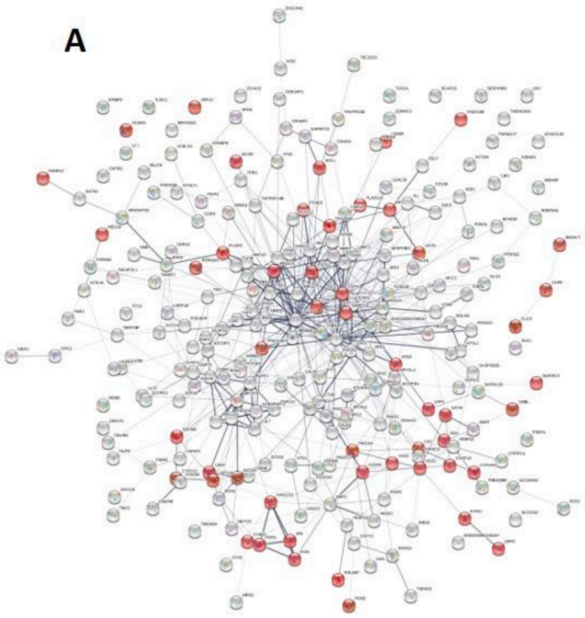


Fig. 1. LC-MS/MS analysis of OE33P and OE33R cell lines revealed 251 significantly modulated proteins. OE33P and OE33R cells (n=4 biological replicates) were lysed and the intracellular proteins were subjected to Multi-Enzyme Digestion Filtered Aided Sample Preparation (MED-FASP) procedure for LC-MS/MS analysis. Identified proteins were quantified using MaxQuant software. Bioinformatic analysis including STRING, DAVID Bioinformatics Resources, and IPA platforms were used to generate networks and perform pathways analysis. **(A)** Volcano plot displaying 5785 proteins identified in our dataset. The y-axis corresponds to the log10 (p-value), and the x-axis shows the "difference" values (OE33R-OE33P). The colored dots represent the 251 significantly modulated proteins; blue dots represent the proteins downregulated in OE33R and red the proteins upregulated in OE33R when compared to OE33P ($p<0.05$, FDR 0.1, Difference>1). Grey dots show the proteins whose statistical analysis did not reach significance. **(B)** Graph showing the difference level of the 251 significantly modulated proteins. Blue bars represent the 154 decreased proteins in OE33R and the red bars the 97 significantly increased proteins in OE33R, when compared to OE33P.



B

Pathway analysis

	Count	%	FDR
Metabolic pathways	38	15.2	0.00004
Proteoglycans in cancer	11	4.4	0.00129
Regulation of lipolysis in adipocytes	6	2.4	0.00191
Terpenoid backbone biosynthesis	4	1.6	0.00491
TNF signaling pathway	7	2.8	0.00713
VEGF signaling pathway	5	2	0.01610
Arachidonic acid metabolism	5	2	0.01610
Focal adhesion	9	3.6	0.01700
Ether lipid metabolism	4	1.6	0.03470
MicroRNAs in cancer	10	4	0.03890
Fc gamma R-mediated phagocytosis	5	2	0.04510

