Systems Biomedicine of Colorectal Cancer Reveals Potential Targets for CRC Treatment.

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Abstract

Colorectal cancer is one of the leading causes of cancer death worldwide. Patients' survival at the time of diagnosis depends largely on the stage of the tumor. Therefore, understanding the molecular mechanisms promoting cancer progression from early stages to high-grade stages is essential for implementing therapeutic approaches. In the present study, we conducted a transcriptomic meta-analysis to identify potential molecular targets for colorectal cancer treatment. We proposed a number of genes that their expression induction or suppression alone or in combination with each other would inhibit tumor progression or metastasis. Furthermore, expressions of a number of them were presented as prognostic biomarkers for colorectal cancer. They are involved in cell proliferation, energy production under hypoxic conditions, epithelial to mesenchymal transition (EMT) and angiogenesis. Employing a network analysis point of view, some genes were discovered that have not been reported noticeably in any kind of cancer neither as a prognostic biomarker nor curative target. As a result, they might have some roles in progression of colorectal cancer.

Introduction

Colorectal cancer (CRC) is a major global medical burden worldwide. It is identified as the third most frequently diagnosed cancer and the fourth cause of cancer death [1]. Approximately more than one million people are diagnosed with CRC each year, and about half of them die of CRC annually [2]. Complex genetic interactions together with environmental factors are recruited to trigger a cell to become cancerous. Among them, aberrant growth factor signals contribute to the uncontrolled proliferation of cells which ultimately leads to metastasis. Metastasis is a dynamic process in which migration of transformed tumor cells from their primary site to other regions in the body occurs [3]. Contrary to early-stage tumor cells, malignant cells have the ability to detach from the stroma as well as acquire motility [4]. This event happens during a process called EMT in which cells lose their epithelial characteristic including adhesion and subsequently dedifferentiate into mesenchymal mobile cells [5]. Genes that are differentially expressed (DEGs) between Primary and Metastatic groups in gene expression datasets, would be the ones that their effects cause different steps of metastasis.

A large number of molecular and pathway targets have been identified for treatment of CRC through the past decades. Growing progresses have been made in the development of chemotherapy and antibody drugs [6]. The first priority in the therapy procedure is to determine whether tumor is primary or metastatic. Besides, it is required to follow a standard procedure to determine mutation status for targeted therapies. For instance, mutations in RAS/RAF/MAPK pathway lead to resistance to anti-epidermal growth factor receptor antibodies [7].

Tyrosine kinases (TKs) targeting using monoclonal antibodies and small molecule tyrosine kinase inhibitors are effective strategies [8]. Targeting cancer-related inflammation biomarkers like IL-6/JAK/STAT3 pathway which inhibits the progression of solid tumors is another beneficial therapeutic strategy [9]. In addition, restraint of cytosolic β-catenin via disturbing hyperactive Wnt/β-catenin signaling pathway could be another treatment approach for colorectal and many other types of cancer [10]. Inhibition of matrix metalloproteinases (MMPs) and TGFβ signaling pathways is a therapeutic approach to prevent liver metastasis [11] [12, 13]. Furthermore, PI3K inhibition suppresses lung

metastasis in CRC patients [14, 15]. Among the known chemotherapy drugs, Cetuximab is one of the popular ones which is a monoclonal antibody against epidermal growth factor receptor (EGFR) [16]. Furthermore, Vascular Endothelial Growth Factor (VEGF) antibody, bevacizumab, is the standard treatment for metastatic colorectal cancer [17].

One way to identify molecular mechanism of pathogenesis in a biological context is to analyze transcriptomic data. In this study, data from two microarray studies each containing three groups of samples were excavated to recognize differentially expressed genes (DEGs) in any two-pair comparison between groups. The goal was to determine DEGs specific to CRC that can be targeted for cancer therapy. Healthy groups came from healthy colon tissue from people with colorectal cancer. Primary group contained gene expression data from a benign tumor and metastatic group contained expression values from metastatic tumor samples in colon or liver and lung metastasis in CRC patients. We hypothesized that these DEGs are involved in cancer progression and their expression could be targeted (suppressed or induced) individually or in combination with one another for CRC treatment. To this end, a systems biology method with focus on network analysis was employed to obtain a number of DEGs from the results of transcriptomic analyses. Then the role of DEGs was interpreted biologically to see whether they could be potential targets for CRC treatment. Moreover, some of these expressions were proposed to be CRC prognostic biomarkers.

Methods

Database Searching and recognizing pertinent experiments

Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) repository was searched to detect experiments containing high-quality transcriptomic samples concordance to the study design. Searches were filtered for Homo sapiens and colorectal/colon cancer, EMT and metastasis are the keywords utilized in the search. Microarray raw data with accession numbers GSE18105 and GSE41258 were downloaded from GEO database. In the first study (GSE18105), all samples were from Metastatic (Stage III and IV of the disease), Primary (non-metastatic, stage II of the disease) and normal colon tissue. In the second experiment (GSE41258), normal, primary tumor samples (samples in stage I and II of cancer) were

selected from colon tissue while metastatic samples were selected from liver and lung metastasis tissues in colon cancer patients.

Meta-Analysis and Identifying Differential Expressed Genes

R software was used to import and analyze data for each dataset separately. Preprocessing step involving background correction and probe summarization was done using RMA method in "affy" package [18]. Absent probesets were also identified using "mas5calls" function in this package. If a probeset contained more than two absent values in each group of samples, that probeset was regarded as absent and removed from the expression matrix. Besides, Outlier samples were identified and removed using PCA and hierarchical clustering methods. Next, data were normalized using Quantile normalization approach [19]. Then, standard deviation (SD) for each gene was computed and SDs median was used as a cut off to remove low variant genes. Many to Many problem [20] which is mapping multiple probesets to the same gene symbol was solved using nsFilter function in "genefilter" package [21]. This function selects the probeset with the highest Interquartile range (IQR) to map to the gene symbol. "LIMMA" package which applies linear models on the expression matrix was utilized to discover DEGs between three groups of samples [22]. Genes with absolute log fold change larger than 0.5 and Benjamini Hochberg adjusted p-value [23] less than 0.05 were selected as the DEGs. Three sets of DEGs were obtained for each dataset. For each set, common DEGs between two datasets were selected as the final DEGs.

Network Construction

STRING database was used to generate Protein-Protein Interaction (PPI) network from all final DEGs. Using "igraph" package in R software [24], the giant component of the weighted network was extracted from the whole network. Next, the weighted adjacency matrix was transformed into a symmetric matrix to get modified into a new adjacency matrix using topological overlapping measure (TOM) function in "WGCNA" R package [25]. To remain with a distance matrix, the new adjacency matrix was subtracted from one.

