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Preliminary proteomic analysis of radiation response markers in rectal cancer patients

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Abstract. – OBJECTIVE: To provide biomarkers related to the radiation response of patients to avoid unnecessary side effects on those who were not sensitive to radiotherapy.

PATIENTS AND METHODS: In the present study, we compared the different four proteins (PDIA3, Vimentin, Galectin3, Dhe3) patterns in rectal tumor tissue before and after radiation therapy by using 2-D PAGE, mass spectrometry, and bioinformatics analysis.

RESULTS: The protein level of Galectin3 and PDIA3 were downregulated in rectal cancer patients before and after radiotherapy (1.42 folds); while Dhe3 protein and Vimentin were upregulated (1-2 folds), and we also revealed Vimentin as its role in the negative regulation of the well-known transcription factor ATF4.

CONCLUSIONS: Our study showed that four candidate proteins, including PIDA3, Galectin3, Dhe3, and Vimentin, might be the potential biomarkers in the identification of radiation response in rectal cancer.

Key Words:
Rectal carcinoma, Radiation response, Biomarkers, Proteomic analysis, Bioinformatics.

Introduction

Rectal carcinoma is one of the most common tumor types in the developed world[1]. In China, due to the changes in lifestyle and dietary habits, rectal carcinoma is the fifth leading cause of cancer mortality in recent years, and the incidence rate was increasing to 10.25/105 annual. Over the past decades, radiation therapy combined with surgery has become the primary management in rectal cancer patients[2]. However, individual rectal cancer patient reveals wide ranges of radiation response, and parts of patients might not benefit from radiation therapy.

Moreover, a potential side effect of radiotherapy occurs in most cancer patients and it might increase the unnecessary risk and cost burden. Thus, the biomarkers for a more accurate prediction of radiation response are encouraged to distinguish the radiosensitivity of rectal cancer patients to make personal therapy plans and reduce the risk of radiation resistance and side effects. However, there were still no acceptable biomarkers for radiotherapy in rectal cancer for routine clinic application[3].

In the cellular biological process, the protein products of various genes are known as the fundamental functional units. Correspondingly, two-dimensional gel electrophoresis (2-D PAGE), as a high throughput approach to discover different proteins patterns, was used combined with proteomics analysis to identify the post-translational modifications of protein in more and more diseases[4]. As the fast development of bioinformatics database and analysis tools, this microarray technology will provide more reliable information about the modulation pathway and physiological outcomes of diseases and to further elucidate the molecular mechanisms underline the pathological process and therapy targets.

To find out more directly genomic-clinic associations, we collected the rectal cancer samples by biopsies of tumor tissue from patients before and after radiation therapy and applied for 2-D PAGE analysis. Then, it was followed by mass spectrometry to identify individual proteins. At last, the bioinformatics analysis tools were used to predict the potential proteins, related pathway and involved protein-protein interaction network of candidate proteins.

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Patients and Methods

Patients and Treatment

The study was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. All patients included received a total dose of 25Gy (Gray) for 5 weeks. The inclusion criteria consisted: 1) pre-surgery; 2) biopsy-proven rectal adenocarcinoma; 3) clinical stage T3-4 or N1-2, World Health Organization performance status of 0-2, distal tumor extent within 10 cm of the anal verge; 4) age 18 years, no prior (in the last 5 years) or concurrent malignancy (except non melanoma skin cancers or in situ carcinoma of the cervix), no prior RT or chemotherapy.

Sample Collection

Pretreatment biopsies were taken at the time of rectoscopy and immediately frozen. The biopsies were performed at a tumor edge and a vital tumor area. Thus, the protein was obtained from each biopsy in both pre and post-radiation therapy.

Spectrometry Analysis and Database Searching

The digested samples were spotted with the alpha-cyano-4-hydroxycinnamic acid (5 mg/mL, Sigma, Merck, USA) matrix solution onto a MALDI MS plate. The in-gel digested peptides were analyzed with Bruker-Daltonics AutoFlex TOF-TOF LIFT Mass Spectrometer (Bruker Daltonics, Bremen, Germany). The working mode was set with positive ion reflection mode, an accelerating voltage of 20 kV, and 100 ns delayed time. Both MS and MS/MS data were acquired with a UV laser at 25 Hz repetition rate with wavelength of 337 nm. The spectrum masses ranging from 700-4000 Da were acquired with laser shots at 200 per spectrum. The measurements were externally calibrated with a standard peptide mixture of angiotensin I ([M + H] + 1296.6853 Da) and angiotensin II ([M + H] + 1046.5420 Da), and internally recalibrated with peptide fragments arising from autoproteolysis of trypsin. The known contaminant ions (human keratin and tryptic autodigest peptides) were excluded.