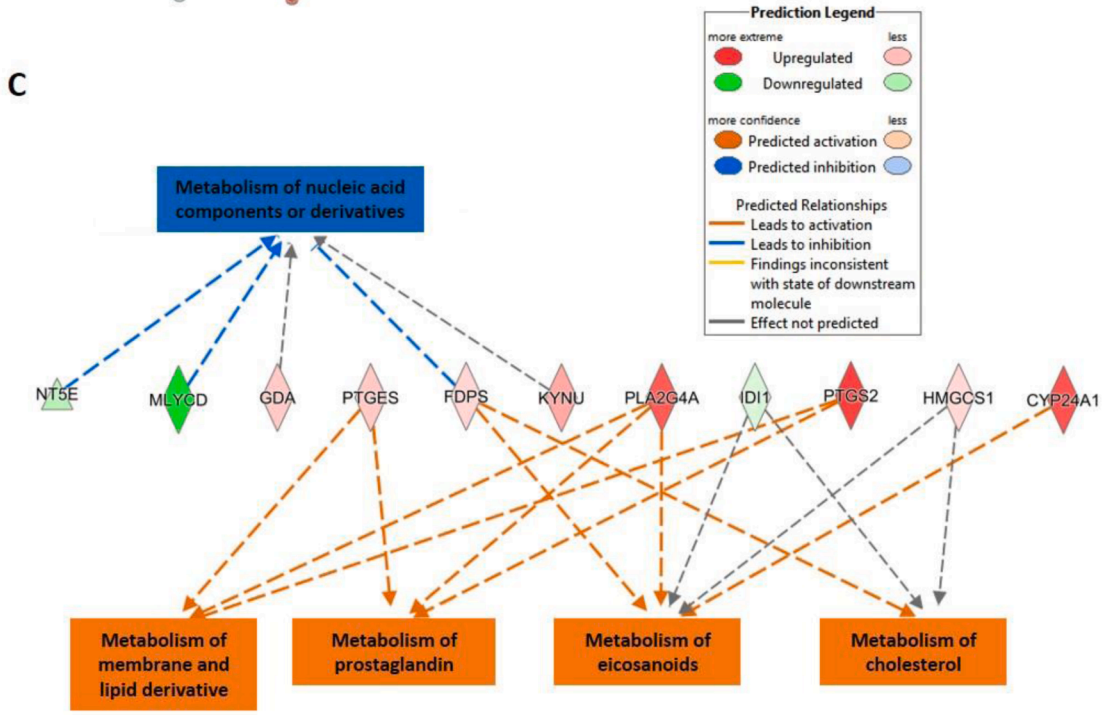


Fig. 2. Network and pathway analysis of the significantly modulated proteins showed that metabolism is increased in OE33R cells. (A) Proteins interaction networks of the 251 identified proteins (showed as nodes in the network) modulated in OE33R compared to OE33P (STRING v11) is illustrated here. Proteins associated with "metabolic pathways" are highlighted in red. **(B)** Significantly modulated proteins were submitted to DAVID for Gene Ontology analysis and the results for KEGG pathway analysis are reported here; "metabolic pathways" is the most significant and enriched pathway in OE33R cells. **(C)** Analysis of the significantly modulated proteins achieved with IPA for "metabolism" pathway. IPA analysis generated regulatory relationships between the decreased (green) and increased (red) proteins in our dataset; the "Molecular Activation Prediction" tool demonstrated that "metabolism of membrane and lipid derivative", "metabolism of prostaglandin", "metabolism of eicosanoids" and "metabolism of cholesterol" are positively regulated in OE33R (orange lines); "metabolism of nucleic acid components or derivatives" is negatively regulated in OE33R (blue lines); grey line illustrates that the effect is not predicted.

altered in OE33R cells. Interestingly, as shown in Fig. 5D, IPA Molecular Activation Prediction tool applied to the 31 mitochondrial proteins predicted that "Apoptosis" is inhibited in OE33R, when compared to OE33P.

4. Discussion

The identification and characterization of alterations in the proteome of radiation resistant cancer cells can provide insights into the mechanisms which regulate the acquisition of a radioresistant phenotype. Radioresistance is a multifactorial process characterised by the adaptation of cancer cell to ionising radiation, by changing cellular processes to enable survival of radiation-induced damages [28]. Despite increasing evidence demonstrating the various cellular processes altered in radioresistant cancers, it is still not clear the cellular mechanisms which regulate these altered processes [29]. For the first time, we performed a comprehensive proteomic characterization of our *in vitro* isogenic cell line model of acquired radioresistance in OAC [17]. Using a