Neighbourhood Ranking to the Seed Genes

Using R software, a matrix of all shortest paths between all pairs of nodes in a weighted network was constructed using using Dijkstra algorithm [26, 27]. Next, a distance score, Dj, for each node in the PPI network was computed. Dj is the average of the shortest paths from all the non-seed genes to reach the node j subtracted from the average of the shortest paths from the seed genes to reach the node j divided by the average of the all shortest paths to reach the node j from the whole network. This scoring system implies how much close each node is to the seed nodes [27, 28].

$$D_j = \frac{\frac{\sum_{i \notin S} SP_{ij}}{|NS|} - \frac{\sum_{i \in S} SP_{ij}}{|S|}}{\frac{\sum_i SP_{ij}}{|S| + |NS|}}$$

Here S is the set of seed nodes and NS is the set of non-seed nodes. A positive score implies that node j is closer to the seed nodes compare to the rest of the nodes. Nodes with positive scores were kept and the rest were removed from the network. It should be noted that the D scores were calculated without imposing any threshold on edge weights.

Enrichment Analysis

Enrichment analysis was performed using Enrichr online tool [29]. Enriched terms for biological processes were obtained from *GO* repository. For pathway enrichment analysis, *wikiPathways* signaling repository version 2019 for humans was used. Enriched terms with the top score and the p-value less than 0.05 were selected.

Results

Data Preprocessing for Microarray Experiments

Each dataset was imported into R separately. Almost 75% of probesets were regarded as absent and left out from the expression matrix to reduce the number of false positives in multiple testing. To be more precise in preprocessing step outlier sample detection was

conducted using PCA and hierarchical clustering. Figure1A illustrates the PCA plot for the samples in GSE18105 study. The same plot was created for the samples in GSE41258 study. Between the three groups of samples, a number of them are away from their sets along the PC1 axis and they should be considered as outliers. To be more specific, a hierarchical clustering method introduced by Oldham MC, et al [30] was used. Pearson correlation coefficients between samples were subtracted from one for measurement of the distances. Figure 1B depicts the dendrogram for the normal samples. Figure 1C normal samples are plotted based on their Number-SD scores. To get this number for each sample, the average of whole distances is subtracted from the distance average of each sample. Then, results of these subtractions are normalized by the standard deviation of distance averages [30]. Samples with Number-SD less than negative two are mainly far apart from their cluster set in the PCA plane and they were regarded as outliers. Fourteen outlier samples for GSE18105 and sixteen outlier samples for GSE41258 were found. Supplementary file1 contains information about the groups of samples.

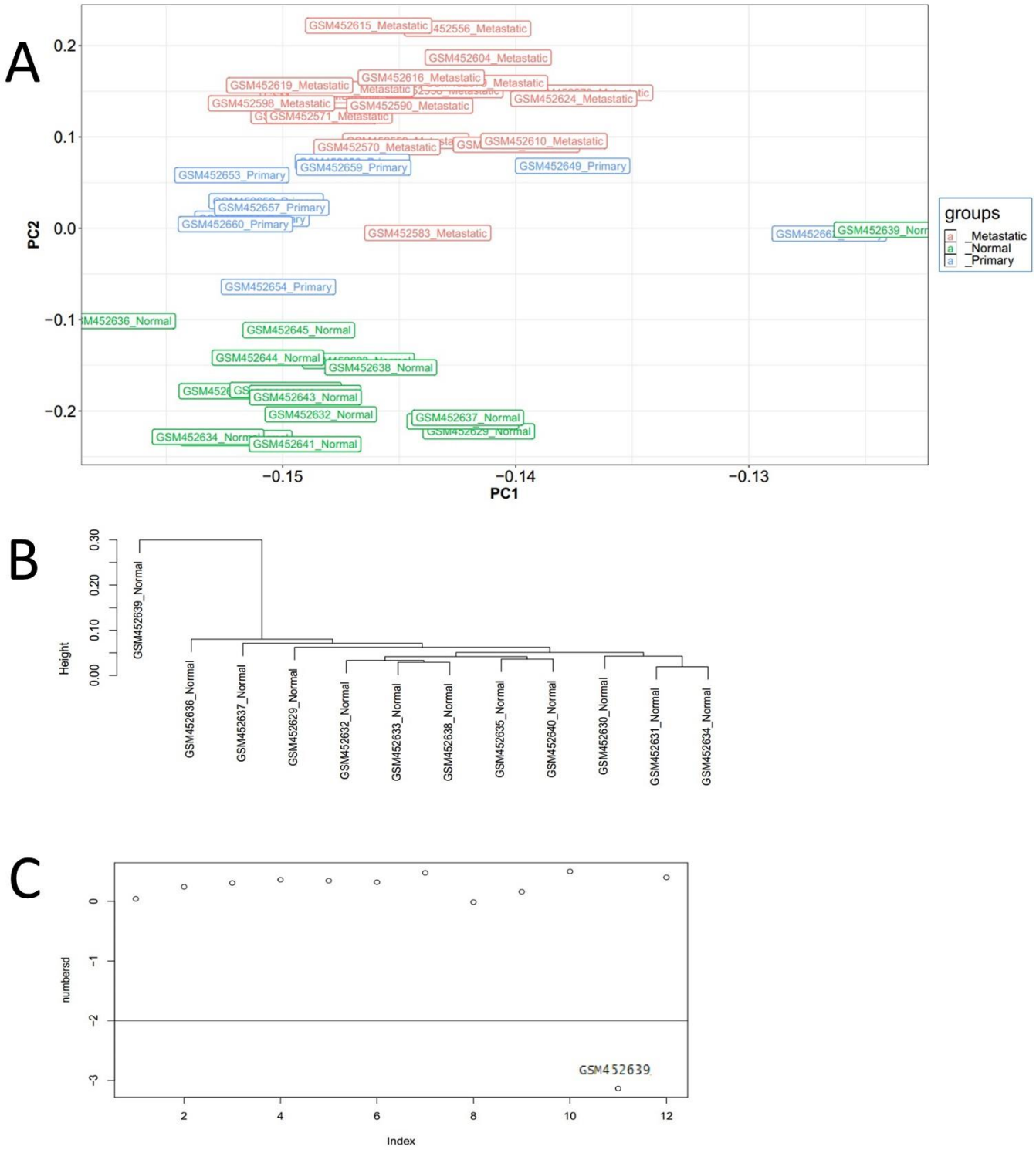


Figure 1: Illustration of outlier samples in GSE18105 study. A, is the PCA plot, B is the dendrogram for the normal samples and C is the Number-SD plot for same samples.

Figure2A illustrates the expression values for some housekeeping genes and a number of DEGs with absolute log fold change (LogFC) larger than 2 in GSE18105 dataset. Common DEGs between lung-primary analysis and liver-primary analysis with absolute LogFC larger than 1 in GSE41258 dataset are illustrated in Figure 2B. Housekeeping genes are situated on the diagonal of the plots whilst DEGs are located above or under the diagonal. This demonstrates that the preprocessed dataset is of sufficient quality for the analysis. SPP1 gene is not DEG in metastatic colon samples while it has a high LogFC in metastatic liver and metastatic lung samples comparing to the primary group which are 2.6 and 3.2 respectively.