Database Searching and Data Analysis

PMF data from AutoFlex MALDI-TOF/TOF mass spectrometer were processed with FlexAnalysis 2.4 software in a default mode. PMF data were submitted by BioTools 3.0 software to MASCOT (V2.1, Matrix Science, UK) for protein identification against the NCBI Homo sapiens (human) protein database (updated on Feb 24, 2007), which contained 191507 sequences. The software MetaCore version 6.2 (Bioinformatics Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, CAS.) were used to further analyze the protein molecular process, potentially involved pathways, and protein-protein interaction network based on the GeneGo database.

Results

Patient Characteristics

Four patients were collected in this study. There were 3 females and 1 male. The mean age was 45 years (45.6 ± 3.14). The pretreated tumor stages were determined by rectoscopy/colonoscopy and histologically biopsy, and CT scan result. There were 4 of grade III (T3-4, N1-2).

Detection of Candidate Proteins in RP

The comparison of the protein profiles of rectal cancer tissue of patients before and after radiation therapy was made. Automatic gel comparison revealed several spots with differential expression (with at least a 1.3 fold difference in the percentage of the volume) before and after radiotherapy. Twenty-three spots were picked and showed the altered pattern of expression in rectal cancer patients (Figure 1). One protein was chosen when there were the duplicate entries. The proteins with a CI% less than 95% were abandoned. In this case, only monotonically changing feature intensities between pre-radiotherapy and end of radiotherapy from the same patients were selected. As a result, 12 unique proteins were identified and further together fed into the MetaCore software. Table I shows a list of selected proteins with more than 1.3 fold different expression of rectal tissues in response to radiation therapy, including 6 over-expression proteins and 7 downregulation proteins. As the highest peak, G3p was more than 3.0 folds overexpression compared with pre-radiation therapy. The canonical pathway maps, cellular, molecular processes, and biological networks were further analyzed by using the MetaCore software. Experimental data were visualized on the maps drawn from scratch by GeneGo annotators and were manually curated and edited.

The canonical pathway maps represent a set of about 650 signaling and metabolic maps covering human biology (signaling and metabolism) in a comprehensive way. In Figure 2, the height of the histogram corresponds to the relative expression value for a particular protein. The top-scored map

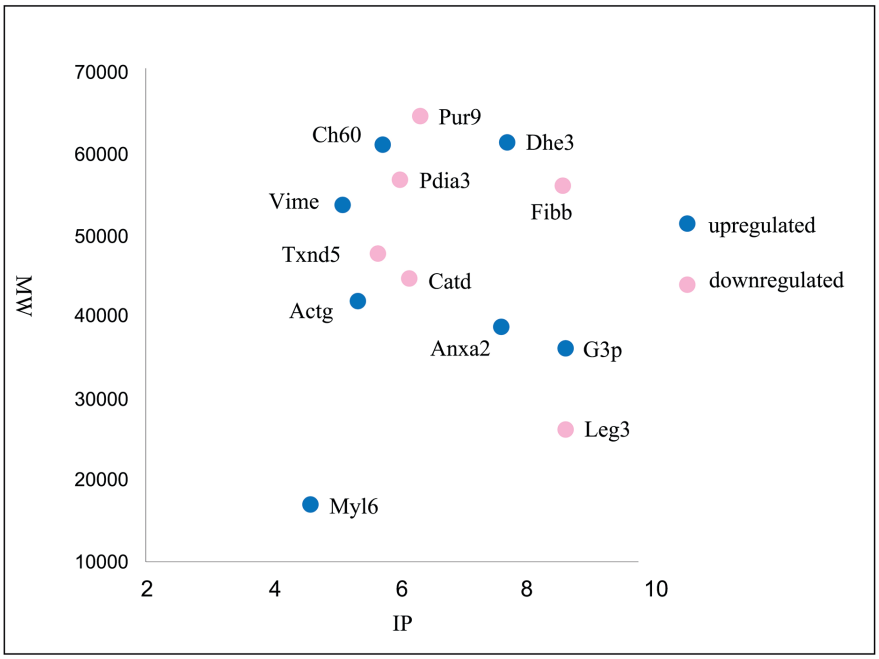

Figure 1. Theoretical 2-DE maps of cortical and cuticular KIFs and KAPs expected after gel MS analysis. Predicted 2-DE patterns (computing with pI/MW tool available online on ExPASy proteomic server). X axis: IP (isoelectric point); y axis: MW (molecular weight).

(map with the lowest *p*-value) is identified as the "cytoskeleton remodeling-regulation of the actin cytoskeleton by Rho GTPase", which involve the upregulated proteins MELC, Myosin II, and Actin.