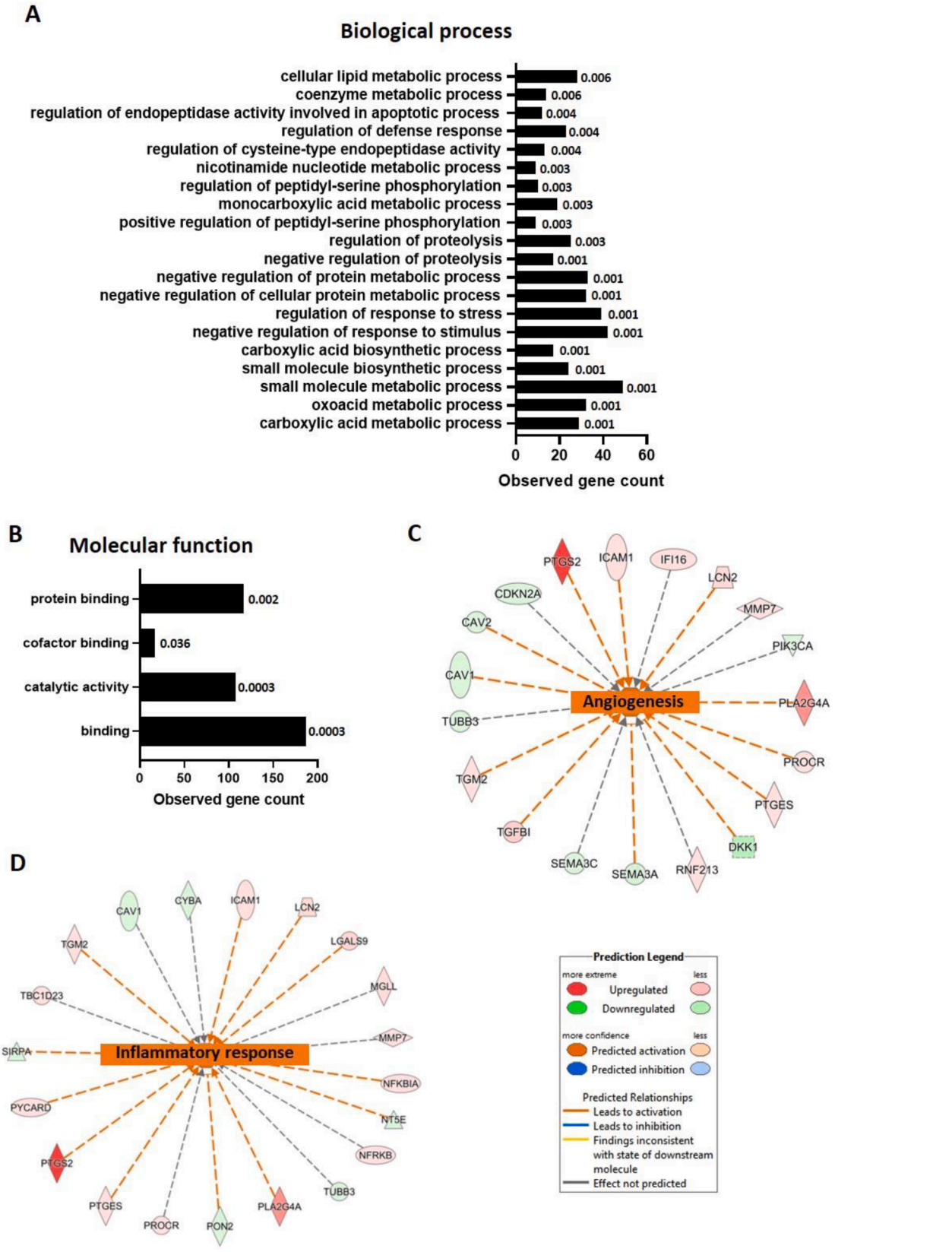


Fig. 3. Gene Ontology and pathway analysis of the 251 significantly modulated proteins in OE33R, when compared to OE33P. Biological process **(A)** and Molecular function **(B)** of the significantly modulated proteins were obtained using DAVID Bioinformatics Resources. Numbers represent the FDR values. **(C, D)** Pathway analysis of the significantly modulated proteins showed that angiogenesis (C) and inflammatory response (D) are activated in OE33R. IPA analysis generated regulatory relationships between the decreased (green) and increased (red) proteins in the "angiogenesis" and "inflammatory response" processes; the "Molecular Activation Prediction" tool demonstrated that "inflammatory response" and "angiogenesis" were positively regulated in OE33R (orange lines), when compared to OE33P; grey line illustrates that the effect is not predicted.