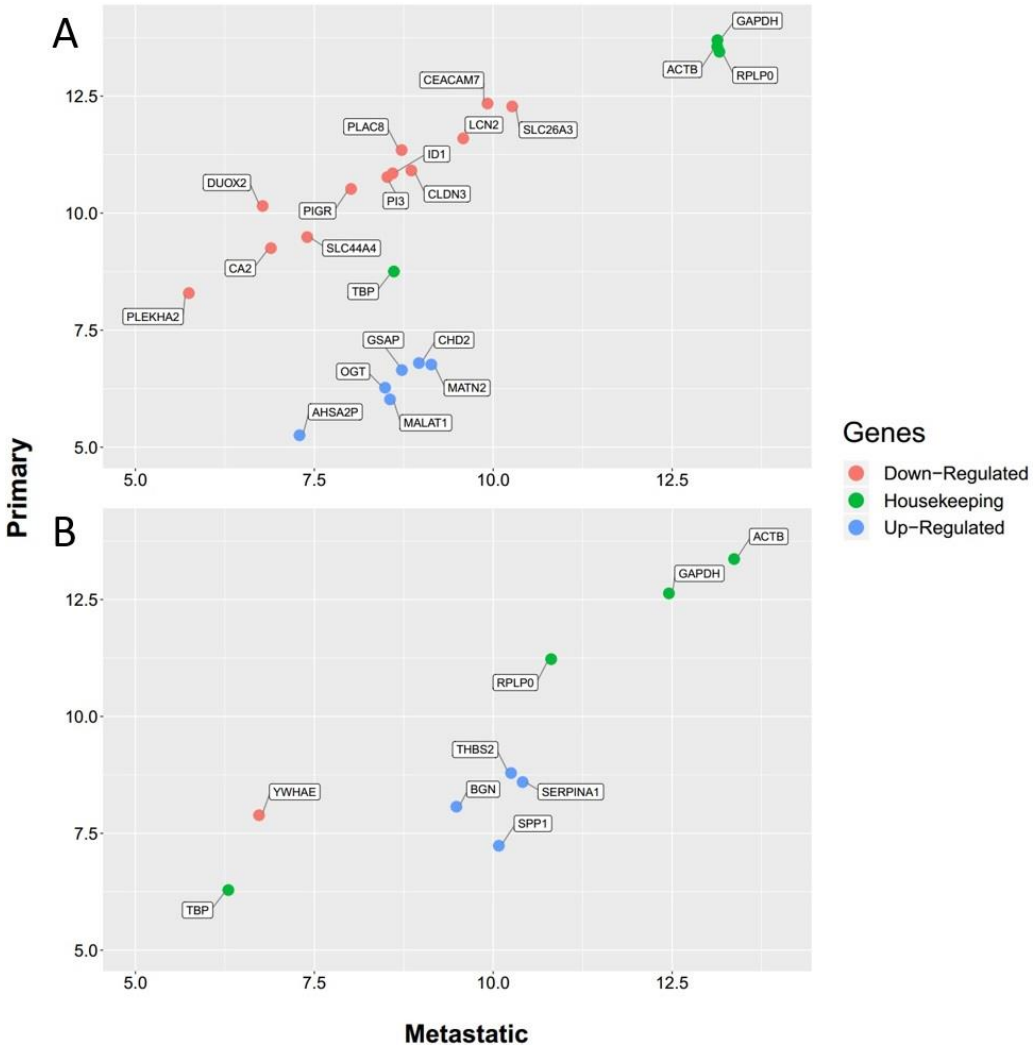


Figure 2: The quality Control plots. A, illustrates DEGs and housekeeping average values between Primary and Metastatic colon samples. B, illustrates common DEGs and housekeeping average values between Primary and Metastatic lung and liver samples. These charts exhibit metastatic group versus primary group.

Undirected Protein-Protein Interaction Network

4118 unique DEGs (differentially expressed genes) with adjusted p-value < 0.05 and absolute log fold change > 5 were achieved from seven sets of DEGs. Sets are liver metastasis vs normal, liver metastasis vs primar, lung metastasis vs normal, lung metastasis vs primary and primary vs normal for GSE41258. For GSE18105 they are metastasis vs normal, metastasis vs primary and primary vs normal. In the next step, common DEGs between all metastasis vs normal analyses in two datasets were 123 number. Common DEGs between all metastasis vs primary analyses in two datasets were 13 and Common DEGs between all primary vs normal analyses in two datasets were 210. There were 242 unique DEGs between these three groups and they were considered as the final DEGs. All DEG sets are presented in supplementary file 2. These DEGs were utilized to construct the Protein-Protein-Interaction (PPI) network. STRING database was employed to generate the Interactions based on seven filtrations namely, Neighborhood, Text mining, Experiments, Databases, Co-expression, Gene fusion and Co-occurrence. STRING combined scores were used as the edge weights. The giant component of the weighted network with 205 nodes and 554 edges is presented in figure 3.

