Journal of Proteomics 262 (2022) 104600

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Proteomic profiling of metabolic proteins as potential biomarkers of radioresponsiveness for colorectal cancer

Md Zahirul Islam Khan, Shing Yau Tam, Zulfikar Azam, Helen Ka Wai Law *

Department of Health Technology and Informatics, Faculty of Health and Social Sciences, The Hong Kong Polytechnic University, Hung Hom, Hong Kong, China

ARTICLE INFO

Keywords:
Colorectal cancer (CRC)
Radiation therapy (RT)
Metabolic proteins
Radioresponsiveness
Biomarkers

ABSTRACT

Surgery, radiation therapy (RT), and chemotherapy are commonly used treatment modalities for CRC management. The locally advanced CRC is managed with preoperative RT or in combination of chemoradiotherapy whereas palliative RT is recommended for metastatic CRC patients to enhance overall survival and reduce distressing symptoms. There are many biomarkers established based on tumour staging, grading and molecular characteristics of patients (e.g., mutation, DNA methylation, and gene expression profiling). Interestingly, none of these markers are adequately validated for RT scheme. In order to establish the radioresponsive biomarker in CRC, we established a mouse xenograft tumour model and applied radiation to the tumours. We identified 9 metabolic proteins, namely PGK1, PGAM1, ENO1, PKM, TKT, GLUD1, LDHA, GAPDH, and MDH2, which are differentially expressed in tumours with different radioresponsiveness. Furthermore, we validated their expression in tumours from the unirradiated, poorly responded and highly responded tumour groups. In addition, we analysed their expressions in clinical samples from the public database. Extensive literature studies shown that these metabolic proteins are associated with key biochemical pathways including, glycolysis, ammonia detoxification, carcinogenesis, and drug responses. Further studies are needed to translate our findings into clinical use.

Significance: With the increasing incidence of colorectal cancer (CRC) globally, it is crucial to establish strategic treatment protocol by personalizing cancer treatment. Despite the well-established treatment protocols for CRC in the past decades, the mortality remains high. There is a trend of applying personalized treatment to improve patient survival. It has been reported that biomarkers may be used to predict treatment outcomes or to adjust individual treatment protocols. This project aims to identify specific metabolic proteins as biomarkers for CRC radioresponsiveness. Using bioinformatical analysis, we have identified 9 metabolic proteins which could be used as potential biomarkers for radiation therapy in CRC tumours.

1. Introduction

Globally, colorectal cancer (CRC) is the third most commonly diagnosed malignancy and ranked second for cancer mortality [1]. GLOBOCAN and World Health Organization cancer statistics reported 1.8 million new CRC cases with approximately 0.9 million death worldwide [1,2]. The rate of new cases and mortality are increasing due to delayed diagnosis, development of metastasis and recurrence. The CRC is commonly classified into several stages according to the American Joint Committee on Cancer (AJCC)/ Union for International Cancer Control (UICC) tumour, node, and metastasis (TNM) system. In addition, the subtypes of CRC can be classified by tumour anatomical sites in the proximal colon, distal colon, and rectum [3]. Majority of CRC cases are adenocarcinoma with cancer invaded deeper layers of the colon or rectum and has spread to nearby lymph node system. Among the newly diagnosed cases, 23.5% have already metastasized to other organs (Stage IV) [4].

Surgery remains the primary treatment option for CRC however the role of adjuvant RT should not be undermined. Many advanced staged rectal or sigmoid colon cancer patients require chemoradiotherapy treatment along with primary surgical treatment [5,6]. Currently, standard RT for rectal or sigmoid colon cancer is 3-dimensional

* Corresponding author at: Department of Health Technology and Informatics, Faculty of Health and Social Sciences, The Hong Kong Polytechnic University, Hung Hom, Hong Kong, China.
E-mail addresses: zahir.islamkhan@connect.polyu.hk (M.Z. Islam Khan), marco-shing-yau.tam@polyu.edu.hk (S.Y. Tam), zulfikar.azam@connect.polyu.hk (Z. Azam), hthelen@polyu.edu.hk (H.K.W. Law).

https://doi.org/10.1016/j.jprot.2022.104600
Received 7 December 2021; Received in revised form 25 April 2022; Accepted 1 May 2022
Available online 6 May 2022

conformal RT (3DCRT) locally, which allows target localization and dose analysis of target volumes and organs at risk via 3D planning and dose volume histograms. Recently, the intensity-modulated radiotherapy (IMRT), volumetric arc therapy (VMAT), helical tomotherapy, and proton therapy are also widely used in medium risk, locally advanced and inoperable rectal cancer [7]. Due to the advances in RT equipment and treatment planning system, conformation of radiation dose to target tumour and radiation dose reduction to surrounding normal tissues could be achieved. Hence, tumour control could be potentially improved by dose escalation with reduced incidence of acute and late radiation toxicities to the bowel.

RT significantly boost up the treatment outcomes of surgery or chemotherapy by extending the overall survival and diseases free survival of the patients [8]. In addition, palliative RT is also effective in reducing the symptoms and extending survival of metastatic patients [9]. Equally, advanced techniques of RT for inoperable and elderly patients have been developed for enhancing the prognosis and disease control [7].

Tumour response to RT largely depends on the tumour heterogeneity even if they are diagnosed with the similar cancer type [7,10]. Despite of significant improvements have been made in RT techniques associated with precise delivery of radiation to localized tumours [11], there are still some obstacles for establishing personalized RT due to heterogeneity in the location, physiology, and genomic features of the tumours [12]. The tumour radiophenotype is regulated by a number of factors including clonogene number, rate of DNA damage, ratio of cell growth, immunogenicity of cells, and oxygenation of cells [12]. Precise genomic biomarkers for RT could reflect a universal radiophenotype for a distinct tumour type yet and long-term prospective study is need to establish it [13]. Over the past decade, gene mutation-based prognostic biomarkers have been proposed for systemic single-agent-targeted treatment approach. For example, KRAS/BRAF/NRAS are the most commonly used mutation-based biomarkers for metastatic CRC or metastatic melanoma [14–16]. Moreover, cancer antigen 125 (CA125), carbohydrate antigen 19–9 (CA19–9), carcinoembryonic antigen (CEA), prostate-specific antigen (PSA) are currently used clinical biomarkers for ovarian cancer, pancreatic cancer, CRC and prostate cancer respectively but they are not RT specific [17,18].

In proteomic studies, researchers perform quantitative mass spectrometry (MS)-based techniques for the identification and validation of differentially expressed protein (DEPs) to establish biomarkers for diagnosis, prognosis, and treatment of diseases [19,20]. Moreover, the translational proteomics emphasizes the advancement of proteomics and its powerful quantitative analysis to determine pathogenic protein expression profiles and its associated phenotypes that can be contributed to the clinical practice [20]. The rapid development in high-throughput omics technologies has identified numerous biomarkers candidate for cancer diagnosis, prognosis, and treatment. In CRC, the KRAS and NRAS mutations are the most conventionally used prognostic biomarkers for anti-EGFR therapy, and chemotherapy in metastatic CRC patients [21–24]. Yet, none of these studies has concluded the correlation of their molecules with RT response. In addition, many biomarker candidates have been used to predict the individual treatment outcome however they were established mainly based on the TNM stages [25]. Despite of the advancement in technology, these biomarkers have not been adequately validated for RT planning. Most importantly, there is no marker for predicting the radioresponsiveness of CRC tumours to RT treatment.

The application of high-throughput proteomics generates big datasets. Therefore, it is mandatory to use various powerful bioinformatics tools to categorize the proteins for their experimental validation. The application of bioinformatics pipelines are highly depends on the type of disease and nature of the study. In addition, the selection of appropriate single or combined bioinformatics tools required previous literature studies, robust consensus of the generated data, and experimental validation of overall findings. In the current study, we used several bioinformatics tools to establish RT responsiveness biomarkers for CRC and validated our findings by western blotting and analysing public database. Herein, using a mouse model inoculated with human CRC cells, we have shortlisted some specific metabolic proteins that are differentially expressed in between tumours that responded well to RT and those with poor response. We propose that specific metabolic proteins may be used as predictive biomarkers for radioresponsiveness. The findings could provide new insight in the development of prospective clinical trials for improving the efficacy of RT for CRC patients and enhance their survival.

2. Materials and methods

2.1. Ethical statement

The study was approved by the Animal Subjects Ethics Subcommittee (ASESC) of Centralized Animal Facility, The Hong Kong Polytechnic University (18–19/69-HTI-R-GRF). The animal experiments were carried out in guidance with the licensing agreement of the Centralized Animal Facility, The Hong Kong Polytechnic University, and Department of Health (DH/SHS/8/2/4 Pt.7), Hong Kong SAR. There was no cruelty of animals during the experiments. Proper procedure was followed in mice sacrifice.

2.2. Cell line and culture condition

Human CRC HT-29-Red-Fluc cell line was acquired from PerkinElmer, Inc. (Waltham, USA). HT-29 cells were maintained with 10% fetal bovine serum (FBS), (Gibco, USA) in Dulbecco's modified eagle medium (DMEM, Gibco, USA). Cell culture was maintained at 37 °C in 5% CO_2 in 100% humidity.