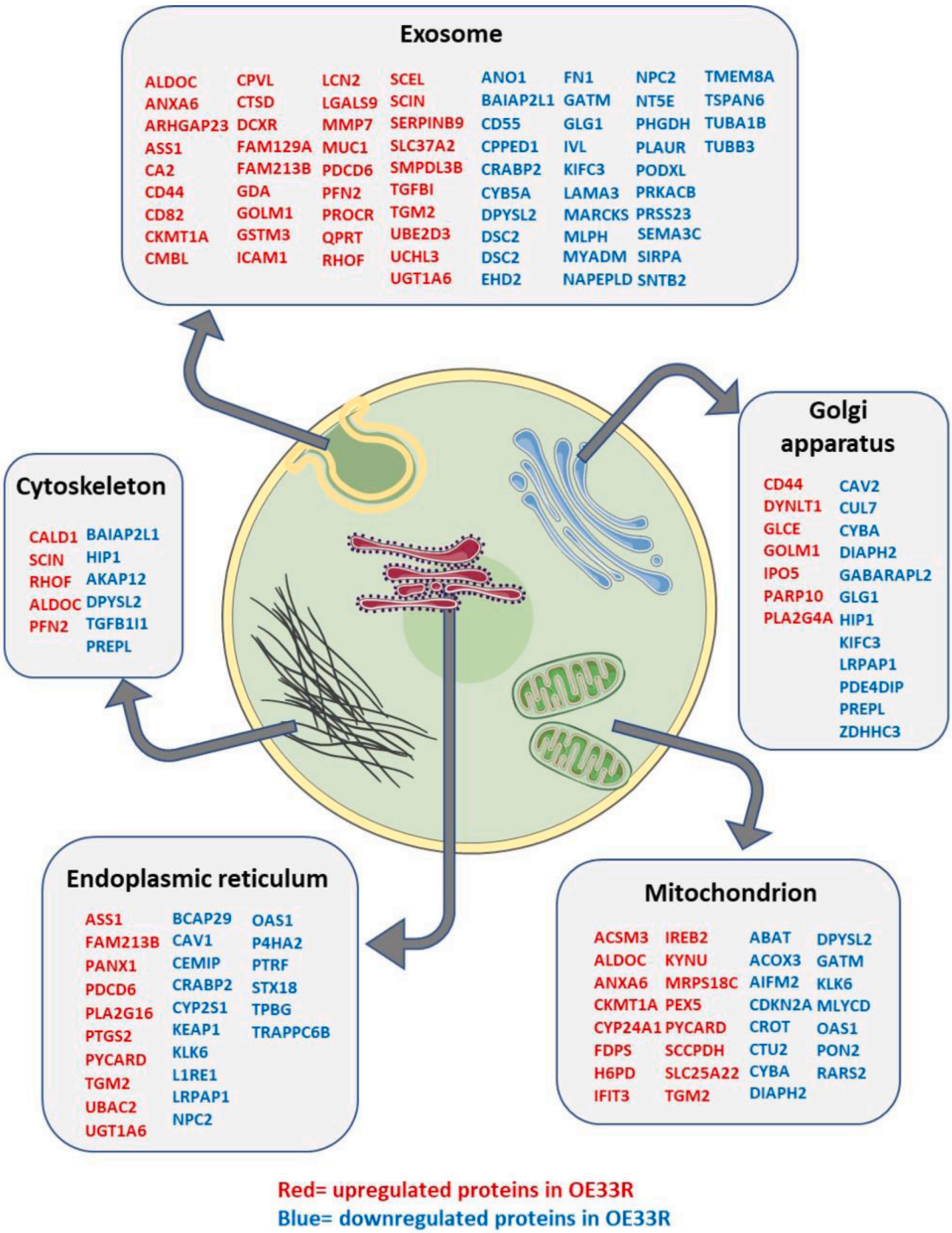


Fig. 4. Cellular compartment analysis of the 251 significantly modulated proteins in OE33R, when compared to OE33P. Cellular component analysis was obtained using DAVID Bioinformatics Resource. Down-regulated proteins in OE33R, when compared to OE33P, are shown in blue and upregulated proteins in red in the various cellular organelles.