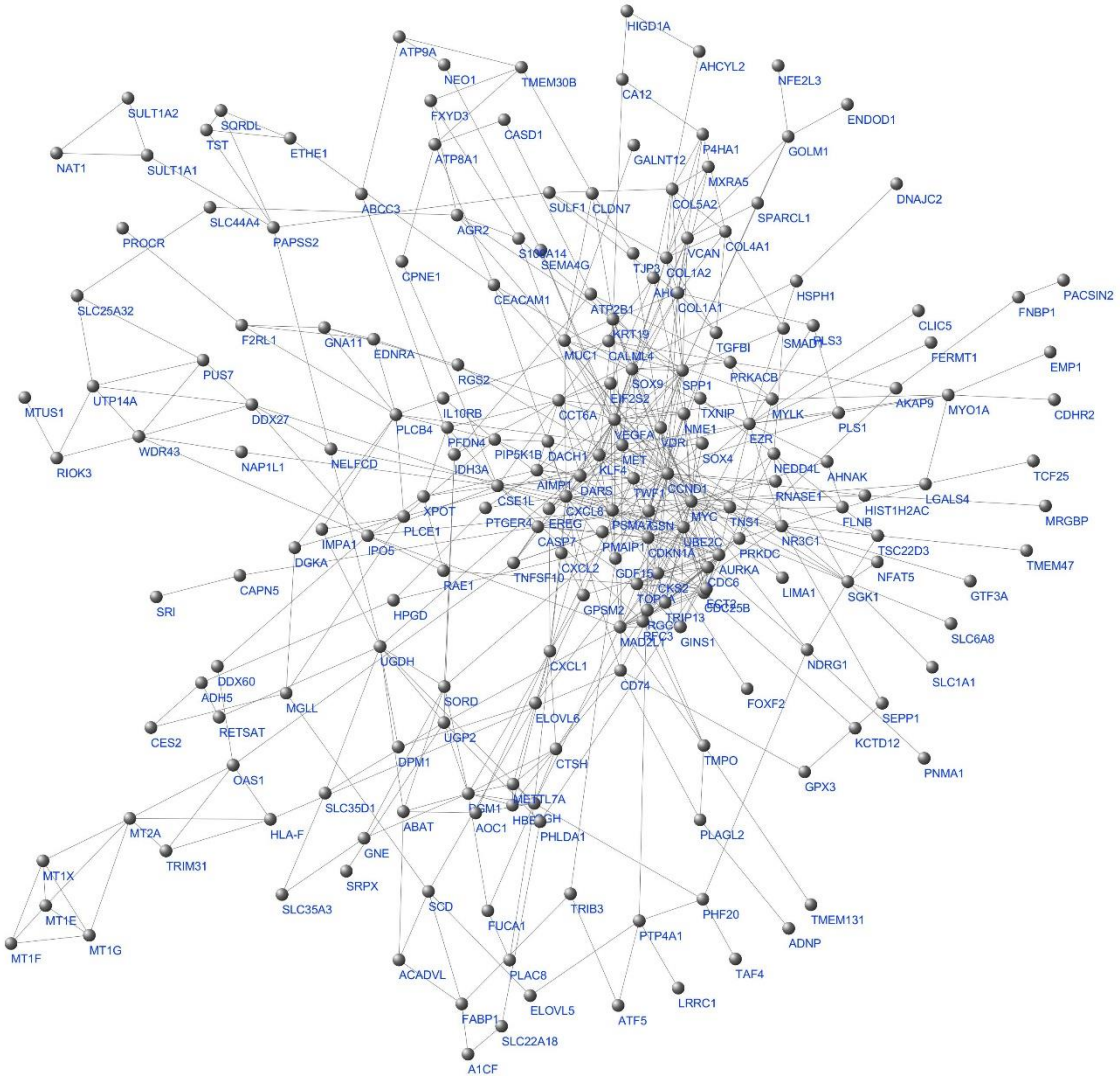


Figure3: The whole network giant component. Labels are protein/gene symbols. This is a scale free network [31] which follows a power law distribution (most of the network nodes have a low degree while there are few nodes with high degree).

Determination of Seed Genes Neighborhood through Shortest Path-Based Scoring System

In this step, we used weights between nodes as the estimation of distances in the weighted adjacency matrix. Nodes with shorter distances from the seed genes were selected and a smaller network was extracted from the main network. Computing the shortest path score for the non-seed genes led to a network with 39 nodes comprising 12 seed nodes and 27 neighbors. This multi-component graph is called Core network and is illustrated in figure 4A.

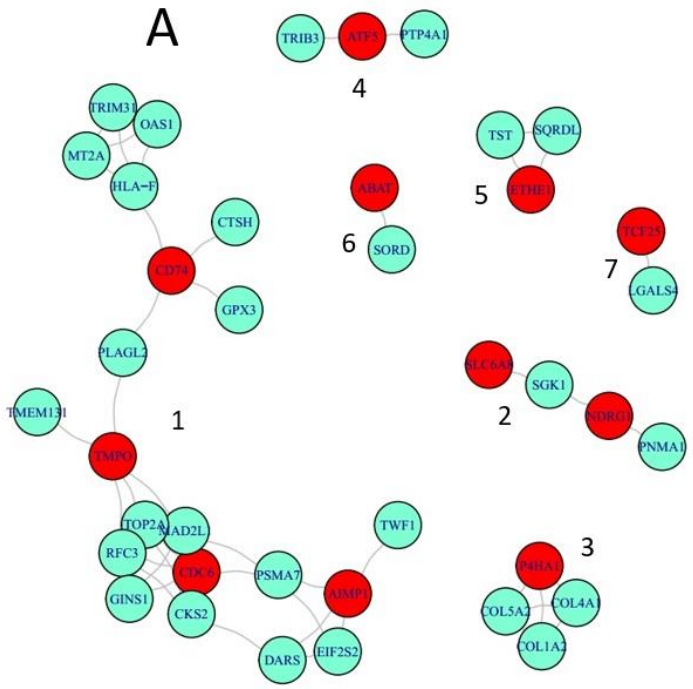
The expression states for these genes between any pair-wise comparison are depicted in Table1. For the three Metastatic-Normal comparisons (MvsN set) in the two microarray datasets, only TWF1status is not the same between the three groups. For the Primary-Normal analyses (PvsN set), all the genes witness a similar status between the two datasets. Status for the three Metastatic-Primary comparisons (MvsP set) are more challenging. In this set, the liver-primary and lung-primary are more similar to one another than the metastatic-primary comparison. They differ only in CD74 and ABAT genes.

MvsN			
DEGs	GSE18105	GSE41258	
	metastatic-normal	liver-normal	lung-normal
ETHE1	Down	Down	Down
DARS	Up	Up	Up
SGK1	Down	Down	Down
TMEM131	Down	Down	Down
TST	Down	Down	Down
LGALS4	Down	Down	Down
TRIB3	Up	Up	Up
COL5A2	Up	Up	Up
COL4A1	Up	Up	Up
PTP4A1	Up	Up	Up
COL1A2	Up	Up	Up
SORD	Up	Up	Up
SQOR	Down	Down	Down
OAS1	Down	Down	Down
TRIM31	Down	Down	Down
TWF1	Up	Down	Down

MvsP			
DEGs	GSE18105	GSE41258	
	metastatic-primary	liver-primary	lung-primary
TCF25	Down	Up	Up
CD74	Down	Down	Up
ETHE1	Down	Down	Down
ATF5	Down	Up	Up
AIMP1	Up	Down	Down
CDC6	Down	Down	Down
ABAT	Up	Up	Down
SLC6A8	Down	Up	Up
HLA-F	Down	Down	Down
TMPO	Up	Down	Down
NDRG1	Down	Up	Up
P4HA1	Up	Up	Up

PvsN		
DEGs	GSE18105	GSE41258
	primary-normal	
SGK1	Down	Down
EIF2S2	Up	Up
TRIB3	Up	Up
DARS	Up	Up
RFC3	Up	Up
ETHE1	Down	Down
TOP2A	Up	Up
CKS2	Up	Up
SORD	Up	Up
PSMA7	Up	Up
GPX3	Down	Down
MAD2L1	Up	Up
SQOR	Down	Down
TST	Down	Down
GINS1	Up	Up
LGALS4	Down	Down
PNMA1	Down	Down
PTP4A1	Down	Down
PLAGL2	Up	Up
COL1A2	Up	Up
CTSH	Up	Up
MT2A	Down	Down
COL5A2	Up	Up

Tabe1: The practical information for the core network components. The State column refers to the logFC (log fold change) column and Up means gene is upregulated and Down means gene is Downregulated.