The gene content of the uploaded files is used as the input list for the generation of biological networks using Analyze Networks (AN) algorithm with default settings. This is a variant of the shortest paths' algorithm with main parameters of 1. relative enrichment with the uploaded data, and 2. relative saturation of networks with canonical pathways. These networks are built on the fly and unique for the uploaded data. In this workflow the networks are prioritized based on the number of fragments of the canonical pathways on the network. Five top-scored pathway networks were identified, including regulation of biological quality, response to biotic stimulus, tissue development, glutamate deamidation, glutamate biosynthetic process, glutamate catabolic process, and positive regulation of cell communication. Four proteins (Pdia3, Vimentin, Galectin3, Dhe3) involved are picked as potential biomarkers in response to radiation therapy of rectal cancer.

Pdia3

Protein disulfide isomerase family A, member 3 (PDIA3) known as ER60, the glucose-regulated protein, was mainly involved in the formation of the major histocompatibility complex (MHC) class I peptide-loading complex and played a role in the tumorigenesis process in several kinds of cancers. In ovarian cancer[5], prostate cancer[6], breast cancer[7], cholangiocarcinoma[8], and uveal melanomas[9], the elevated expression of PDIA3 in both mRNA and protein levels, was associated with the tumor proliferation and metastasis process. In our proteomic results, after five-week radiation therapy, the PDIA3 was downregulated in rectal cancer patients (Table I and Figure 3). We have also shown that the PDIA3 protein appears to be linked to radiation response of rectal cancer and therefore provides novel potential biomarkers in the prognosis of rectal cancer.

Galectin-3 and TITF1 Pathway

Galectin3, also known as Leg3, an endogenous lectin that belongs to the beta-galactoside-binding gene family, was involved in a variety of biological activities, including cell cycle regulation, tumor cell adhesion, proliferation, progression, and metastasis. In recent studies, Galectin3 ranged higher levels in colon cancer patients, and serum Galectin3 might represent a biomarker to characterize colon cancer transformation. In our results, the protein level of Galectin3 was downregulat-

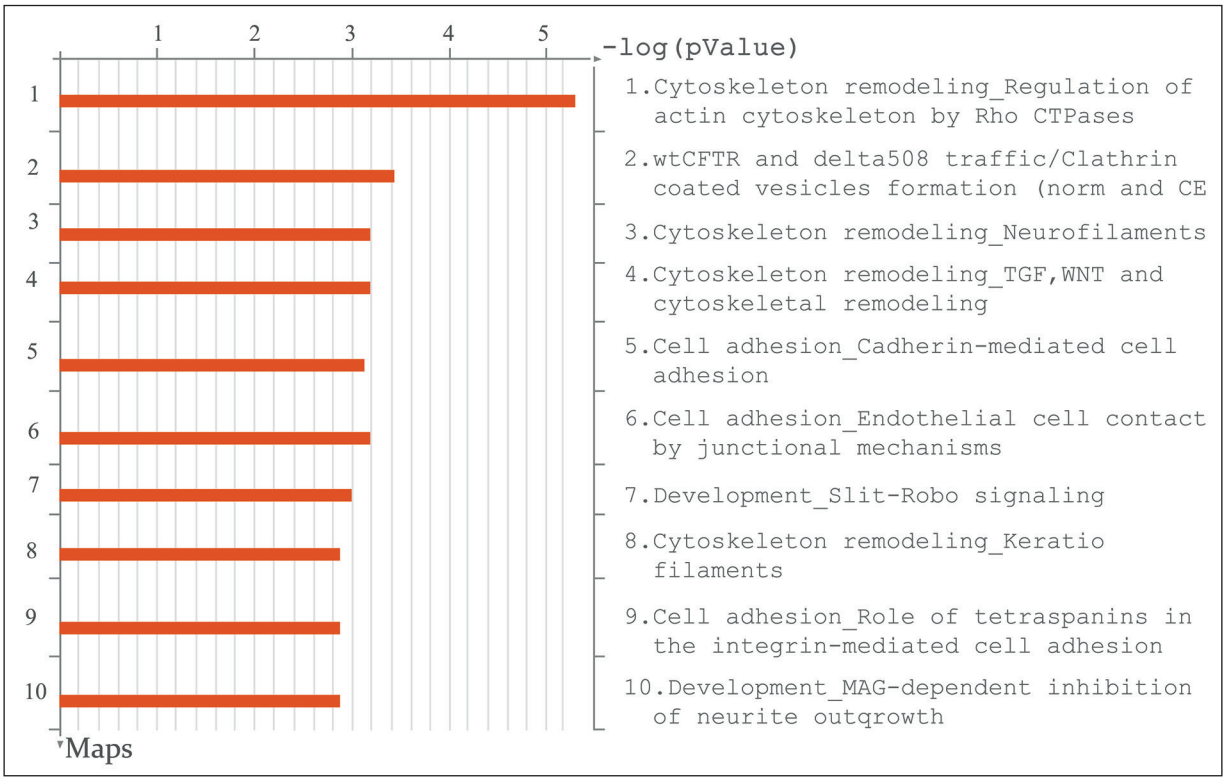

Figure 2. The biological functions found in the MetaCore software using the 13-radiation response related proteins. The height of the histogram corresponds to the relative expression value for a particular gene/protein. The top four processes are all associated with cytoskeleton remodeling.