2.3. Mice model

Six to eight weeks old male nu/nu mice were purchased from the Laboratory Animal Services Centre of The Chinese University of Hong Kong. During the project, the mice were housed in Centralized Animal Facility, The Hong Kong Polytechnic University. They were being kept in individually ventilated cages with appropriate food and water supply. Before starting of the experiments, the mice were adopted with the living environment and sacrificed if they failed to thrive after tumour inoculation.

2.4. Tumour inoculation and measurement of tumour size

Half million of HT-29-Red-Fluc cells were suspended in 100 μL of complete medium. The cell suspension was then injected subcutaneously to the hind leg region. To increase the sample size and reduce the number of total mice used, cells were injected into both hind legs of mice for developing two individual tumours.

A high accuracy (0.1 mm) digital caliper was used to measure the mice tumour size. After visualization of prominent tumour growth, measurements of length and width (longer and shorter side) were counted two times a week and the tumour size was calculated by using the formula described by Sapi and colleagues [26].

$$\text{Volume } (mm^3) = \text{Length (mm)} \times \text{Width}^2\ (mm^2) \div 2$$

2.5. Irradiation and selection of tumours for proteomics

After the tumour volume reached 200–300 mm^3 (about 3–4 weeks post CRC inoculation), irradiation that mimicked RT treatment was conducted with 140 kV and 3 mA setting. The mice received a single postero-anterior field RT treatment of 15 Gy in the tumour site. Unirradiated mice were used as control. After irradiation, the tumour size was measured two times a week for 4 weeks to monitor the RT effectiveness (Fig. 1A-B). The RT response was defined by the reduction of

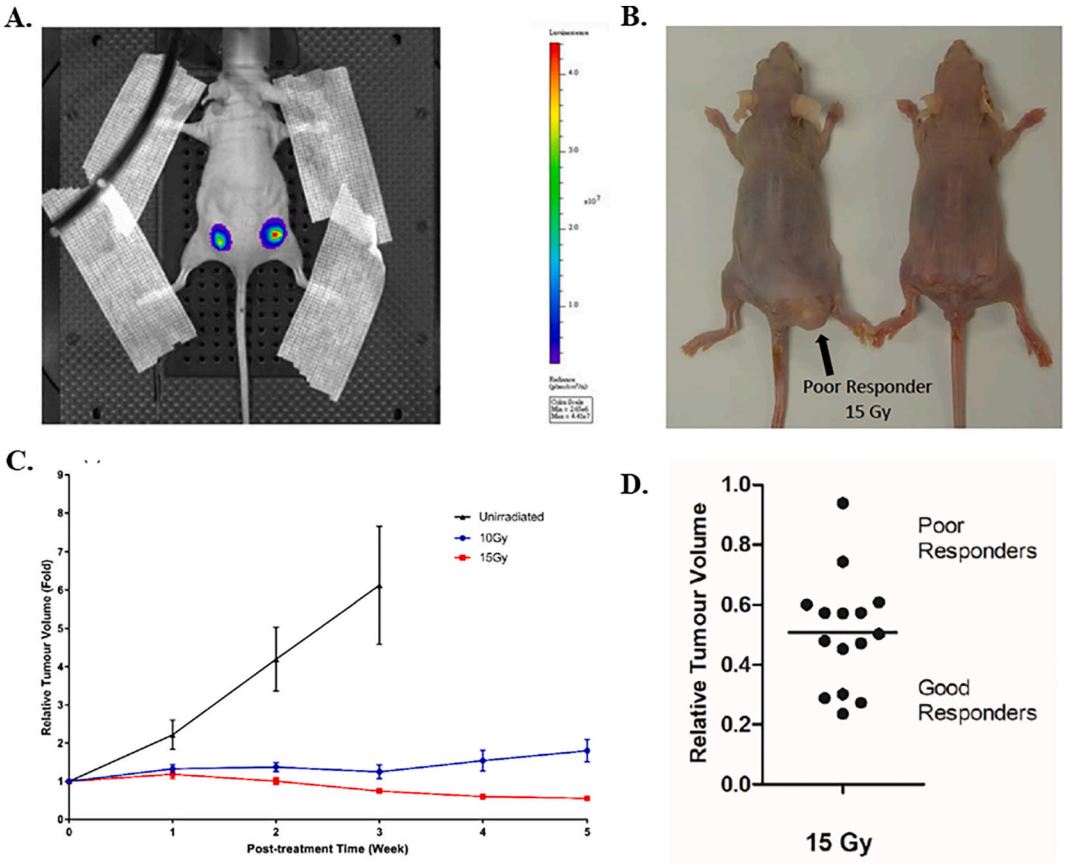

Fig. 1. Establishment of HT-29 CRC tumour xenograft model and irradiation protocols. (A) The CRC tumours can be detected by bioluminescence imaging or digital caliper measurement. (B) About 4–5 weeks after post irradiation, mice were sacrificed administrating an over-dose of anesthetics. As shown by the black arrow, tumour responsiveness against radiation therapy vary even the same mouse. (C) The fold changes in tumour size relative to the initial tumour volume immediately before irradiation in mice receiving no radiation, 10 Gy or 15 Gy irradiation was plotted against time. (D) Tumours were collected from the post 15 Gy irradiated ice were ranked according to their relative size to the original tumours.

tumour size from the initial irradiation treatments day to the last day of the experiments (Fig. 1C). After 4 weeks, the mice were sacrificed. The tumour actual size and metastatic natures were further verified after dissecting the tumour site and the tumour samples were used for further analysis.

After collecting the tumour samples, we ranked the samples into three groups based on shrinkage of tumours size against RT. The three groups are (1) control group (unirradiated), (2) poor RT response, and (3) good RT response (Fig. 1D). Higher tumour size reduction was considered as better response. At least three samples from each group were chosen for conducting the proteomics study.

2.6. MS sample preparation for proteomics analysis

MS samples were prepared using EasyPep Mini MS Sample Prep Kit (Thermo Scientific, USA) according to manufacturer's protocol. Briefly, 5 mg of tissues were taken in a centrifuge tube and disrupted with tissue homogenizer after adding 100 μL lysis solution. Universal nuclease was added along with lysis solution for complete digestion of nucleic acid contents in the mixtures. The purified proteins were collected after centrifugation and concentrations were measured using Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Then, 50 μg of proteins were obtained, and final volume adjusted to 100 μL with lysis solution. Afterwards, reduction and alkylation were performed by using reduction solution and alkylation solution. To block the reduction and alkylation, 10 min of heat incubation was performed. For digestion, reconstituted enzyme solution (Thermo Scientific, USA) was added to the preparation and incubated at 37 °C for overnight and digestion was stopped by adding Digestion stop solution (Thermo Scientific, USA). Thereafter, the peptide solutions were desalted and cleaned by using a series of washing in a C18 Peptide Clean-up column. Lastly, the cleaned peptides were collected by 70% acetonitrile in water with 0.1% trifluoroacetic acid elution and were dried by Refrigerated CentriVap Centrifugal Concentrator (Labconco Corporation, USA).

2.7. LC-MS/MS analysis

The peptides fractioning and comparison were conducted on Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher Scientific, USA) in University Research Facility in Chemical and Environmental Analysis, The Hong Kong Polytechnic University. For the fractioning, Dionex Ultimate 3000 RSLCnano System decorated with Acclaim PepMap RSLC analytical columns (NanoViper, C18) (Thermo Fisher Scientific, USA) and Trap Column Cartridges Holders with nanoViper Fittings (Thermo Fisher Scientific, USA) were used. This unique combination provides high resolution separation at a flow rate 20 nL/min and improved sensitivity. The proteomics buffer concentration for all samples were maintained by 98% water with 2% acetonitrile and 0.1% formic acid. 1 μL of samples were injected for fractioning and collected peptides data from described experiments were analysed by Orbitrap Fusion Lumos Mass Spectrometer. The acquisition parameters of the instrument was: scan number: 1–10, mass range: m/z 50–6000, resolution: 500,000 FWHM at m/z 200, and mass accuracy: less than 1 ppm using internal and less than 3 ppm using external calibration. The raw data has been submitted to the publicly available MassIVE database (Ref: MassIVE MSV000089307, Center for Computational Mass Spectrometry,

University of California, San Diego).

2.8. Data analysis

MS and MS/ MS peptides spectra were analysed by Progenesis QI for proteomics (Version 4.2, Nonlinear Dynamics, Newcastle, UK) software compared with Swiss-Prot dataset (Sequence of *Homo sapiens*, Release date: June 1, 2018). The parameters for analysis were as follows: tolerance level: 10 ppm, maximum mass cleavage: 1, peptides charge: (2–4+), modification: both fixed and variable, Oxidation (M). The 1% False Discovery Rate (FDR) was applied to identify the peptides from complex mixtures. A fold change (FC) more than 2, $p \leq 0.05$, minimum two unique peptide contents, and replicated proteins in all samples were counted to complete the analysis.