B

DEGs	Degree	Betweenness
MAD2L1	7	59.33
CDC6	6	6
TOP2A	6	13.83
RFC3	6	13.83
TMPO	5	107
CKS2	5	17
AIMP1	4	19.5
HLA-F	4	51
CD74	4	93
PSMA7	4	43
GINS1	4	0
TRIM31	3	0
OAS1	3	0
EIF2S2	3	0.5
DARS	3	5
MT2A	3	0
PLAGL2	2	91
CTSH	1	0
TWF1	1	0
GPX3	1	0
TMEM131	1	0

Figure4: The Core network and centralities. This network presented in A contains seven components and seed genes are in red. B, Illustrates the Degree and Betweenness centralities for the nodes in the giant component. DEGs are sorted based on the highest Degree.

Network Descriptive

The network diameter is eight containing TRIM31, HLA-F, CD74, PLAGL2, TMPO, MAD2L1 PSMA7, AIMP1 and TWF1. In the following, the giant component descriptive is explained. Transitivity is around 60%, edge density is about 18% and the mean distance is 3.48. Two important centralities, Degree and Betweenness, for the giant component, are provided in figure 4B. MAD2L1 gene has the highest Degree and a relatively high betweenness. TMPO has the highest Betweenness and a rather high degree. Similar to TMPO, its direct neighbour, PLAGL2 has a pretty high Betweenness. This gene links the two parts of the giant component together. A common feature of the graph components is that all of them contain at least one seed gene. This would suggest that each component might have a role in metastasis process. Other centralities for the nodes and the average distances between each node and the other nodes are provided in supplementary file3 (S3).

Enrichment Analysis

Gene sets in the components 1 to 4 were given to *Enrichr* online tool. Tables 2A and 2B depict the enrichment analysis for the core network giant component. Enriched terms are sorted based on the highest p-value. Gastric Cancer Network2 is of the highest p-value containing TOP2A and RFC3 genes which are involved in DNA replication process. Involvement of the same genes in retinoblastoma cancer (WP2446) proposes the potential importance of these genes in different cancers. GPX3, TOP2A, and RFC3 are present in the highest number of pathways. Top2A also is involved in Gastric Cancer Network1.

In 2B, PSMA7 and MAD2L1 genes have monopolized the most GO terms to its self. After them, OSA1 and HLA_F possess the highest number of terms. GO:0060333, GO:0071346 and GO:0019221 terms contain the highest number of genes. These terms are related to interferon gamma and cytokine-mediated signaling which presents the role of cytokine mediated signaling cascades in colon cancer progression. The enrichment analysis for the core network components of 2, 3 and 4 are presented in Supplementary file 4.

Component 2, SGK1 contains the most number of pathways. NDRG1 and SGK1 are of the maximum number of GO terms and all four genes in component 2 are engaged in the regulation of apoptosis process. In component 3, all the collagen genes are engaged in Focal Adhesion-PI3K-Akt-mTOR-signaling pathway and extracellular matrix organization biological process. TRIB3 in component 4, is engaged in three enriched pathways especially insulin signaling and together with ATF5 enriches the negative regulation of transcription GO terms.

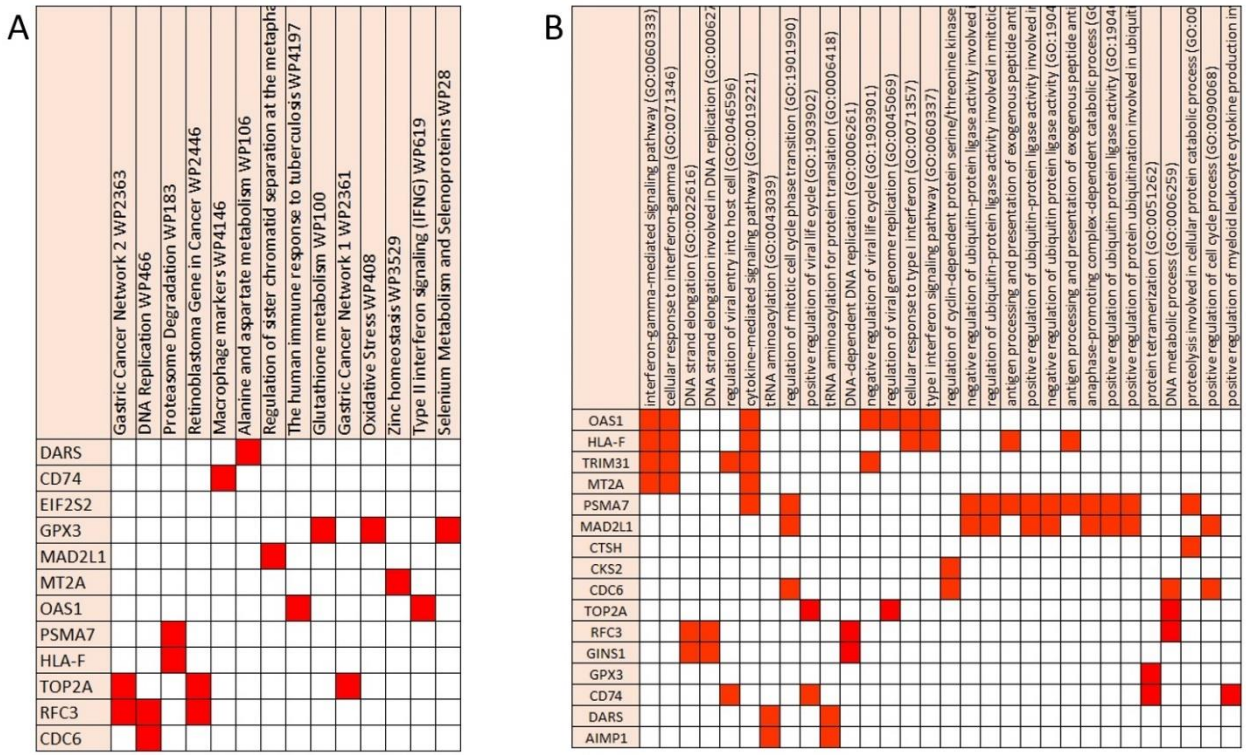


Table 2: Enrichment terms for the giant component. 2A illustrates the enriched pathways from WikiPathways signaling datasets. 2B shows the enriched terms for biological processes from GO repository. Red cells reveal the involvement of the genes in the enriched elements.

Discussion

The core network GC is composed of an up and a down parts attached via PLAGL2 transcription factor (TF). The lower part is engaged mostly in cell cycle and DNA replication and the upper part is engaged in cellular apoptosis. Component 2 contains genes involved in ECM remodeling, component 3 produce ECM proteins, component 4 is composed of genes involved in transcription inhibition, and Component 5 is composed of mitochondrial genes playing important roles in controlling cellular redox homeostasis. Table1 illustrates the expression results for obtained genes in all components of GC.