ed (1.42 folds than controls) in rectal tissues after radiation treatment in cancer patients (Table I and Figure 4). As shown in the protein-protein network (Figure 4), Galectin3 was involved in the positive regulation of TITF1, one of the lineage-specific oncogenes in lung cancers[10]. It is suggested that Galectin3 might be acting as a useful biomarker in predicting radiation response in rectal cancer prognosis.

Dhe3

Another potential biomarker of the radiation response was Dhe3 protein. It was upregulated in our proteomics result (Table I) and involved in the

Table I. Different expression level of selected proteins of rectal tissues in response to radiation therapy.

Protein Score	Proteins identified	Accession No.	Level
69	Pur9	P31939	-
170	Pdia3	P30101	-
150	Fibb	P02675	-
63	Txnd5	Q8NBS9	-
132	Catd	P07339	-
58	Leg3	P17931	-
81	Anxa2	P07355	+
82	My16	P60660	+
167	Dhe3	P00367	+
231	Vime	P08670	+
100	Ch60	P10809	+
70	Actg	P63261	+
123	G3p	P04406	++

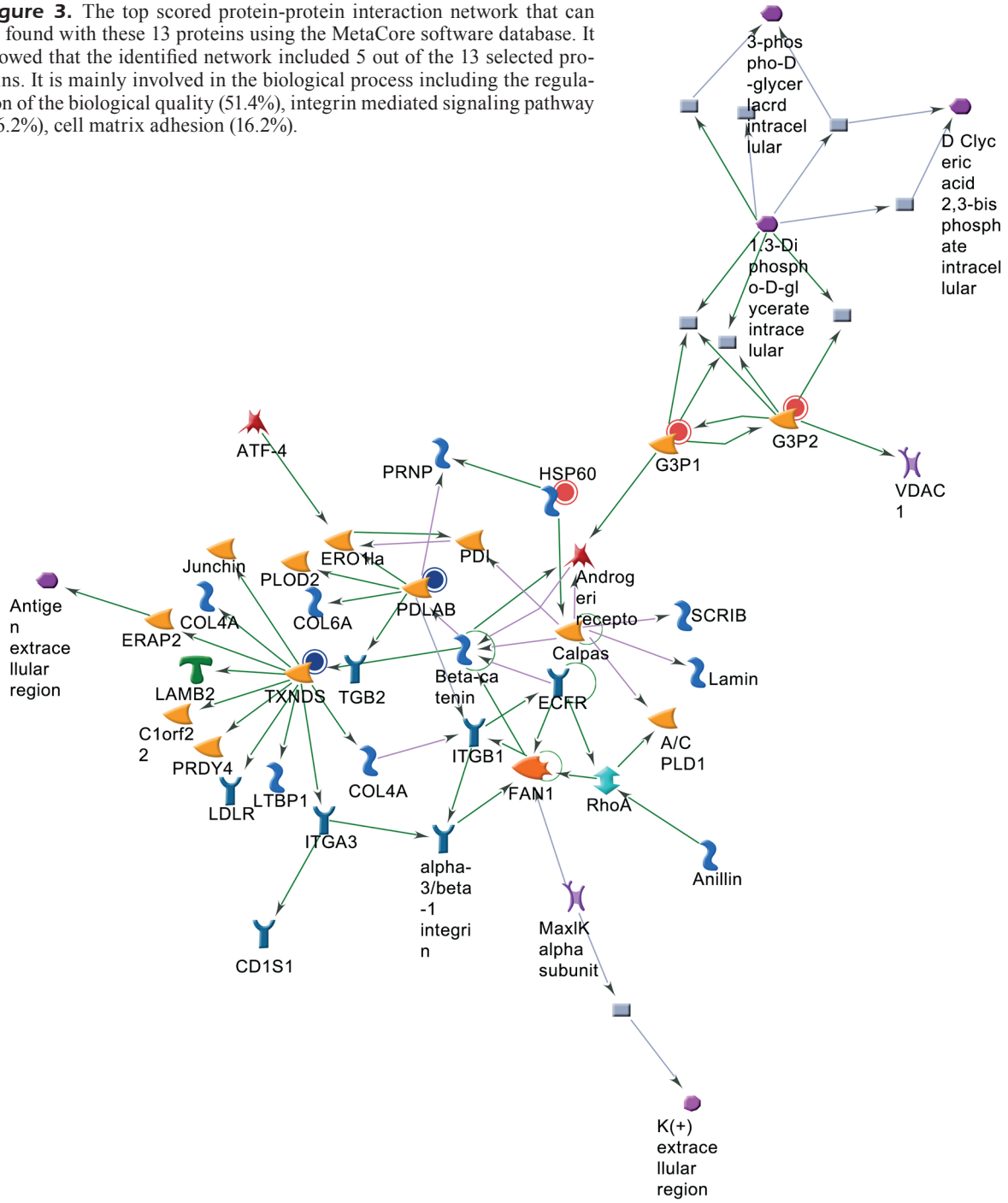

Figure 3. The top scored protein-protein interaction network that can be found with these 13 proteins using the MetaCore software database. It showed that the identified network included 5 out of the 13 selected proteins. It is mainly involved in the biological process including the regulation of the biological quality (51.4%), integrin mediated signaling pathway (16.2%), cell matrix adhesion (16.2%).