bioinformatic approach to interrogate our proteomic dataset and obtain an in depth knowledge of the cellular pathways altered in the OAC radioresistant cell line, OE33R. We found that metabolic pathways, angiogenesis and inflammation were key players in the development of a radioresistant phenotype, and these findings were supported by our previous published data [8]. Interestingly, metabolism, angiogenesis and inflammation are emerging hallmarks of cancer [30], which play a crucial role in the response to ionising radiation [28]. For the first time, we identified proteomic signatures of radioresistance in OAC, including altered level of PTGES, PTGS2, PLA2G4A, IDI1, CYP24A1, FDPS, MLYCD and NT5 for the metabolic pathway; altered level of ICAM1, LCN2, IFI16, MMP7, PIK3CA, PLA2G4A, PROCR, PTGES, RFN213, DKK1, SEMA3A, SEMA3C, TGFBI, TGM2, TUBB3, CAV1, CAV2, CDKN2A, PROCR, PTGS2 for the angiogenesis pathway; altered level of ICAM1, LCN2, LGALS9, MGLL, MMP7, NFKBIA, NFRKB, PLA2G4A, PTGES, PTGS2, PYCARD, SIRPA, TGM2, TUBB3, TBC1D23, PROCR, CAV1, CYBA, PON2 and NT5E for the inflammatory response pathway. Noteworthy, ten of these proteins are common to two or more pathways including ICAM1, LCN2, MMP7, PLA2G4A, PROCR, PTGES, PTGS2, TGM2, TUBB3 and CAV1 thus indicating that the proteins have more