2.9. Bioinformatics analysis

Graphpad Prism Version 8.0.1 was used to generate the volcano plot data from raw proteomics. We considered the average gene expression for Log2 fold change calculation and didn't perform multiple test correction. Heat map data was produced by Morpheus software (https://software.broadinstitute.org/morpheus/), a web-based versatile matrix visualization and analysis software. Raw protein expression data were imported and analysed by Morpheus tool with default parameter for Fig. 1C. Next, hierarchical clustering was performed with following parameters for Fig. 1C: metric-Euclidean distance, linkage method-Average, and cluster: Rows and columns. Rest of the heat maps were generated from Log2 fold change data in Morpheus tool with default parameter. Gene ontology (GO) and pathway data of significantly enriched protein of each group ($p \leq 0.05$, $-2.0 \leq \text{Log2FC} \geq 2.0$) were generated by a web-based tool called Enrichr (https://amp.pharm.mssm.edu/Enrichr/) with default settings. Gene set enrichment analysis (GSEA, version 4.1.0) software (https://www.gsea-msigdb.org/gsea/index.jsp) was employed to identify the hallmark gene sets of the significantly enriched protein of each group. Web-based GEne Set AnaLysis Toolkit 2019 (WebGestalt, 2019) was used to generate rank-based pathway of proteins significantly deregulated between good vs poor responders (http://www.webgestalt.org/). Wikipathway of Functional Database and GSEA of Method of Interest category were used respectively with default settings in this purpose. Protein-protein interaction (PPI) network of selected proteins were generated by STRING (https://string-db.org/) software. All the bioinformatics analysis were performed between September and October 2021.

2.10. Western blotting

Western blotting was performed using standard, established protocol as previously published [27]. Briefly, the protein samples were gently mixed with 2× loading dye and heat incubation was performed for 5 min at 95 °C to denature the proteins. Similar amounts of proteins were loaded and run on 12% SDS-PAGE at ambient temperature. Proteins were then transferred onto Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Inc., USA), and followed by two hours blocking in 5% bovine serum albumin (BSA) (Hyclone BSA, GE Healthcare Life Science, USA) in Tris-buffer saline with a supplement of 0.1% tween 20 (TBST). Then the blocked membrane was incubated overnight with primary antibodies: Acetyl-α-Tubulin (#5335, Cell Signaling Technology, Inc., (CST, USA)), β-Actin (#8457, CST), GAPDH (#2118, CST), GLUD1 (#12793, CST), LDHA (#3582, CST), and PGK1 (#ab38007, Abcam, UK) at 4 °C. The membrane was then incubated by the secondary anti-rabbit IgG, Horseradish peroxide (HRP)-linked or anti-mouse IgG-HRP-linked (#7076, CST) antibodies for two hours. Western Lightning Plus-Electrochemiluminescence (ECL) (PerkinElmer, Inc., USA) was added to the membrane according to manufacturer's instruction to visualize protein bands in ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc., USA). The relative protein expressions were quantified using ImageJ software (NIH) with internal control of acetyl-α-Tubulin or β-actin. All antibodies were diluted in a ratio of 1:1000 to make the working solution.

2.11. Public data mining and analysis

The differential expression of selected genes and proteins were analysed from publicly available dataset: The Cancer Genome Atlas (TCGA) ((http://gepia.cancer-pku.cn/detail.php) programme under National Cancer Institute (NCI). From TCGA-colon adenocarcinoma (TCGA-COAD) analysis, there were 275 tumour samples compared with 349 adjacent normal tissues for the expression profile analysis. $P < 0.01$ and fold change Log2FC >2.00 were considered as cut-off values to plot the box plot. The overall survival of CRC was determined by using TCGA-COAD dataset with a medium cut-off, 95% confidence interval, counting the number of transcripts per million (TPM), and considering the hazards ratio (HR).

3. Results

3.1. Overview of proteomics data

In order to analyse the protein expression changes among good responders, poor responders, and unirradiated control tumours, we employed high throughput MS proteomics analysis. MS analysis identified a total of 1416 proteins. Among the identified proteins, 106 DEPs between control and poor responders ($-2.0 \leq \text{Log2FC} \geq 2$, $p \leq 0.05$) where 39 and 67 proteins were up and downregulated respectively (Supplementary Table 1, Fig. 2A). On the other hand, 570 proteins were DEPs between control and good responders ($-2.0 \leq \text{Log2FC} \geq 2$, $P \leq 0.05$) of which 253 and 317 proteins were up and downregulated respectively (Supplementary Table 1, Fig. 2B). The overall expression pattern among groups were displayed in heat map (Fig. 2C). This showed the characteristic expression patterns of each sample.

3.2. GO and pathway analysis of significantly altered proteins

We employed Enrichr, a functional enrichment analysis database, to classify the genes according to their respective GO terms. GO biological process function of Enrichr showed significantly altered proteins in both good and poor responders group enriched for neutrophil degranulation and neutrophil related immunity with regard to control (Fig. 3A, D). At the molecular function level, secretory granule lumen processes are enriched in poor responders group, whereas focal adhesions term was in the top GO molecular function category in good responders group (Fig. 3B, E). GO interpretation of cellular component revealed that, most of the altered proteins are associated with RNA binding function in both group (Fig. 3C, F). The GO terms for each group are associated with similar terms but the degree of association is different.

We further examined the association of these altered proteins with pathways by Enrichr and the results showed that, KEGG proteasome are enriched in poor responders followed by spliceosome pathways (Fig. 4A) but in case of good responders, KEGG ribosome pathway and glycolysis (Fig. 4B) are enriched, suggesting that a number of radio-resistance pathways are activated in poor RT responders.

Next, we identified hallmark pathways associated with altered proteins in good responders group compare to control group by GSEA and the data revealed that, epithelial-mesenchymal transition (EMT) was deregulated (Fig. 4C). Among the deregulated proteins associated with EMT, CALU, CAPG, GAGLN, PPIB, TPM2, and VIM are found to be downregulated in good responders but not the poor responders and the control (Fig. 4D).

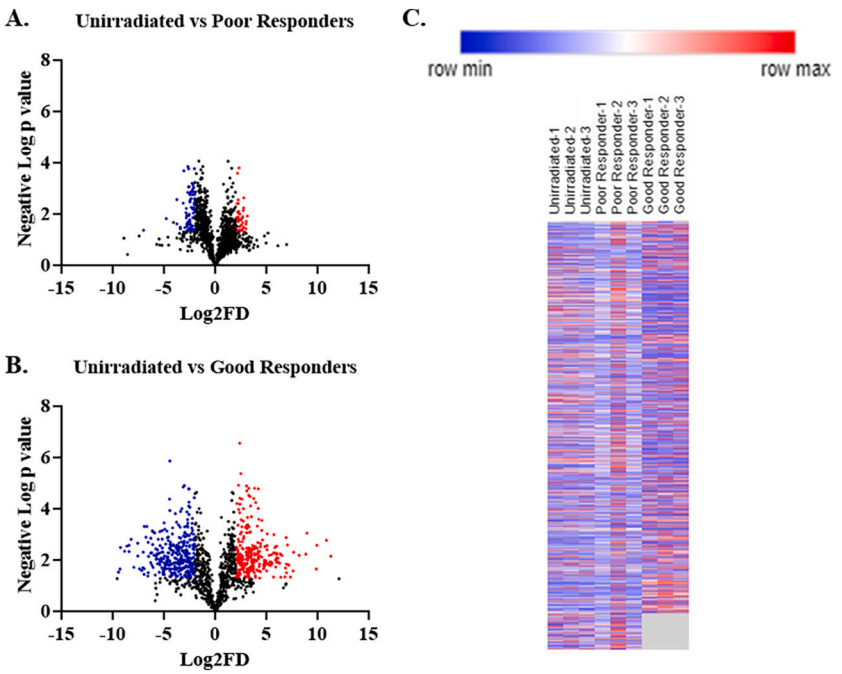

Fig. 2. Overview of proteomics data. (A) Volcano plot analysis of DEPs between unirradiated vs poor responders, (B) Volcano plot analysis of differential expressed proteins between unirradiated vs good responders. The blue and red points represent significantly down and up-regulated differentially expressed proteins respectively in unirradiated group ($p \leq 0.05$, $-2.0 \leq$ Log2FC ≥ 2.0). (C) The hierarchical heat map analysis of differential expressed proteins among groups. Each row corresponds to one protein, blue and red indicate down and upregulation of proteins respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