top four protein-protein network maps which was about the glutamate deamidation and biosynthetic process (Figure 5). In two previous studies, the Dhe3 was reported as a candidate biomarker in Clostridium difficile Toxin A treated coloncytes[11] and one of the congenital diseases – Smith-Lemli-Opitz syndrome (SLOS)[12]; however, its biological functions were still not clear.

Vimentin-ATF4 Pathway

Vimentin was a commonly reported protein in several proteomics studies. As the type III intermediate filament protein in mesenchymal cells, the main functions in cellular biological functions were to play a role in transepithelial-mesenchymal transition (EMT)[13]. In recent studies, increasing evidence proved that it was involved

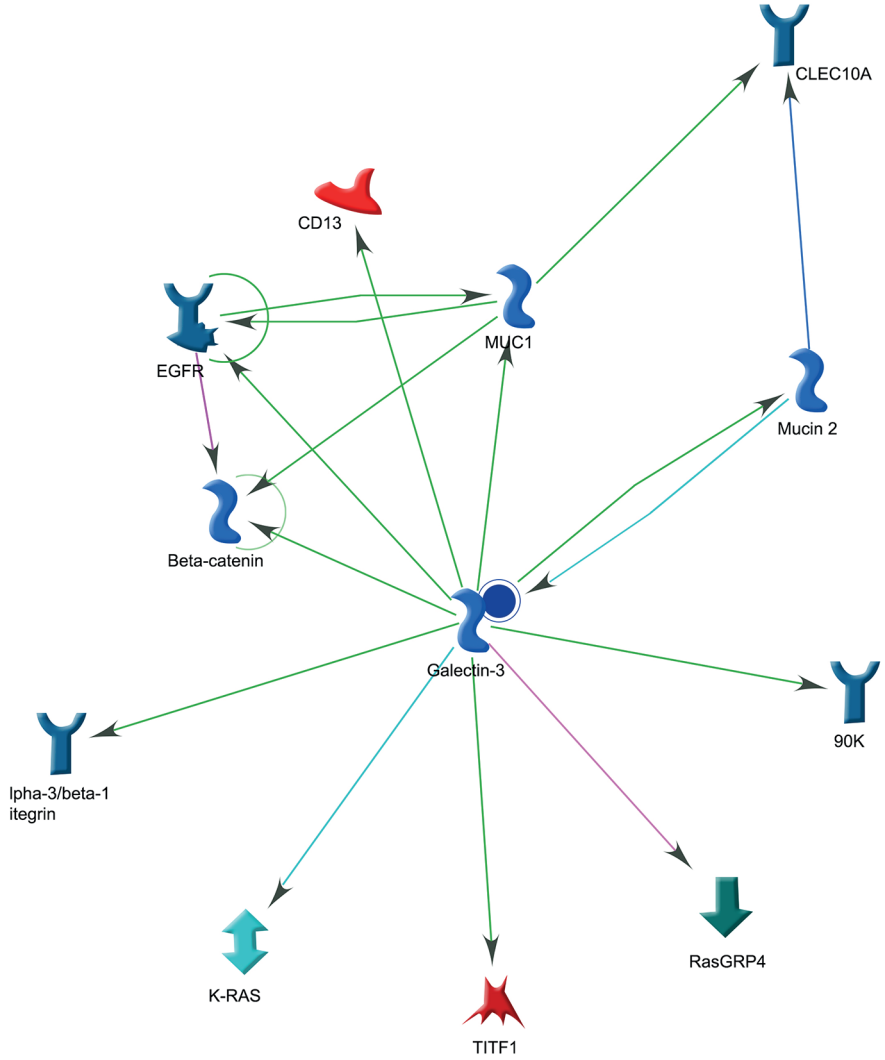

Figure 4. The fifth scored protein-protein interaction network that can be found with these 13 proteins using the MetaCore software database. It is mainly associated with the biological process including positive regulation of cell communication (41.7%), glial cell differentiation (25.0%), cell fate specification (25.0%).

in the tumorgenesis of various types of cancer, which suggested the directions toward future cancer therapy utilizing Vimentin as a potential molecular target[13]. In our study, Vimentin was upregulated (Table I) in the top two-pathway network, which was involved in response to biotic stimulus tissue development and multi-organism process (Figure 6). The main interesting function of Vimentin in our proteomic data analysis was revealed as its role in negative regulation of the well-known transcription factor ATF4.