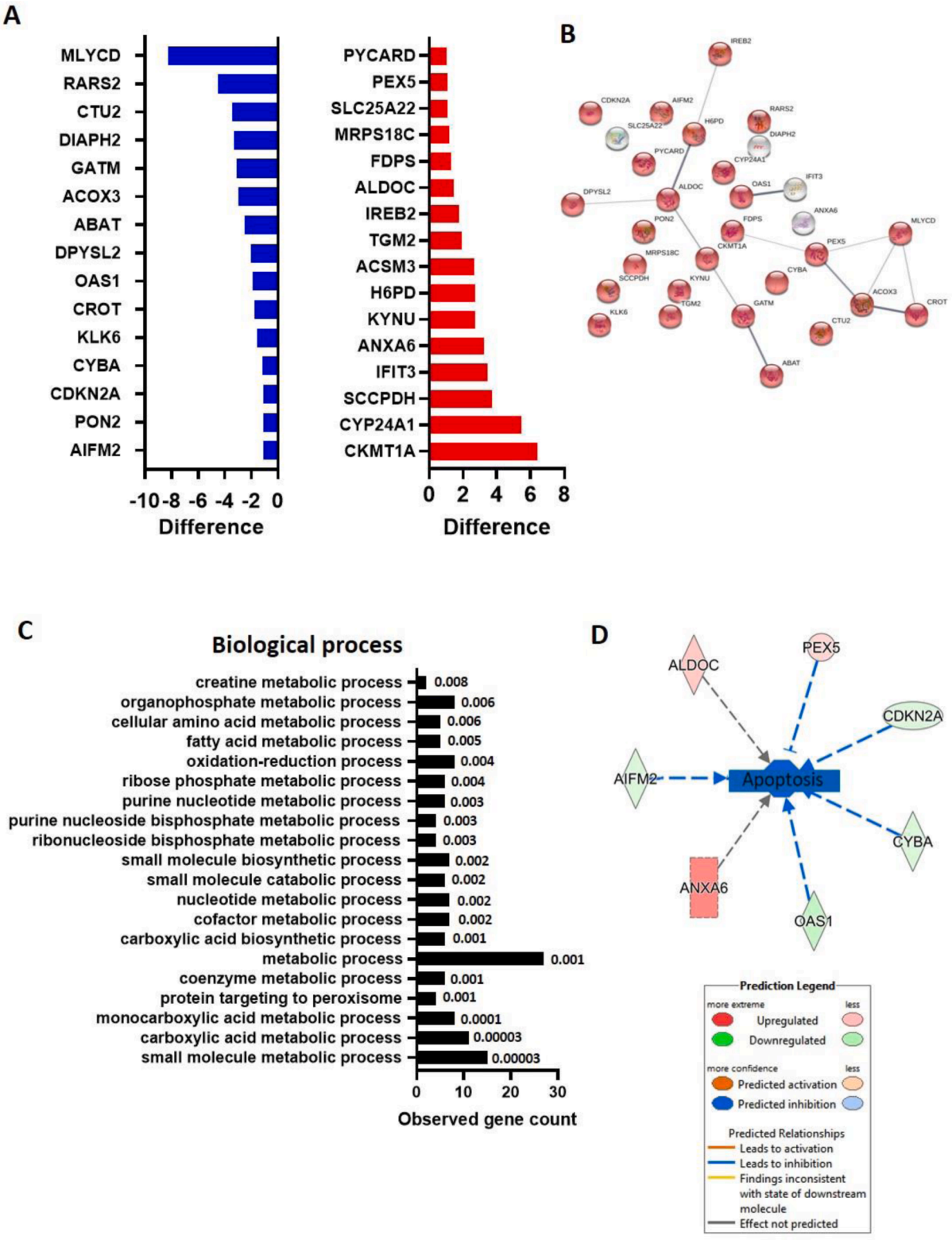


Fig. 5. Pathway analysis of the mitochondrial proteins significantly modulated in OE33R showed that "metabolic processes" are enriched and that "apoptosis" is inhibited in OE33R cells. (A) Graph showing the "Difference" level of the 31 significantly modulated proteins in the mitochondria. Blue bars represent significantly decreased proteins, and the red bars increased proteins in OE33R, when compared to OE33P. **(B)** Proteins interaction networks of the 31 mitochondrial proteins (showed as nodes in the network) significantly modulated in OE33R vs OE33P (STRING v11) is illustrated here. Proteins associated with "metabolic processes" are highlighted in red. **(C)** Enriched biological process of the significantly modulated mitochondrial proteins were obtained using DAVID Bioinformatics Resources. Numbers indicate FDR values. **(D)** IPA analysis generated regulatory relationships between the decreased (green) and increased (red) mitochondrial proteins in our dataset; the "Molecular Activation Prediction" tool demonstrated that "Apoptosis" is predicted to be inhibited in OE33R (blue lines); grey line illustrates that the effect is not predicted.