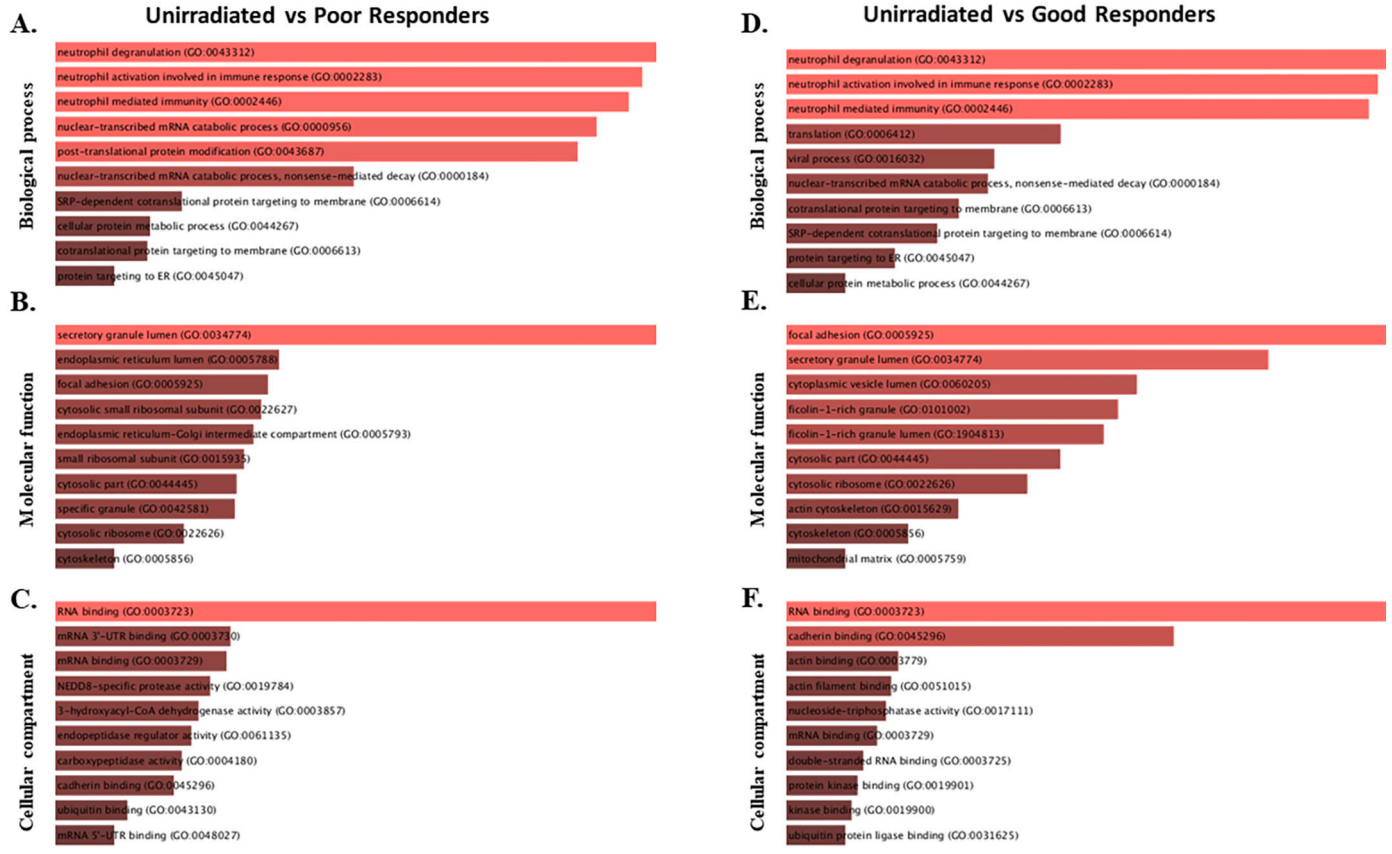

Fig. 3. GO analysis of DEPs. (A-C) Bar chart showed the top 10 GO terms for biological process, molecular function and cellular component respectively between unirradiated vs poor responders with significant DEPs. (D—F) Bar chart showed the top 10 GO terms for biological process, molecular function and cellular component respectively between unirradiated vs good responders with significant DEPs. Bar charts length and colour represents the significance of respective term.

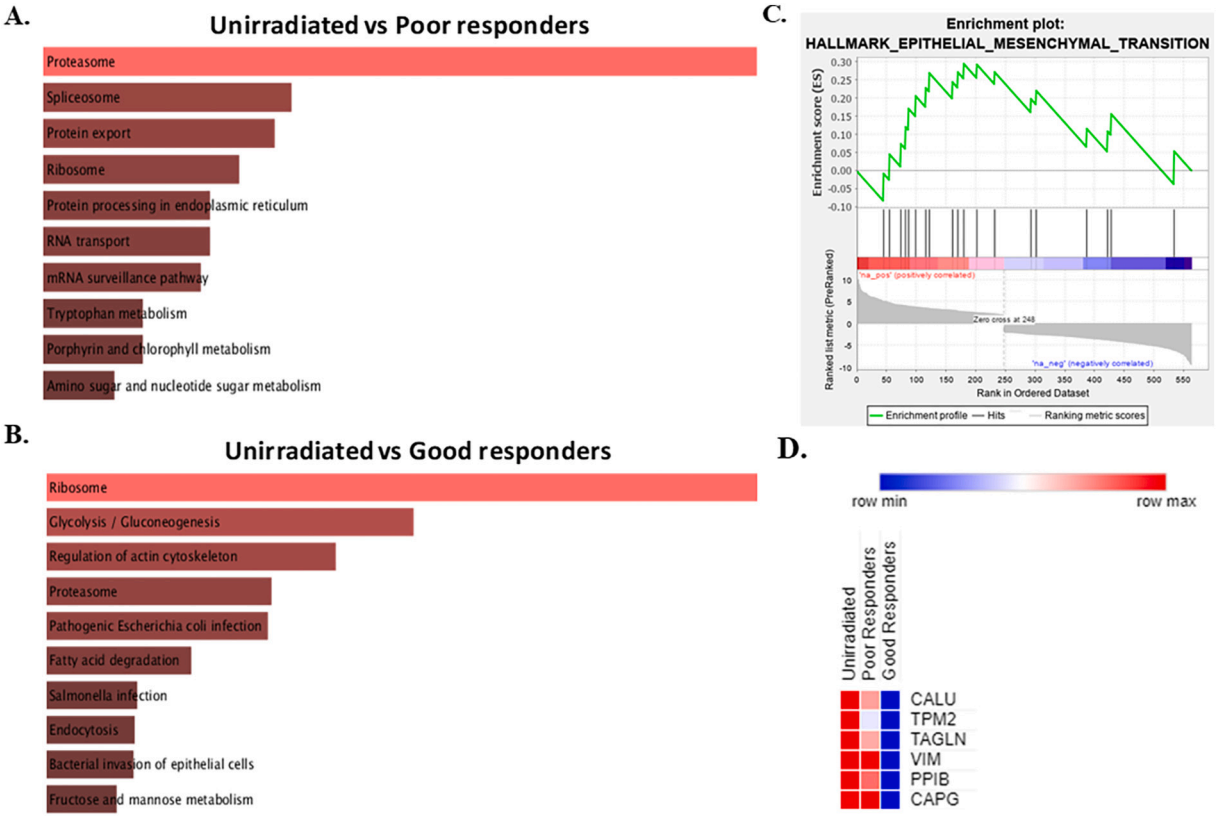

Fig. 4. Pathway and GSEA of significant DEPs. (A) KEGG pathway analysis between unirradiated vs poor responders group with significant DEPs. (B) KEGG pathway analysis between unirradiated vs good responders group with significant DEPs. Bar charts length and darkness represents the significance of the respective terms. (C) GSEA of significant differential expressed proteins data with hallmark gene sets between unirradiated vs good responders group. Data related to EMT are shown here. The green curve represents the enrichment score. (D) Heat map analysis of key EMT proteins among groups. The expressions of these 6 proteins in the good responders group are significantly different from the poor responders and control groups. Each row represents one protein, blue and red indicate down and upregulation of proteins respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. High expression of phosphoglycerate kinase 1 (PGK1) and other metabolic proteins might serve as a prognostic marker for radio-resistance of CRC

We employed WEB-based Gene SeT AnaLysis Toolkit (WebGestalt), a functional enrichment analysis web tool to generate rank-based pathways based on expression changes among groups. Based on WebGestalt results, a number of pathways are dysregulated between poor and good responders (according to Wikipathway) (Fig. 5A). Interestingly, the metabolic reprogramming of CRC was highly depreciated in the good responders group. Particularly, the proteins involved in this particular pathway were downregulated in good responders compared to poor responders (Fig. 5A, B) but the protein expression patterns were almost similar between control and bad response group (Fig. 5B). Literature search of metabolic reprogramming of CRC associated proteins in our dataset showed that they are all associated with CRC pathogenesis and upregulation of these proteins are linked to poor CRC diagnosis/ prognosis (Table 1). Moreover, STRING based PPIs data revealed that, these proteins are highly interconnected with each other (Fig. 5C).

Next, we investigate whether any of these proteins has predictive value for CRC. To do this, we performed extensive literature search and found proteins in our list have been well studied in CRC. To validate the proteomics data, we performed western blot analysis on the top three proteins of the same tumour samples used for proteomics analysis. Our results indicated that PGK1 was significantly downregulated in response to RT and PGK1 sits on top of our metabolic reprogramming in CRC tumours. In addition, we performed extensive literature study and found that PGK1 is associated with CRC progression and metastasis (Table 1). Overall, our results and previous demonstration indicated that PGK1 could be used as novel RT responsiveness biomarkers for CRC tumours. (Fig. 6A-B). As GAPDH use as an internal control and we found that it was dysregulated in our computational analysis. So, we checked GAPDH expression in tumour samples and found similar expression pattern as of computational analysis indicating that our computational analysis is valid. However, the expressions of LDHA and GLUD1 were not reached statistical significance, but the trend was similar in all experiments (Fig. 6A, C).