The expression of PLAGL2 gene in Balb/c3T3 cell lines leads to induction of Nip3 gene which can bind to Bcl-2 causing cellular apoptosis [32]. However, it is considered as an oncogene in different cancers. PLAGL2 anti-apoptotic role is carried out when it binds and prevents Pirh2 proteasomal degradation because Pirh2 promotes proteasomal degradation of P53

protein [33]. PLAGL2 suppresses neural stem cell (NSC) differentiation by interfering in Wnt/β-catenin signaling resulting in glioblastoma [34]. Besides, PLAGL2 regulates actin cytoskeleton and cell migration by promoting actin stress fibers and focal adhesion [35]. Results of PvsN analysis manifests that this gene is induced in colon cancer and have a high betweenness centrality. Since this gene connects the two parts of the GC, it would be a pertinent target for disturbing colon cancer network.

TRIM31 (a ubiquitin ligase) was downregulated in MvsN sets while there are contradictory results in different studies where it is shown to be reduced in lung cancer cells [36] and stepped up in gastric [37] and colorectal cancer [38]. MT2A gene is an antioxidant involved in the process of autophagy and apoptosis which protects cells against hydroxyl radicals and plays an important role in detoxification of heavy metals [39] [40]. Inhibition of this gene expression results in proliferation inhibition of CRC cells [41]. However, this gene was downregulated in PvsN analyses. It is also downregulated in pancreatic cancer [42] and its silencing promotes the effects of chemotherapy drugs on osteosarcoma primary tumors [43]. OAS1 expression is downregulated in breast ductal carcinoma and prostate cancer (PCa), at both mRNA and protein levels. In addition, OAS1 expression is negatively correlated with the progression these cancers [44]. Our analysis shows that expression of this gene is suppressed in CRC like the other two cancers. HLA-F is a MCH class 1b gene a surface marker of activated lymphocytes [45]. Presence of anti-HLA-F antibodies in cancer patients serums is a diagnostic method that implies the activated lymphocytes against cancerous cells [46] [47]. HLA-F is inducible by NF-κB and is responsive to IFN-γ similar to OAS1. Enriched GO terms also support the fact that these genes are engaged in interferon gamma and cytokine-mediated signaling (Table 2B). This gene was suppressed in MvsP analysis and could be a diagnostic marker of CRC.

CD74 (HLA class II histocompatibility antigen gamma chain) one of the seed genes exhibited a mixed trend of expression in MvsP analysis in Table1. CD74 serves as a survival receptor expressed on normal colon epithelial cells and colon carcinoma cells. CD74 stimulation by MIF results in enhanced cell survival by induction of Bcl-2 and Akt phosphorylation [48].

CTSH gene is a lysosomal cysteine protease upregulated in PvsN analysis. This protease plays an important role in cytoskeletal protein Talin maturation. Talin promotes integrin activation and focal adhesion formation leading to cell migration [49]. As a result, suppression of CTSH expression could be a choice of metastasis inhibition. Glutathione peroxidase GPX3 is an important antioxidant enzyme that defends cells against Reactive Oxygen Species (ROS) and its downregulation occurs in many cancers. For instance, its expression is suppressed in human colon carcinoma Caco2 cell lines, resulting in augmented ROS production [50]. It reduces H_2O_2 and lipid peroxides to water and lipid alcohols, respectively, and in turn oxidizes glutathione to glutathione disulfide [51]. Downregulation of GPX3, happened in PvsN analysis, leading to ascending of H_2O_2 level which is positively correlated with the tumor progression [52]. As a result, induction of GPX gene families would be a therapeutic approach.

Thymopoetin (TMPO/ Lamina-associated polypeptide 2 (LAP2)) gene was downregulated in liver and lung metastasis in MvsP analysis. This gene encodes a number of separate LEM domain containing protein isoforms via alternative splicing [53, 54]. The proteins are located in the nucleus of the cells which helps to form nuclear lamina and maintenance of the nucleus membrane structure [55]. TMPO prevents the depolymerization of nuclear laminas and excessive activation of the mitosis pathway. TMPO-AS1 is an antisense lncRNA for TMPO that is overexpressed in CRC patients. Expression of this gene is positively correlated with pathological features of the patients and distant metastasis [56]. TMPO-AS1 overexpression results in depolymerization of nuclear laminas and augmentation of mitosis pathways [57]. Regarding the fact that TMPO is one of the seed genes that has the greatest Betweenness centrality, it connects the top part of the Giant component engaged in cellular apoptosis to the bottom part engaged in cell cycle and DNA replication. Based on the given information it would be suggested that inhibition of TMPO-AS1 may result in the induction of TMPO gene and this would help to hinder the tumor growth.

TMEM131 is a transmembrane protein which was downregulated in MvsN analysis. No documentation was found to connect this gene to cancer. Therefore, this gene would be CRC specific. Enrichment analysis shows that this gene is engaged in IFN-γ signaling pathwy.

TOP2A gene was upregulated in PvsN analysis. In breast cancer (BC), HER-2 along with its adjacent gene TOP2A the molecular target for several anticancer drugs, are frequently coamplified together [58]. Moreover, Copy Number Variations (CNVs) in TOP2A gene have been identified as biomarkers in colorectal cancer [59]. DNA topological structure is controlled by this nuclear enzyme and upregulation of this gene is a cell proliferation marker in both normal and neoplastic tissues [60]. TOP2A mRNA expression is an independent prognostic factor in patients with (Estrogen Receptor) ER-positive breast cancer and could be useful in the assessment of the ER-positive breast cancer risk [61].Therefore, rather than a target for cancer therapy, this gene could be a possible prognosis biomarker.

Replication Factor C subunit 3 (RFC3) roles in DNA replication, DNA damage repair, and cell cycle checkpoint control. Hepatocellular carcinoma (HCC) and cell proliferation of ovarian tumors are suppressed by shRNA-mediated silencing of RFC3 gene [62] [63]. This gene was upregulated in PvsN analysis and it is also upregulated in Triple-negative breast cancer (TNBC) [64]. Since expression inhibition of this gene at both mRNA and protein level suppresses the migratory and invasive ability of MCF-7 cell lines [65], this gene would be a therapeutic target for colorectal cancer treatment. Moreover, TOP2A and RFC3 were shown to be engaged in the Gastric Cancer Network2 pathway in the enrichment analysis which also could indicate the importance of these two genes in CRC.

Mitotic Arrest Deficient 2 Like1 (MAD2L1) was upregulated in PvsN and is a mitotic spindle assembly checkpoint component that is responsible for preventing anaphase initiation until precise and complete metaphase alignment of all chromosomes takes place. Increase in the level of MAD2L1 transcripts was detected in a large number of samples of the ductal breast carcinoma [66]. MAD2L1 inhibits APC (anaphase-promoting complex), but is absent when chromosomes are correctly aligned on the metaphase plate [67]. Its upregulation in our analysis would provide the evidence that cancerous cells were dealing with mitotic deficiencies.