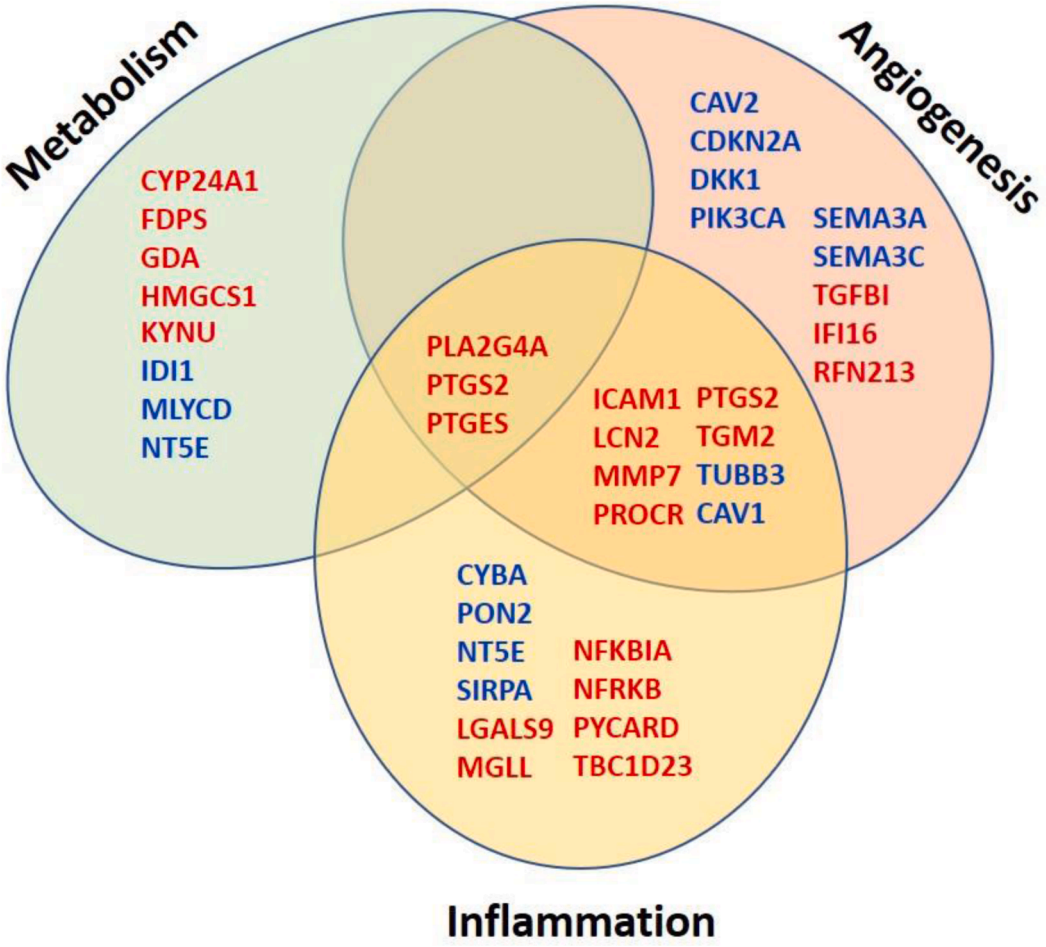


Fig. 6. Cross-pathway proteomic signatures of radioresistance in OAC. IPA analysis revealed that metabolism, angiogenesis and inflammation are altered pathways in OE33R cells. Here, we highlighted the proteomics signatures identified for the three pathways and the relationships between them. Down-regulated proteins in OE33R, when compared to OE33P, are shown in blue and upregulated proteins in red.

than one function and that they are included in the same hierarchical cluster that comprise these pathways. Therefore these proteins may provide a potential cross-pathway proteomic signature of radioresistance in OAC (Fig. 6). Interestingly, some of these proteins have been already subject of investigation for their role in cancer and radioresistance. For example, protein-glutamine gamma-glutamyltransferase (TGM2) increased 3.5-fold in OE33R in our analysis, and is associated with both inflammatory and angiogenesis pathways. Protein-glutamine gamma-glutamyltransferase is a stress responsive protein that has been found increased in many cancer types, particularly in treatment resistant cancers [31-33]. Additionally, it has been reported that TGM2 expression is increased in breast cancer and its overexpression had a validity as a prognostic marker for metastatic and treatment resistant breast cancer [32]. It is well known that ionising radiation triggers inflammatory response, and our results indicate that a number of proteins related to inflammation are altered in our radioresistant subline. A significant increase in ICAM1, PLA2G4A, PTGS2 was observed in the OE33R cell line similar to previously published studies in radioresistant glioblastoma, demonstrating the potential predictive value of these genes [34]. The role of prostaglandin E synthase (PTGES), which we identified is overexpressed in OE33R cells and is common to all 3 pathways of metabolism, inflammation and angiogenesis, was previously found to be dysregulated in non-small cell lung cancer cells and linked to the promotion of tumour migration and metastasis [35]. Furthermore, it has been reported that targeting the synthesis of PGE_2 by inhibitors of PGES or the modulation of the activity of its receptors EP2 and EP4 represent a useful strategy to overcome treatment resistance to radiotherapy [36]. The use of isogenic radioresistant cell lines represents a valid approach to investigate the cellular processes regulating resistance too IR in cancer cell [29]. For example, it has been demonstrated that a comparative analysis of the levels of certain genes in breast cancer isogenic cell lines showed that CDKN1A and SOD2 are significantly increased in the radioresistant cells, and the higher expression of these genes was predictive of a poor prognosis [37]. Interestingly, in our dataset we found that Cyclin-dependant kinase inhibitor 2A (CDKN2A) together with other two members of cyclin-dependant kinase pathway, CDK12 and CDK2AP1, were significantly altered in OE33R cells when compared to OE33P. Previous studies using isogenic models of radioresistance have shown that resistance to apoptosis, and high DNA repair capacity, are potential mechanisms of radioresistance in cancer [17, 38-42]. Here, using a proteomic approach we found that apoptosis is significantly diminished in our radioresistant OAC model and we provided a potential signature of mitochondrial proteins which inhibited apoptosis by increasing the levels of ALDOC, PEX5 and ANXA6 and decreasing the levels of AIFM2, OAS1, CYBA and CDKN2A. These findings are consistent with our previously published data in which we demonstrated that OE33R have enhanced clonogenic survival [8]. In addition, other studies using *in vitro* models of radioresistance, showed an association of radioresistance with other cellular mechanisms such as increased cell migration [38,43], altered antioxidant balance [37,44], epithelial-mesenchymal transition [45] and immune systems [46].