3.4. Cross-validation of prognostic markers with TCGA dataset

We further validated the gene expression of our RT prognostic marker proteins with publicly available dataset TCGA-COAD and web-based platform the Gene Expression Profiling Interactive Analysis (GEPIA). The TCGA gene expression analysis showed that *PGK1, LDHA, MDH2, ENO1, GAPDH, PKM*, and *TKT* expressions were significantly upregulated in CRC tissues compared to adjacent normal samples (Fig. 7A-C). The expressions of *GLUD1*, and *PGAM1* were not significantly upregulated but they followed the same upregulation trends with other proteins (Fig. 7A-C). We also explored the association of these proteins with patient survival. The curve was plotted using expression levels using TCGA-COAD dataset. We found that the upregulation of genes are associated with reduced overall survival of CRC patients (Fig. 7A-C).

4. Discussion

Despite of the advancement in RT delivery technology, recurrence and development of metastasis remains the main causes for poor

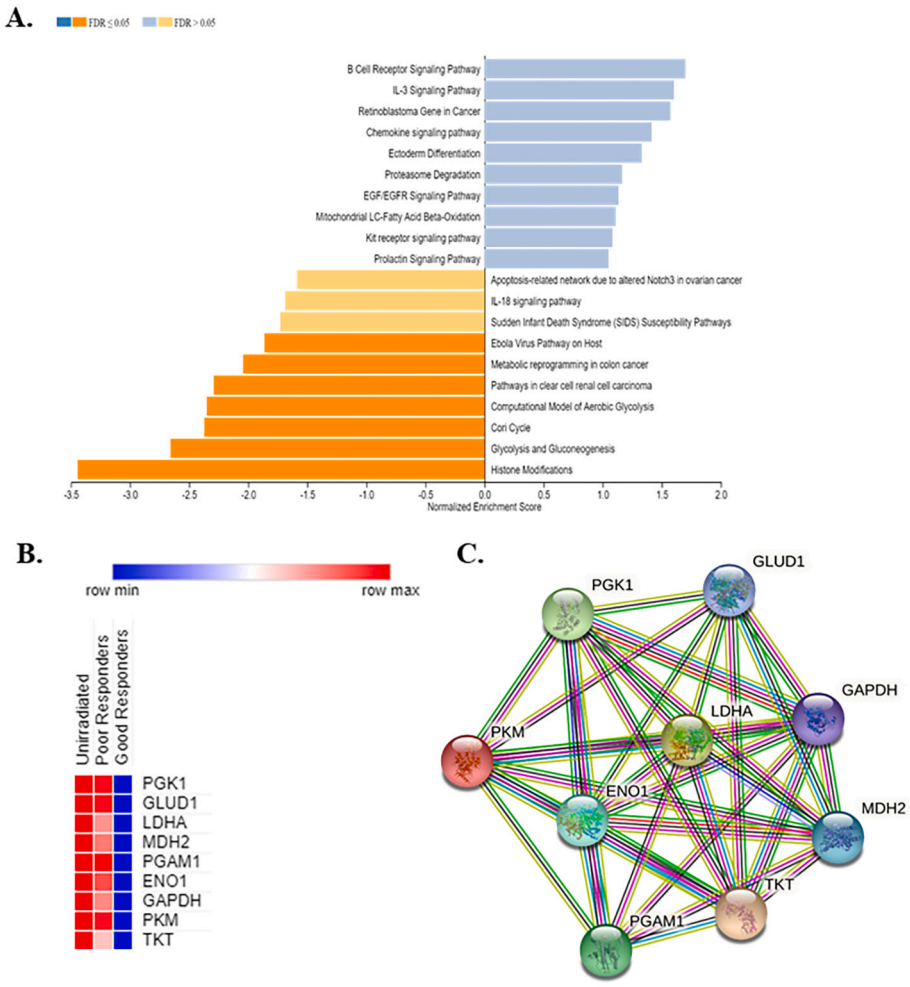

Fig. 5. Pathway and functional analysis of significant DEPs between good and poor responders groups. (A) Wikipathway analysis of between good vs poor responders group with significant DEPs. Pathways having FDR smaller than or equal to 0.05 are darker shades and while light shades are the opposite. (B) Heat map analysis of metabolic regulation of CRC proteins among groups. Each row represents one protein, blue and red indicate down and upregulation of respective proteins. (C) PPI network generated by STRING software of proteins related to metabolic regulation of CRC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prognosis and high mortality of CRC patients [7,49,50] . In this project, we aim to identify biomarkers that may be used to predict radioresponsiveness of CRC. By using mouse xenograft model, a list of candidate proteins was identified (Table 1). The Response Evaluation Criteria in Solid Tumour (RECIST) theory has been well established and commonly applied in clinical settings. RECIST theory subdivides the tumours into groups of complete disappearance, partial disappearance, unchanged or progressive disappearance. However, the underlying molecular biology is much less studied. In addition, tumours that were nonresponsive to RT were only distinguished from responsive tumours after the end of the whole treatment period. Therefore, the delayed in selecting alternative treatment strategies including RT dose modification, application of chemotherapy, and application of chemoradiotherapy resulted in reduced overall survival of those nonresponsive patient cohorts [11,51,52].

It has been reported that abnormal expression of proteins are key regulatory components in cell death process induced by RT [53,54]. Similarly, our study proposed that the radioresponsiveness is associated with a group of differentially expressed proteins in CRC tumours. Our GO study revealed that RT has altered many biological functions of CRC including metabolism, neutrophil degranulation, focal adhesion, RNA binding functions, and EMT.

The poor prognosis for most cancers is due to the development of metastasis where EMT enhances the cellular migration properties. In fact, EMT is one of the most important hallmarks of carcinogenesis. It is very crucial; not only for tumour growth and metastasis, but also poor prognosis of treatment outcome due to development of resistance [55,56]. EMT reduces tight junction between cells, basal polarity, and cytoskeletal structure to induce cellular motility, resulting development of invasiveness or metastatic phenotype of CRC [57,58]. GSEA analysis demonstrated that the hallmark EMT proteins are significantly downregulated in the tumours of good RT response but upregulated in both unirradiated and poor responses tumours. Our list of EMT regulatory proteins included CALU, CAPG, PPIB, TAGLN, TPM2, and VIM, they regulated the EMT process in CRC tumours. The recent findings indicated that CALU play key roles in CRC development and metastasis [59], TPM2 is implicated with potential roles in CRC development [60]. Similarly, transgelin (TAGLN) is found to be a key regulatory factor associated with later stages CRC where it promotes CRC carcinogenesis through TGFβ signaling [61]. Another EMT marker protein vimentin (VIM) expression was upregulated to induce EMT, resulting poor prognosis and reduced survival of CRC patients [62]. Moreover, the overexpression of capping actin protein (CAPG) contributes to CRC migration and peptidylprolyl isomerase B (PPIB) promotes chemoresistance in CRC by reducing p53 and binding with MDM2 [63,64]. These literatures supported our notion that specific EMT proteins may be used as prognostic markers for tumour radioresponsiveness. Therefore, our results indicated that downregulation of hallmark EMT may promote RT outcomes of CRC tumours.

In order to establish RT-prognostic biomarkers, web-based functional analysis was performed and we identified a number of pathways dysregulation between poor responding and good responding groups. Interestingly, the metabolic reprogramming of CRC is highly depreciated in good RT responses group compared to unirradiated control and poor RT responses groups. The metabolic reprogramming is a process regulated by oncogene or tumour suppressive genes which provide

Table 1
Roles of shortlisted metabolic proteins in CRC.

Gene symbol	Name	Reported functions in CRC	References
PGK1	Phosphoglycerate kinase 1	PGK1 secreted from CRC tumours promotes cell proliferation, angiogenesis, metastasis, and facilitate fluorouracil (5-FU) resistance	[28,29]
GLUD1	Glutamate dehydrogenase 1	GLUD1 regulates cellular energy generation under hypoxic condition, act as a prognostic and metastatic biomarker in CRC.	[30,31]
LDHA	Lactate dehydrogenase A	Critical enzyme LDHA promotes production of ATP by regulating glycolysis in both normal and hypoxic conditions. With significant correlation of LDHA with HIF1α, HIF2α, GLUT-1, VEGFA, and VEGFR1, LDHA is proposed to be a potential prognostic biomarker in CRC.	[32–34]
MDH2	Malate dehydrogenase 2	MDH2 is one of the key regulatory enzymes of tricarboxylic acid (TCA) cycle of NAD/NADH coenzyme system. MDH2 inhibitor could be a potential anticancer target in CRC management.	[35–37]
PGAM1	Phosphoglycerate Mutase 1	The upregulation of PGAM1 associated with glycolysis process and dysregulation of PGAM1 promotes metastatic process of CRC.	[38,39]
ENO1	Enolase 1	ENO1 involves in glycolysis and promotes CRC growth, migration, and metastasis through AMPK/mTOR pathway.	[38,40,41]
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	The dysregulation of GAPDH is commonly associated with various carcinogenesis including CRC where Vitamin C selectively destroys mutated CRC cells (KRAS and BRAF) through targeting GAPDH.	[42–44]
PKM	Pyruvate kinase M1/2	PKM catalyzes in glycolysis process and glucose consumption whereas, PKM promotes cell growth and migration in CRC cells.	[45,46]
TKT	Transketolase	TKT is one of glycolytic and pentose-phosphate pathway enzymes associated with CRC metabolic reprogramming.	[47,48]