Discussion

Rectal cancer is known as one of the main causes of cancer patients around the world[1]. Radiotherapy has become the standard therapy for local advanced rectal cancers. To date, in order to identify potentially non-sensitive patients to radiation therapy and avoid unnecessary side effect, a wide range of predictive tools have been used to investigate the molecular biomarkers to predict the response to radiotherapy. However, the results

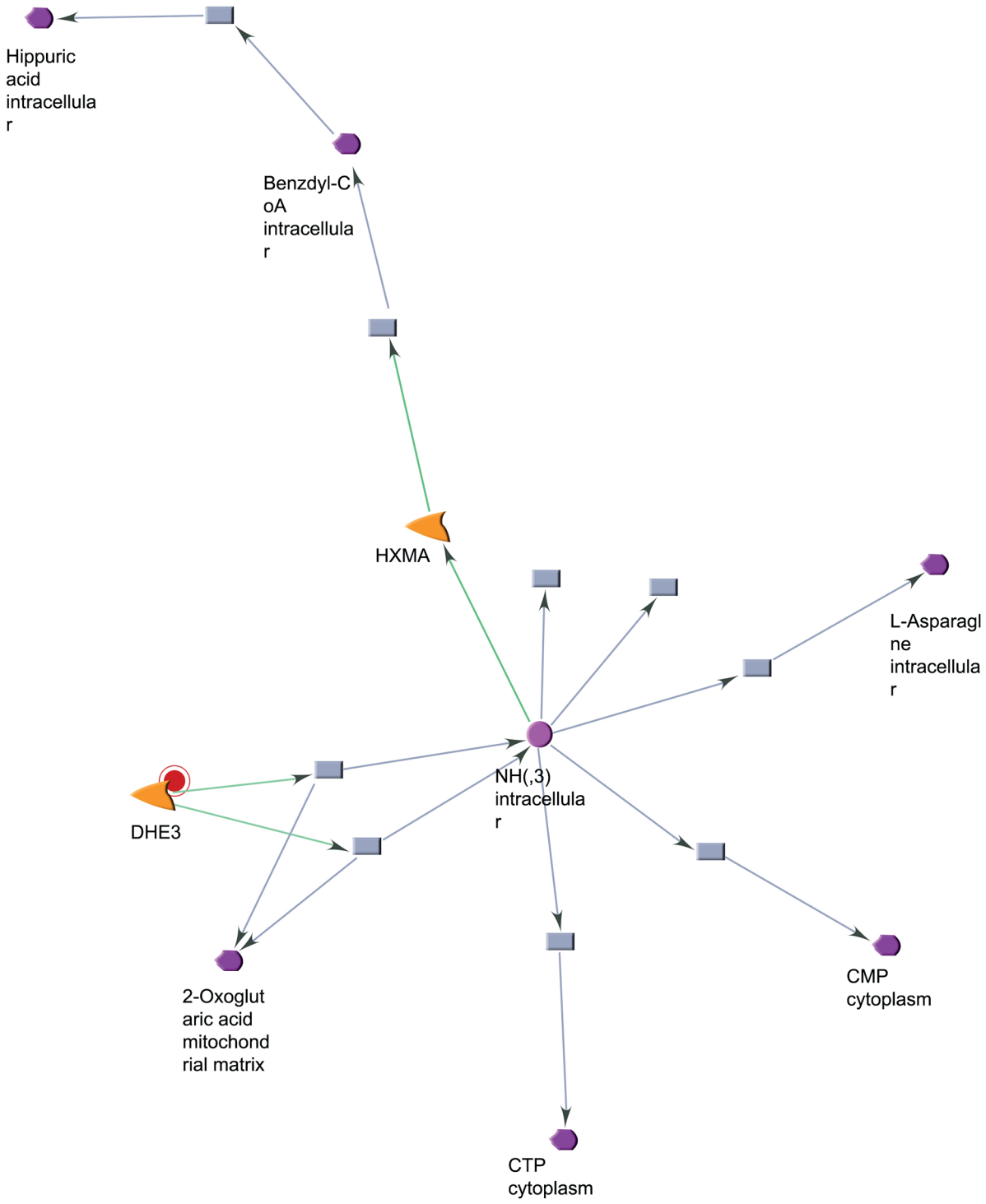

Figure 5. The fourth scored protein-protein interaction network. It is mainly associated with the biological process including glutamate deamidation (50.0%), glutamate biosynthetic process (50.0%), glutamate catabolic process (50.0%).