The GINS complex is a DNA replication machinery component in the eukaryotes and is a requirement for the replication initiation and progression of DNA replication forks [68]. GINS1 (PSF1) mRNA level is positively correlated with tumor size in CRC patients and is a

prognostic marker of CRC [69]. This gene has been recently introduced as a targeted oncogenic agent for inhibition of synovial sarcoma meaning that expression inhibition of this gene promotes apoptosis [70]. It was upregulated in PvsN analysis, therefore, its expression inhibition would be a potential target for inhibition of tumor growth by disturbing DNA replication machinery.

CDC6, one of the seed genes, plays a critical role in regulation of the eukaryotic DNA replication onset and its downregulation has been demonstrated in prostate cancer [71]. Its transcription is regulated by E2F or androgen receptor (AR) alone or in combination, in PCa cells [72]. Transfection of CDC6 siRNA results in not only decreased level of ovarian cancer SKOV3 cell proliferation but also increased apoptosis rates [73]. Expression of Cdc6 and Cdt1 are upregulated in ER-positive and ER-negative breast cancer cells. In addition, Cdc6 is considered as a potential therapeutic target in in BC patients [74]. Results for this gene in MvsP analysis are totally contradictory to the BC data. No study directly measured the expression level of this gene in CRC samples. Therefore, down-regulation of this gene might be a CRC specific biomarker but it requires further investigation.

CKS2 (CDC28, Cyclin-Dependent Kinases Regulatory Subunit 2) protein interacts with the catalytic subunit of the cyclin dependent kinases and its downregulation results in suppressing the expression of p-Akt and p-mTOR. Therefore, one of CSK2 oncogenic roles would be played by Akt/mTOR oncogenic pathway [75]. Compared with normal tissues, CKS2 is expressed at much higher level in CRC tissues and it has revealed that increased CKS2 expression is highly correlated with enhanced metastatic stage [76]. CKS2 upregulation has been found in BC tissues at both mRNA and protein levels. Importantly CKS2 is considered as a potential biomarker and therapeutic target for the BC treatment due to the fact that its inhibition suppresses cell proliferation and invasion ability in vitro and decreases tumor growth in vivo [77]. In the PvN analysis, this gene was upregulated which would be a therapeutic target for CRC treatment.

PSMA7 gene encodes a protein that is one of the essential subunits of 20S proteasome complex [78]. Overexpression of PSMA7 both at the mRNA and protein levels has been reported in gastric cancer [79]. Depletion of PSMA7 by shRNA-transfected RKO CRC cell lines

mediates inhibition of cell growth and migration. Consequently, inhibition of PSMA7 could be a beneficial therapeutic strategy for colorectal cancer patients [80].This gene was upregulated in PvsN analysis.

AIMP proteins form a complex with aminoacyl-tRNA synthetases. AIMP1 is a precursor of inflammatory cytokine EMAPII which mediates growth inhibition of PCa cells. In addition, it sensitizes tumor vessels to tumor necrosis factor α (TNFα) [81]. Furthermore, Down-expression of AIMP1, AIMP2 and AIMP3 genes in CRC tissues has been reported which suggest the tumor-suppressive roles of AIMP proteins [82]. AIMP1 gene depicted a mixed expression trend in MvsP analysis.

DARS was found to be upregulated in MvsN analyses. This gene encodes a member of a multi-enzyme complex that its role has been proved in mediating the amino acids attachment to their cognate tRNAs. So far no study has investigated the role of this gene in cancer progression. Only two studies have reported that DARS-AS1 gene is positively associated with the pathological stages in thyroid and ovarian cancer by targeting mir-129 and mir-532-3p respectively [83, 84]. It might be a CRC prognostic marker or a curative target.

Eukaryotic Translation Initiation Factor 2 Subunit Beta (EIF2S2/EIF2B) acts in the early steps of protein synthesis. GTP-bound EIF-2 transfers Met-tRNAi to the 40S ribosomal subunit. EIF-2 consists of three subunits, alpha, beta, and gamma. The hydrolysis of GTP to GDP takes place at the end of the initiation process that leads to release of the inactive eIF2·GDP from the ribosome. Exchange of GDP for GTP is performed by beta subunit so that active EIF-2 is ready for another round of initiation [85]. In one study, EIF2B has been proposed as a potent therapeutic target in lung cancer [76]. Moreover, elimination of natural killer cell cytotoxicity via promoted expression of natural killer (NK) cell ligands is done by pSer535-eIF2B following by expression of pSer9-GSK-3β (inactive GSK3β) and generation of ROS which promotes breast cancer growth and metastasis [86]. Since, Tyr216-GSK-3β (Active GSK3β) has the inhibitory effects on the EMT process contrary to the inactive form, induction of active GSK-3β together with EIF2B would be a therapeutic approach to prevent EMT [87]. EIF2B stepped up in PvsN analysis.

Twinfilin Actin Binding Protein 1 (TWF1/PK9) gene encodes twinfilin, an actin monomer-binding protein that promotes EMT in pancreatic cancer tissues [88]. siRNAs of TWF1 dramatically inhibited F-actin organization and focal adhesions formation and promotes the mesenchymal-to-epithelial transition (MET) with a round cell shape in BC MDA-MB-231 cell lines. Inhibition of TWF1 expression by both miR-30c and siRNA in MDA-MB-231 cells increases sensitivity of the cancer cells to doxorubicin and paclitaxel drugs. Furthermore, expression levels of EMT markers, VIM and SNAI2, are reduced as a result of miR-30c action [89]. In MvsN analysis, this gene witnessed a mixed trend. This may suggest that metastatic biopsies from colon were undergoing the EMT while biopsies from other tissues are not. Therefore, TWF1 might be an EMT biomarker of CRC. Moreover, inhibition of this gene would be a good therapeutic approach since this inhibition bolsters the sensitivity to chemotherapy drugs and prevents EMT.

Creatine metabolism has been implicated in colon cancer progression and metastatic colonization of the liver. CRC cells induce and release creatine kinase-B (CKB) into the extracellular space where it phosphorylates creatine to generate the high-energy metabolite phosphocreatine. Phosphocreatine is then actively imported into metastatic cells via a specific creatine transporter SLC6A8, one of the seed genes and a member of component 2 in the core network. Intracellular phosphocreatine is converted into ATP to provide the fuel for the survival of metastatic cancer cells within the hypoxic microenvironment. Consistent with these findings, a significant reduced liver colonization in mouse xenograft models has been observed due to genetic depletion of SLC6A8 in colon and pancreatic cancer cell lines. Regarding important function of this gene for cancer cell survival, expression inhibition of this gene or preventing import of phosphocreatine into the cells would be a therapeutic method to inhibit energy production of cancer cells [90]. This gene has a mixed trend in MvsP analysis. What's more, SLC4A4 is one of the most upregulated and SLC6A6 is one the most dowregulated DEGs in GSE18105 dataset (Figure 2).