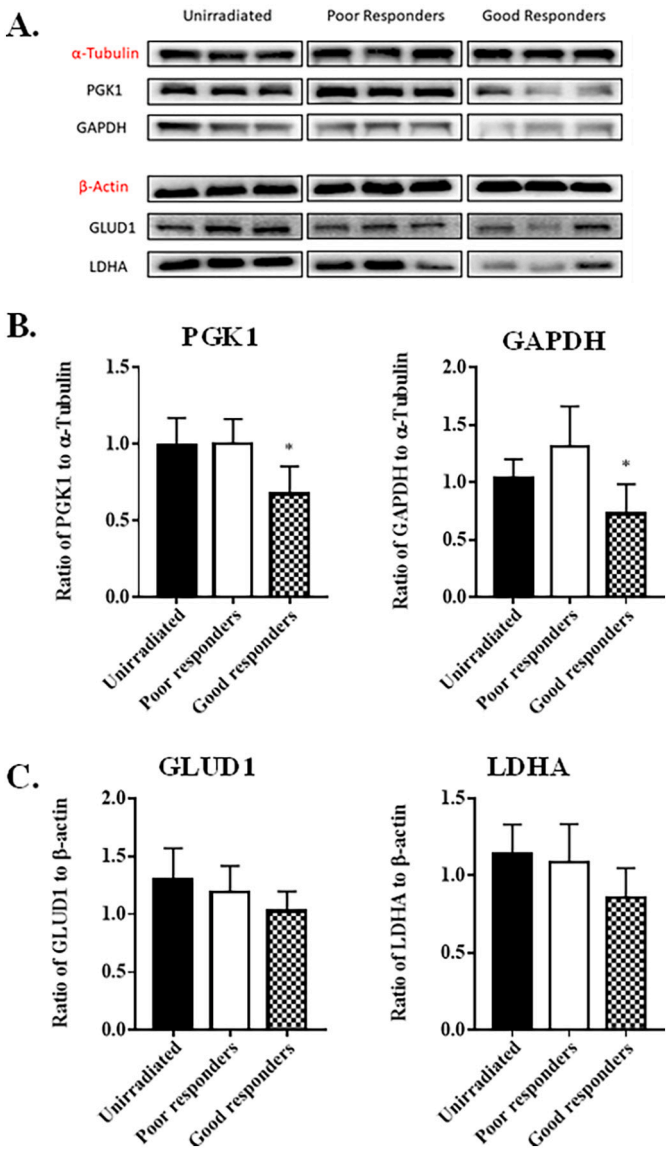

Fig. 6. Western blot analysis of PGK1, GAPDH, GLUD1, and LDHA between unirradiated, poor responders and good responders group. The expressions of PGK1 and GAPDH were significantly downregulated in good responders compared to unirradiated and poor responders (A-B). However, downregulation of GLUD1 and LDHA did not reach statistical significance (A, C). The data are shown as mean ± SEM compared to the control ($*p < 0.05$, $n = 4$).

adequate energy to the cells to maintain tumour microenvironment for rapid tumour growth and progression [65]. Importantly, metabolic reprogramming is key in tumour progression and shaping its responses to the RT or chemotherapies [65,66]. Our results shortlisted 9 metabolic reprogramming of CRC assocaited proteins namely, PGK1, GLUD1, LDHA, MDH2, PGAM1, ENO1, GAPDH, PKM, and TKT, are found to be upregulated in unirradiated control and poor responses groups but downregulated in good responses group. In addition, these proteins are highly connected with each other.

We performed extensive literature studies on the shortlisted metabolic proteins and revealed that PGK1, the top proteins on our list, is the first ATP-generating enzyme in glycolysis, associated with CRC progression and development of metastasis [67]. In addition, PGK1 glycosylation promotes CRC proliferation and growth by regulating glycolysis and TCA cycle [68]. Similarly, GLUD1 is a crucial catalytic enzyme of glutamine metabolic pathway and related to poor prognosis of CRC. It is proposed that GLUD1 might be a novel prognostic marker for metastatic CRC and new therapeutic target in refractory CRC [30,31]. Equally, LDHA is another critical regulator of glycolysis and ATP production. It is upregulated in many cancer types. The association of LDHA with HIF-α, GLUT-1, NRP1, VEGFA, and VEGFR1 may serve as surrogate markers in CRC [32–34]. MDH2 is another regulatory enzyme of mitochondrial TCA cycle. It is shown that MDH2 inhibitor could be a potential therapeutic strategy of CRC [35–37]. Research showed that PGAM1 upregulation promoted glycolysis and development of metastasis in CRC [38,39]. ENO1 is another conserved glycolytic enzyme which is upregulated in many cancer types including CRC. The upregulation of ENO1 promotes CRC growth, migration, and metastasis via regulating RAB1A/AMPK/mTOR [40,41]. The widely accepted housekeeping gene GAPDH is another essential glycolytic regulatory enzyme. It is commonly upregulated in 21 cancer classes, and is a key regulator of mitochondrial membrane permeabilization in pro-apoptic stages [44,69]. Interestingly, vitamin C selectively destroy KRAS and BRAF mutated CRC cells

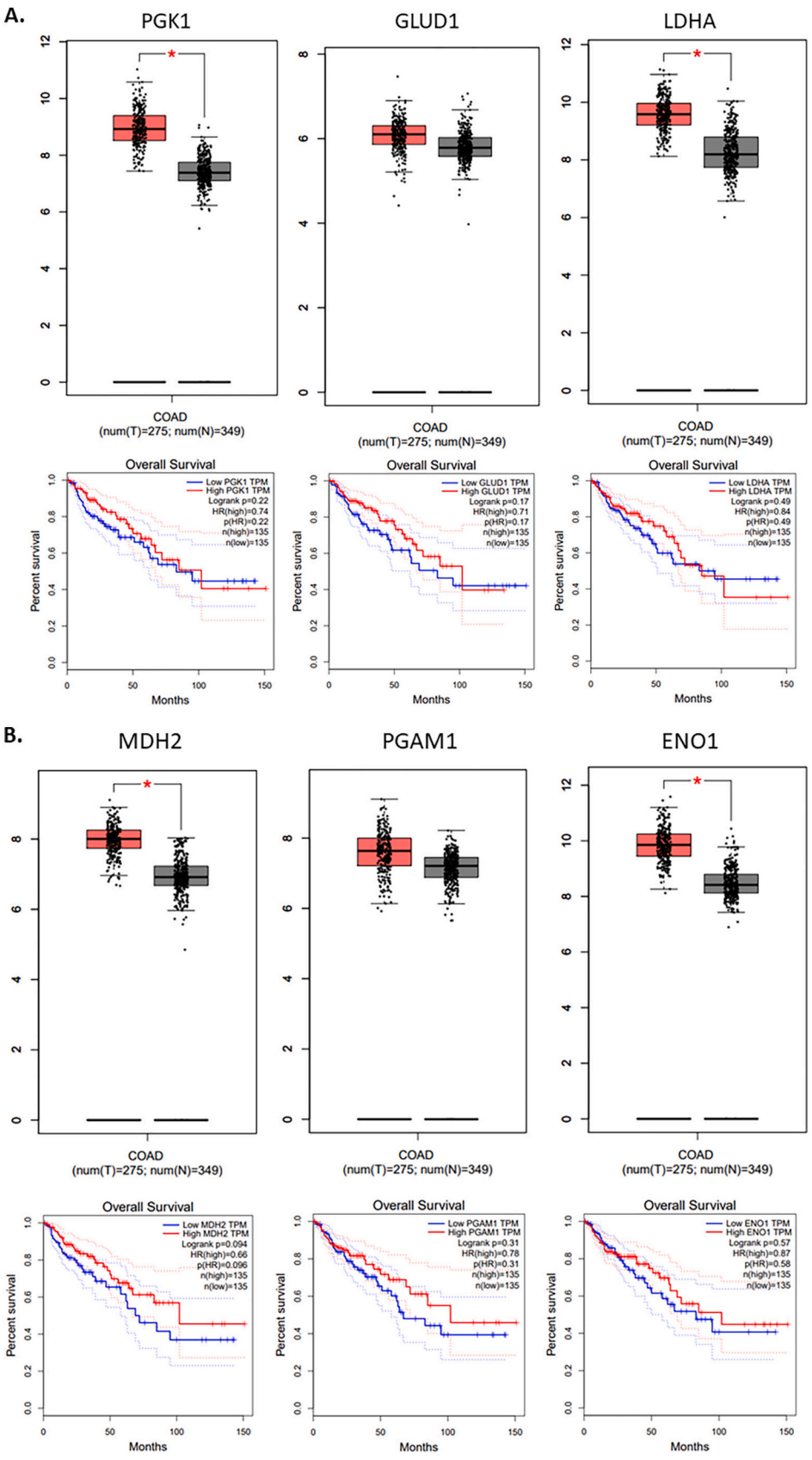

Fig. 7. Box-plot of key metabolic proteins associated with RT responsiveness. The expressions of metabolic proteins were extracted from TCGA-COAD dataset. Number of tumour samples: 275, number of normal tissues: 349, $^{*}P < 0.01$. TPM, and HR.