Due to a lack of available radiosensitisers that can be used to overcome resistance, there is an unmet need to identify mechanisms regulating this radioresistance and target them therapeutically. Increasing

evidence is showing that drugs affecting cellular bioenergetic such as metformin [47,48] and pyrazinib [49] have a potential to be used as radiosensitisers, highlighting the importance of altered metabolism in the resistance to IR. Metabolic reprogramming is defined as an altered cellular metabolic profile in cancer cells, and it is classified as an hallmarks of cancer [50]. Mitochondria have a crucial role in the metabolic reprograming in cancer. In fact, we have previously demonstrated that mitochondria have the ability to change their metabolic profile in response to the enhanced energy level required by the cancer cells [8]. In fact, mitochondria can modulate the energy production profiles in the cells. Interestingly, resistance to IR has been linked to the modification of mitochondrial energy metabolism, morphology and functions in cancer cells [8]. In this study, we identified for the first time 31 proteins significantly modulated in the mitochondria of radioresistant cells that may represent the potential biomolecules responsible for this radioresistant phenotype through rewiring of mitochondrial functions in OAC.

While this study demonstrates for the first time a comprehensive proteomic profile of the established isogenic cell line model of radioresistant OAC, the authors acknowledge that there are limitations to the study design. The study has been conducted in a single cell line and as such the results may only be specific to OE33 cells. However, the oesophageal cancer cells used here represent a well established and characterised isogenic model of radioresistance, and the proteomic analysis reported here provided an in-depth insight on the altered proteins in radioresistant cells and signatures of OAC radioresistance. In addition, this study provides a valid approach for the analysis of cancer tissue proteome.Translating the findings from this *in vitro* cell line model to proteomic analysis of tumour samples from OAC patients could result in a significant resource of novel predictive biomarkers of disease and response to treatment.

Additionally, while the shotgun proteomic approach used in the current study enabled the identification of 5785 proteins, it is limited in the detection of very low abundant proteins and specific subcellular structure proteins, such as membrane proteins. Physicochemical fractionation of whole cell lysates may be required to reduce the sample complexity prior to LC-MS/MS in order to achieve identification of low-abundance proteins or integral membrane proteins.

In conclusion, analysis of multifaceted signatures of radioresistance, such as the OE33R proteome reported here, provides an essential biological ground for deciphering the cellular mechanisms regulating radioresistance in OAC. In this study, we identified alteration of inflammation, angiogenesis and metabolism-related factors as key mechanisms regulating radioresistance in OAC. Cross-validation of these results is necessary to develop a robust biomarker signature panel of radioresistance that can be translated to the clinic.

CRediT authorship contribution statement

Simone Marcone: Investigation, Data curation, Formal analysis, Visualization, Writing - review & editing. **Amy Buckley:** Investigation. **Colm J. Ryan:** Methodology, Supervision, Formal analysis, Writing - review & editing. **Mark McCabe:** Investigation. **Niamh Lynam-Lennon:** Resources, Data curation, Writing - review & editing. **David Matallanas:** Methodology, Supervision, Writing - review & editing. **Jacintha O`Sullivan:** Conceptualization, Supervision, Writing - review & editing. **Susan Kennedy:** Conceptualization, Investigation, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ctarc.2021.100376.

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