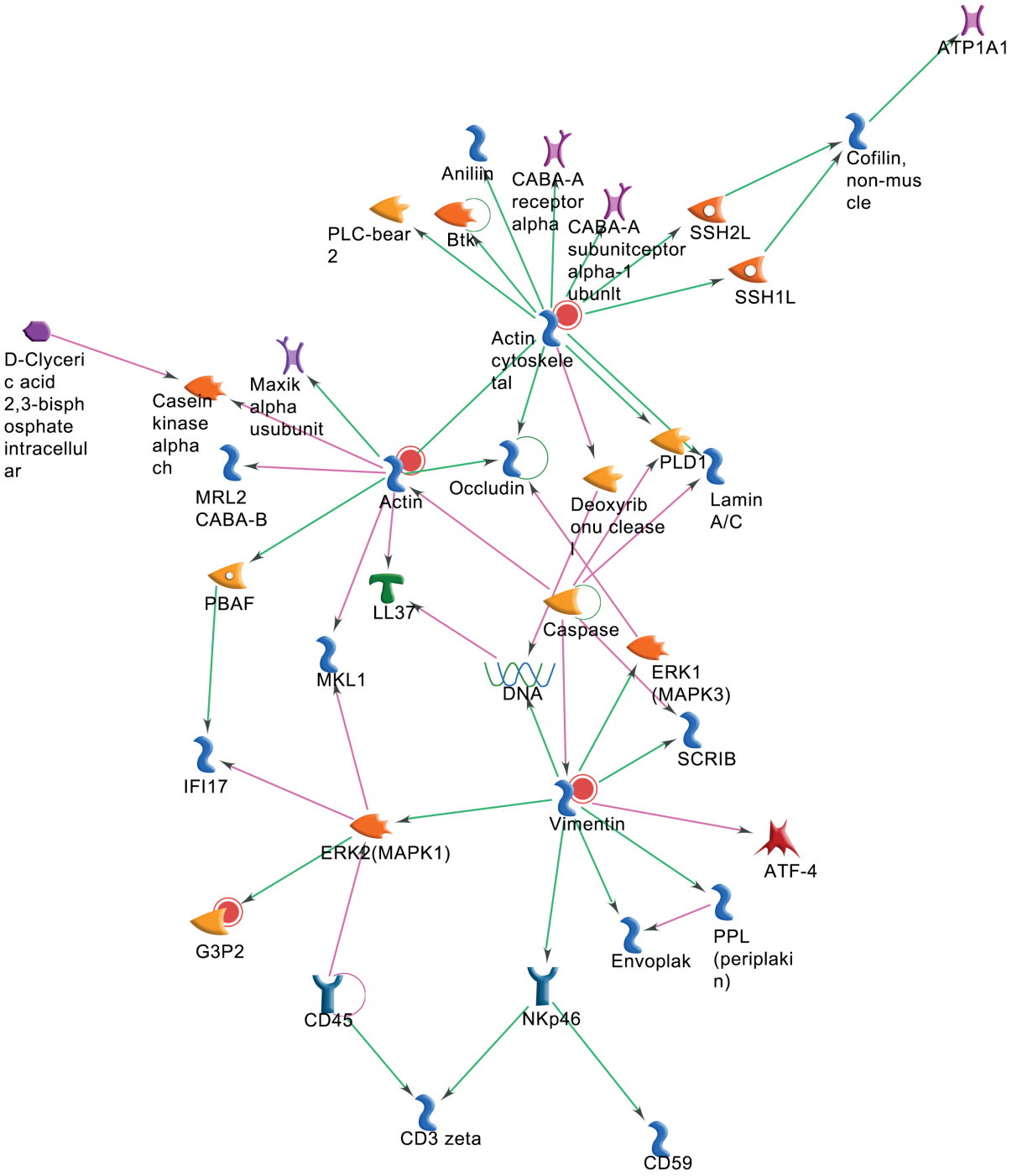

Figure 6. The second scored protein-protein interaction network. It is mainly involved in the biological process including response to biotic stimulus (28.6%), tissue development (31.4%), multi organism process (31.4%).

were controversial since the technological limitations, and till now they suggested no specific biomarkers to predict the radiotherapy of rectal cancer. In cellular radiobiology, radiation effect is mainly identified as the capacity of cells in molecular response to exhibit infinite proliferation, also known as clonogenicity[14]. Events of radiation therapy may vary at both transcription and post-transcription levels. Therefore, the proteomics approach was used to identify the protein regulation patterns as molecular marker of radiation response of cancers since the 1990s.