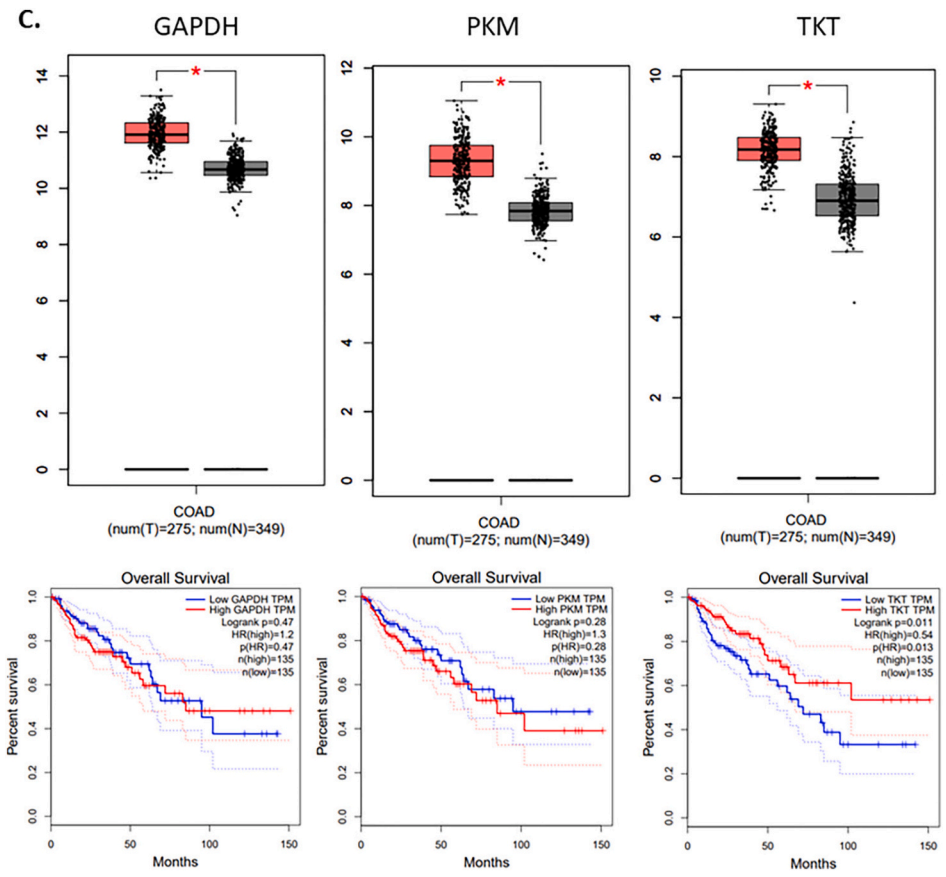

Fig. 7. (*continued*).

by regulating GAPDH [43]. The metabolic enzyme PKM was found to regulate metabolism process of CRC cells whereas the upregulated PKM enhances migration and adhesion of CRC cells by targeting STAT3 [45,46]. Apart from this, pentose phosphate pathway regulatory enzyme TKT can effectively distinguish the carcinoma and normal tissues in colorectal tissues [47,48]. We believe these proteins are upregulated in CRC tumours and effective RT reduced their expressions along with reduction of tumour size. Thus, these proteins may serve as potential biomarkers for CRC of RT management.

5. Conclusion

Overall, using high through-put proteomics analysis, we isolated 9 metabolic proteins which regulate pathways such as glycolysis (PGK1, PGAM1, ENO1, PKM, TKT), ammonia detoxification (GLUD1), carcinogenesis (LDHA, GAPDH), and drug responses (MDH2). Future projects may explore the expression of specific metabolic proteins in biopsy samples collected before, after, and at midcourse of RT and determine their association with radioresponsiveness. If poor response is predicted, additional personalized treatment may be prescribed to improve the treatment outcome. However, because it is an invasive intervention along with associated complications. So more concrete scientific evidence is needed before proceeding into large-scale clinical trials. In addition, our study is limited to the xenograft generated by using a single cell line HT-29. The in vivo experiments may be repeated using different CRC cell lines. Besides, it is important to verify the expression of our proposed markers in patient's sample and determine their association with tumour size and treatment outcome after RT.

Author contributions

ZIK and HKL conceived and designed the project. ZIK conducted the experiments, analysed data, and wrote the manuscript. S.Y. TAM assisted in RT application and generating data. Z AZAM assisted in analysing data. HKL interpret the results and reviewed the manuscript. The authors read, approved and finalized the manuscript.

Funding

This project is partially supported by: (1) Research grant to HKL including Departmental Seeding Fund and Internal Institutional Research Fund (P0031318-UAHS). (2) Postgraduate studentship from The Hong Kong Polytechnic University for ZIK.

Data availability statement

The data presented in this study will be available on request from the corresponding author.

Declaration of Competing Interest

There were absences of commercial or financial relationship involved this research. The authors declared no potential conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

We thank Central Animal Facility of The Hong Kong Polytechnic University for their support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2022.104600.

References

[1] F. Bray, et al., Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (6) (2018) 394–424.
[2] World Health Organization, Cancer. 2020, Available from, https://www.who.int/news-room/fact-sheets/detail/cancer, 2018, 12 September.
[3] N. Keum, E. Giovannucci, Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies, Nat. Rev. Gastroenterol. Hepatol. 16 (12) (2019) 713–732.
[4] M. Fleming, et al., Colorectal carcinoma: pathologic aspects, J. Gastrointest. Oncol. 3 (3) (2012) 153–173.
[5] M. Chen, et al., Neoadjuvant chemoradiation for locally advanced rectal cancer: a systematic review of the literature with network meta-analysis, Cancer Manag. Res. 11 (2019) 741–758.
[6] M. Lupattelli, et al., Preoperative intensity-modulated radiotherapy with a simultaneous integrated boost combined with Capecitabine in locally advanced rectal cancer: short-term results of a multicentric study, Radiat. Oncol. 12 (1) (2017) 139.
[7] S.Y. Tam, V.W.C. Wu, A review on the special radiotherapy techniques of colorectal cancer, Front. Oncol. 9 (2019) 208.
[8] O. Hatcher, A.A. Kumar, Chemotherapy and radiotherapy for colorectal cancers, Surgery (Oxford) 32 (4) (2014) 179–184.
[9] A. Barrett, et al., Practical Radiotherapy Planning, CRC Press, 2009.
[10] E.J. Moding, M.B. Kastan, D.G. Kirsch, Strategies for optimizing the response of cancer and normal tissues to radiation, Nat. Rev. Drug Discov. 12 (7) (2013) 526–542.
[11] J. Meehan, et al., Precision medicine and the role of biomarkers of radiotherapy response in breast cancer, Front. Oncol. 10 (2020) 628.
[12] S.V. Bratman, et al., Genomic biomarkers for precision radiation medicine, The Lancet Oncol. 18 (5) (2017), e238.
[13] S. Eschrich, et al., Systems biology modeling of the radiation sensitivity network: a biomarker discovery platform, Int. J. Radiat. Oncol. Biol. Phys. 75 (2) (2009) 497–505.
[14] A.H. Ree, K.R. Redalen, Personalized radiotherapy: concepts, biomarkers and trial design, Br. J. Radiol. 88 (1051) (2015) 20150009.
[15] S. Sethi, et al., Clinical advances in molecular biomarkers for cancer diagnosis and therapy, Int. J. Mol. Sci. 14 (7) (2013) 14771–14784.
[16] N. Carlomagno, et al., Diagnostic, predictive, prognostic, and therapeutic molecular biomarkers in third millennium: a breakthrough in gastric cancer, Biomed. Res. Int. 2017 (2017) 7869802.
[17] V. Kulasingam, E.P. Diamandis, Strategies for discovering novel cancer biomarkers through utilization of emerging technologies, Nat. Clin. Pract. Oncol. 5 (10) (2008) 588–599.
[18] S.M. Hanash, C.S. Baik, O. Kallioniemi, Emerging molecular biomarkers—blood-based strategies to detect and monitor cancer, Nat. Rev. Clin. Oncol. 8 (3) (2011) 142.
[19] R.A. Alharbi, Proteomics approach and techniques in identification of reliable biomarkers for diseases, Saudi J. Biol. Sci. 27 (3) (2020) 968–974.
[20] B.A. Alves Martins, et al., Biomarkers in colorectal cancer: the role of translational proteomics research, Front. Oncol. 9 (1284) (2019).
[21] H. Adeola, et al., Prospects of 'omics based molecular approaches in colorectal cancer diagnosis and treatment in the developing world: a case study in Cape Town, *South Africa*, Colorectal Cancer Surgery, Diagnost. Treatment 1 (2014) 1–41.
[22] Y. Deng, et al., KRAS as a predictor of poor prognosis and benefit from postoperative FOLFOX chemotherapy in patients with stage II and III colorectal cancer, Mol. Oncol. 9 (7) (2015) 1341–1347.
[23] S.C. Chuang, et al., Effect of KRAS and NRAS mutations on the prognosis of patients with synchronous metastatic colorectal cancer presenting with liver-only and lung-only metastases, Oncol. Lett. 20 (3) (2020) 2119–2130.
[24] H. Blons, et al., Prognostic value of KRAS mutations in stage III colon cancer: post hoc analysis of the PETACC8 phase III trial dataset, Ann. Oncol. 25 (12) (2014) 2378–2385.
[25] T.K. Nikolouzakis, et al., Improving diagnosis, prognosis and prediction by using biomarkers in CRC patients (review), Oncol. Rep. 39 (6) (2018) 2455–2472.
[26] J. Sápi, et al., Tumor volume estimation and quasi-continuous administration for most effective bevacizumab therapy, PLoS One 10 (11) (2015), e0142190.
[27] M.Z. Islam Khan, H.K.W. Law, Cancer Susceptibility Candidate 9 (CASC9) promotes colorectal cancer carcinogenesis via mTOR-dependent autophagy and epithelial–mesenchymal transition pathways, Front. Mol. Biosci. 8 (206) (2021).
[28] S. Shichijo, et al., Two proliferation-related proteins, TYMS and PGK1, could be new cytotoxic T lymphocyte-directed tumor-associated antigens of HLA-A2⁺ Colon Cancer, Clin. Cancer Res. 10 (17) (2004) 5828–5836.
[29] S.S. Ahmad, et al., Phosphoglycerate kinase 1 as a promoter of metastasis in colon cancer, Int. J. Oncol. 43 (2) (2013) 586–590.
[30] M. Miyo, et al., Metabolic adaptation to nutritional stress in human colorectal cancer, Sci. Rep. 6 (2016) 38415.
[31] G. Liu, et al., Glutamate dehydrogenase is a novel prognostic marker and predicts metastases in colorectal cancer patients, J. Transl. Med. 13 (2015) 144.
[32] A.A. Untereiner, et al., H(2)S-induced S-sulfhydration of lactate dehydrogenase a (LDHA) stimulates cellular bioenergetics in HCT116 colon cancer cells, Biochem. Pharmacol. 136 (2017) 86–98.
[33] Y. Mizuno, et al., Intratumoral heterogeneity of glutaminase and lactate dehydrogenase a protein expression in colorectal cancer, Oncol. Lett. 19 (4) (2020) 2934–2942.
[34] M. Azuma, et al., Serum lactate dehydrogenase levels and glycolysis significantly correlate with tumor VEGFA and VEGFR expression in metastatic CRC patients, Pharmacogenomics 8 (12) (2007) 1705–1713.
[35] C.R. Goward, D.J. Nicholls, Malate dehydrogenase: a model for structure, evolution, and catalysis, Protein Sci. 3 (10) (1994) 1883–1888.
[36] H.S. Ban, et al., A novel malate dehydrogenase 2 inhibitor suppresses hypoxia-inducible Factor-1 by regulating mitochondrial respiration, PLoS One 11 (9) (2016) e0162568.
[37] R. Naik, et al., Synthesis and structure–activity relationship study of chemical probes as hypoxia induced factor-1α/malate dehydrogenase 2 inhibitors, J. Med. Chem. 57 (22) (2014) 9522–9538.
[38] M.S. Fedorova, et al., The CIMP-high phenotype is associated with energy metabolism alterations in colon adenocarcinoma, BMC Med. Genet. 20 (Suppl. 1) (2019) 52.
[39] Y. Lei, et al., Proteomics identification of ITGB3 as a key regulator in reactive oxygen species-induced migration and invasion of colorectal cancer cells, Mol. Cell. Proteom. MCP 10 (10) (2011). M110.005397.
[40] Z. Cheng, et al., ENO1 acts as a prognostic biomarker candidate and promotes tumor growth and migration ability through the regulation of Rab1A in colorectal cancer, Cancer Manag. Res. 11 (2019) 9969–9978.
[41] P. Zhan, et al., α-Enolase promotes tumorigenesis and metastasis via regulating AMPK/mTOR pathway in colorectal cancer, Mol. Carcinog. 56 (5) (2017) 1427–1437.
[42] C. Guo, S. Liu, M.-Z. Sun, Novel insight into the role of GAPDH playing in tumor, Clin. Transl. Oncol. 15 (3) (2013) 167–172.
[43] J. Yun, et al., Vitamin C selectively kills KRAS and BRAF mutant colorectal cancer cells by targeting GAPDH, Science 350 (6266) (2015) 1391–1396.
[44] A. Tarze, et al., GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization, Oncogene 26 (18) (2007) 2606–2620.
[45] P. Yang, et al., Pyruvate kinase M2 facilitates colon cancer cell migration via the modulation of STAT3 signalling, Cell. Signal. 26 (9) (2014) 1853–1862.
[46] Y. Kuranaga, et al., SRSF3, a splicer of the PKM gene, regulates cell growth and maintenance of cancer-specific energy metabolism in colon cancer cells, Int. J. Mol. Sci. 19 (10) (2018).
[47] P. Vizán, et al., Modulation of pentose phosphate pathway during cell cycle progression in human colon adenocarcinoma cell line HT29, Int. J. Cancer 124 (12) (2009) 2789–2796.
[48] W.S. Chen, et al., Comparative tyrosine-kinase profiles in colorectal cancers: enhanced arg expression in carcinoma as compared with adenoma and normal mucosa, Int. J. Cancer 83 (5) (1999) 579–584.
[49] F. Roeder, et al., Recent advances in (chemo-)radiation therapy for rectal cancer: a comprehensive review, Radiat. Oncol. 15 (1) (2020) 262.
[50] M. AlQudah, E. Salmo, N. Haboubi, The effect of radiotherapy on rectal cancer: a histopathological appraisal and prognostic indicators, Radiat Oncol J 38 (2) (2020) 77–83.
[51] K. Cheung, Intensity modulated radiotherapy: advantages, limitations and future developments, Biomed. Imaging Intervent. J. 2 (1) (2006) e19.
[52] R. Baskar, et al., Cancer and radiation therapy: current advances and future directions, Int. J. Med. Sci. 9 (3) (2012) 193–199.
[53] J.-S. Wang, H.-J. Wang, H.-L. Qian, Biological effects of radiation on cancer cells, Military Med. Res. 5 (1) (2018) 20.
[54] A. Wahba, S.L. Lehman, P.J. Tofilon, Radiation-induced translational control of gene expression, Translation (Austin, Tex.) 5 (1) (2016) e1265703.
[55] X. Wang, et al., LINC00261 inhibits progression of pancreatic cancer by down-regulating miR-23a-3p, Arch. Biochem. Biophys. 689 (2020), 108469.
[56] T. Brabletz, et al., EMT in cancer, Nat. Rev. Cancer 18 (2) (2018) 128–134.
[57] K. De Bosscher, C.S. Hill, F.J. Nicolás, Molecular and functional consequences of Smad4 C-terminal missense mutations in colorectal tumour cells, Biochem. J. 379 (Pt 1) (2004) 209–216.
[58] T. Vu, P.K. Datta, Regulation of EMT in colorectal cancer: a culprit in metastasis, Cancers 9 (12) (2017) 171.
[59] P. Nasri Nasrabadi, et al., Establishment of a CALU, AURKA, and MCM2 gene panel for discrimination of metastasis from primary colon and lung cancers, PLoS One 15 (5) (2020) e0233717.
[60] B. Zhao, et al., Identification of potential key genes and pathways in early-onset colorectal cancer through bioinformatics analysis, Cancer Control 26 (1) (2019), 1073274819831260.
[61] M. Elsafadi, et al., Transgelin is a poor prognostic factor associated with advanced colorectal cancer (CRC) stage promoting tumor growth and migration in a TGFβ-dependent manner, Cell Death Dis. 11 (5) (2020) 341.
[62] L. Du, et al., High vimentin expression predicts a poor prognosis and progression in colorectal cancer: a study with meta-analysis and TCGA database, Biomed. Res. Int. 2018 (2018) 6387810.
[63] T.-J. Tsai, et al., Capping actin protein overexpression in human colorectal carcinoma and its contributed tumor migration, Anal. Cell. Pathol. 2018 (2018) 8623937.
[64] T.G. Choi, et al., Cyclophilin B induces chemoresistance by degrading wild-type p53 via interaction with MDM2 in colorectal cancer, J. Pathol. 246 (1) (2018) 115–126.

[65] S. La Vecchia, C. Sebastián, Metabolic pathways regulating colorectal cancer initiation and progression, Semin. Cell Dev. Biol. 98 (2020) 63–70.
[66] L. Tang, et al., Role of metabolism in cancer cell radioresistance and radiosensitization methods, J. Exp. Clin. Cancer Res. : CR 37 (1) (2018) 87.
[67] S.S. Ahmad, et al., Phosphoglycerate kinase 1 as a promoter of metastasis in colon cancer, Int. J. Oncol. 43 (2) (2013) 586–590.
[68] H. Nie, et al., O-GlcNAcylation of PGK1 coordinates glycolysis and TCA cycle to promote tumor growth, Nat. Commun. 11 (1) (2020) 36.
[69] M. Tarrado-Castellarnau, et al., Glyceraldehyde-3-phosphate dehydrogenase is overexpressed in colorectal cancer onset, Translat. Med. Communicat. 2 (1) (2017) 6.