Cell cycle is the main target in radio-oncology since there was evidence implicating DNA damage in specific cell cycle stage as the primary cause of cell death[15,16]. Proteomics studies have already suggested that certain proteins may play a role in cell cycle and induction of apoptosis in several kinds of cancers. Recent studies have reported that PDIA3 was highly expressed in ovarian cancer, prostate cancer, uveal melanomas, breast cancer, and cholangiocarcinoma with real time-PCR confirmation about the mRNA expression. It was also reported that the downregulation of PDIA3 protease inhibits cellular proliferation by inducing G1/S cell cycle arrest[17]. In our results, the PDIA3 was firstly identified in a downregulation pattern in rectal cancer after radiation therapy. In previous studies, PDIA3 is involved in tumorigenesis metastasis and also could be a prospective target for cancer therapeutics. The role of PDIA3 was also detected in our proteomic studies and in terms of the response of PDIA3 after radiotherapy, it would appear that PDIA3 could be a valuable candidate biomarker in rectal cancer.

Cell adhesion is reduced in human cancers; it allows cancer cells to disobey the normal growth order, resulting in cell structure disorder, cancer invasion, and metastasis[18]. Okegawa et al[19] showed that some adhesion molecules play a pivotal role in the development of cancer progression, which is a multi-step process, including recurrent, invasive, and distant metastasis. In our proteomics analysis with 13 proteins, it indicated that cytoskeleton remodeling and cell adhesion were the mainly involved biological functions in these radiation response proteins. Galectin3, that binds glycan epitopes of cell membrane, with its carbohydrate-binding properties, might constitute the basis for cell-cell or cell-matrix interaction and tumor progression. Modulation of Galectin3 expression in cancer cell adhesion has been reported in previous studies[20]. These observations led to the recognition of Galectin3 as a useful diagnostic/prognostic biomarker for several cancer types, such as thyroid, prostate, and colon cancer[21-23]. It also reported that it might play a role in the immune escape mechanism in tumor progression by inducing apoptosis of cancer-infiltrating T-cell[23]. It was observed in rectal cancer for the first time in our protein-protein interaction results. Galectin3 was downregulated after radiation therapy and it might involve in TITF1 positive regulation pathways. TITF1, thyroid transcription factor 1, is one of the oncogenes contributes to tumorigenesis[24]. The downregulation of Galectin3 in radiation-treated rectal cancer revealed it might act as an important biomarker to evaluate rectal cancer prognosis.

Dhe3 gene functions were less reported in previous studies. Just in two proteomics studies, it was reported as a candidate biomarker in Clostridium difficile toxin A treated colonocytes and the congenital disease SLOS[12,13]. Dhe3 protein was suggested to be involved in metabolism or oxidative stress response. In our results, Dhe3 was mapped in the top four protein-protein networks, and might be associated with glutamate metabolic process. As its upregulated trend in response to radiation therapy in rectal cancer and response in the colonocytes physiology study, it might be a valuable potential biomarker in the digestive system disease.

As a multifunctional protein that interacts with a large number of proteins, Vimentin acts as a potential regulator in several different physiological processes; however, its true function in cancer progression is yet to be unraveled. There were works directly elucidating the role of Vimentin in various signaling pathways. Activating transcription factor 4 (ATF4) is a member of the activating transcription factor family. In cancer biological process, ATF4 expression is along with cancer cell progression and treatment resistance[25], where it promotes cancer cell survival by transcriptionally regulating metabolic homeostasis and angiogenesis[26,27]. Meanwhile, what interested us was that Vimentin was reported recently as one of the novel negative regulators of the ATF4 in osteoblasts[28]. In our results, Vimentin was significantly downregulated in rectal cancer tissue after radiation therapy. If it mainly acts as a turn-off factor in ATF4 pathway in this radiation response reaction, it might provide number of suggestions that Vimentin serves as an attractive biomarker and therapeutic target in radiation therapy of rectal cancer. Further researches about the *in vitro* regulation of Vimentin in ATF4 pathway in cancer cells must be encouraged and support these clinical data.

Conclusions

This research showed four potential biomarkers in radiation response of rectal cancer, including PDIA3, Galectin3, Dhe3, and Vimentin. However, such proteomics prediction is not focused on both necessary to give evidence of the radiation response in rectal cancer. Further studies on mRNA and protein levels are needed

to observe the overexpression or inhibition. The determination of a useful biomarker in radiation therapy of rectal tumors might be sufficient time and dose effect *in vitro* studies results. We consider the fact that we have found the potential and valuable radiation response-related proteins by using the proteomics approach in patients' rectal cancer samples. Further researches are urgent needed to apply these biomarkers in clinic.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

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