AGC protein kinases consist of more than 60 protein kinases in the human genome, classified into 14 families. SGK1, one of the DEGs in the core network, and AKT are two families of this superfamily. SGK1 is a serine/threonine kinase that activates certain potassium, sodium, and chloride channels [91]. SGK1 is a downstream effector of PI3K, which run pathways

independent of pathways shared with AKT. The two kinases are phosphorylated and activated by PDK1 and mTORC2 complex [92, 93]. In general, PI3K-dependent survival signals can be mediated by either Akt or SGK1 that inactivates the pro-apoptotic proteins, Bad and FKHRL1 [94]. In a study on A-498 kidney cancer cells, they found that survival signal promoted by IL-2 is mediated by SGK1 activation [95]. IL-2 activates Sgk1 but not Akt at the transcriptional level [95], Moreover, the promoter of Sgk1 is under tight control of the tumor suppressor protein p53 [96]. Sgk1 has been shown to mediate cell survival and drug resistance to platinoid and taxane compounds in women with breast cancer [97]. This gene was downregulated in the PvsN analysis and it has been still under discussion to propose a potential curative approach regarding this gene for CRC treatment.

One of the seed genes N-Myc Downstream Regulated 1 (NDRG1) is connected to SGK1 gene. This gene which has a mixed expression trend in MvsP analysis is one of the repressors of the EMT in metastatic CRC by attenuating NF-κB signaling [98]. Inhibition of NDRG1 results in the increase in NF-kB and the reduction in cell-cell junction protein E-cadherin [99]. The promoter region of the NDRG1 gene contains a p53 binding site which is necessary in caspase activation and apoptosis in the case of DNA damage [100]. A transcription factor that plays an important role in response to low oxygen concentrations, or hypoxia is Hypoxia-Inducible Factor (HIF)-1. HIF-1α is translocated from cytoplasm to nucleus, where it binds to HIF-1β to form HIF-1 complex. This complex works as a transcription factor, binds to hypoxia response element (HRE) in the promoter of NDRG1 [101]. Consequently, under hypoxic environment of tumor, induction of NDRG1 by HIF-1 complex is a defensive mechanism against cancer progression. HIF-1α also removes the inhibitory effect of myc complex on this gene [101]. Furthermore, NDRG1 interferes with WNT/ β-catenin pathway by downregulating nuclear β-catenin and prevent metastasis in CRC [102]. In addition, NDRG1 Controls gastric cancer migration and invasion through regulating MMP-9 [103]. Our results would suggest that the tumor suppressive role of this gene would be act on the primary metastatic sit in CRC and might have a contrary role in liver and lung metastasis. As a result, Enhancing NDRG1 signaling as a therapeutic strategy might have a reverse effect on the secondary metastatic sites.

Increase in expression of PNMA1 is observed in pancreatic ductal adenocarcinoma patients (PDAC). In addition, the higher expression of PNMA1 gene, the larger tumor size is observed. Suppressing PNMA1 expression causes a reduction in cell viability which promotes apoptosis. Upregulation of PNMA1 occurs in HCC and higher expression of PNMA1 is associated with more aggressive phenotypes [104]. This gene was downregulated in PvsN, so it is a struggle to decide whether this gene could be a therapeutic target in CRC or not.

Component 3 contains collagen (COL1A2, COL5A2 and COL4A1) and P4HA1 (a collagen hydroxylase) genes inter-connected in the process of ECM remodeling based on enrichment results. All the members presented a similar trend in expression. COL1A2 and COL5A2 were upregulated in MvsN and PvsN analyses. COL4A1 was upregulated in MvsN analysis and P4HA1 which is one the seed genes upregulated in MvsP analysis. P4HA1 is implicated in metastasis of breast and pancreatic cancers [105] [106]. Under hypoxic conditions this gene would trigger HIF1 pathway, cell survival and progression of colorectal cancer cells. HIF-1 induces expression of genes that are encoding collagen prolyl (P4HA1 and P4HA2) and lysyl (PLOD2) hydroxylases. P4HA1 and P4HA2 are required for collagen deposition, whereas PLOD2 is required for ECM stiffening and collagen fiber alignment [107]. These changes in ECM triggered by HIF-1 are necessary for motility and invasion because in focal adhesion junctions actin cytoskeleton is connected to ECM through attachment of integrins to collagens [108]. Besides, there is a positive feedback between P4HA1 and HIF-1 in modulation of ECM. As a result, targeting P4HA1 and P4HA2 expressions would inhibit the progression of EMT via HIF1-Collagen pathway.

PTP4A1 a member of component 4, is a protein phosphatase engaged in p21-activated kinase (PAK) signaling pathway. Inhibition of PTP4A1 gene in MDA-MB-231 breast cancer cells by an increase in miR-944 expression impairs cell invasion [109]. Rac/Cdc42 activates PAKs leading to formation of membrane ruffles, stress fibers and remodeling of focal adhesion complexes [110]. This phosphatase might have a relationship with Src by triggering TGFB1 induction and cancer progression [111]. This gene was down-regulated in PvsN analysis since the role of this gene is more related to migratory behavior of the cancer cells, its expression should be investigated in metastasis-primary cancer analysis. BCL-2 is a target of ATF5 one of the seed genes [112]. ATF5 was induced in the liver and lung metastasis analyses

and there are many evidences linking the role of ATF5 in mitochondrial dysfunction in cancer progression [113]. Survival pathways stimulated by ATF5 would be essential to ignore anchorage-dependent and niche-dependent cell death signals in malignant glioma. That's could be the reason why this gene was upregulated in lung and liver in CRC patients. Targeting this gene might have therapeutic implications in glioblastoma [114]. Therefore, inhibition of this gene might prevent liver and lung metastasis in CRC. TRIB3 which is a colorectal cancer prognosis marker is a scaffold protein activated under hypoxic conditions [115]. Silencing of TRIB3 downregulates VEGF-A expression in gastric cancerous cells, leading to suppression of endothelial cell recruitment and vessel formation. This gene was upregulated in MvsN analysis, therefore, it would be a favorable target for anti-angiogenic therapy [116].

Genes in component 5 are mitochondrial which their role in cancer progression has not been sufficiently investigated so far. All three genes were downregulated in our analysis. These genes are highly expressed in normal colon tissue compared to other tissues based on RNA-seq expression information in the Gene database of NCBI [117] because of the presence of anaerobic bacteria in the digestive tract [118]. ETHE1 (persulfide dioxygenase) and SQOR are antioxidants that convert hydrogen sulfide (H2S) to persulfide and persulfide to sulfite respectively. Therefore, they defend cells against toxic concentrations of sulfide. ETHE1 is the only gene in our analysis which was downregulated in the three analyses while SQOR was downregulated in MvsN and PvsN analyses. Their downregulation is essential for cancer cells proliferation and survival, since it has proved that in the hypoxic environment of CRC tumors, sulfide is a supplementary tool that provides electron for mitochondrial ETC to generate ATP [119]. As a result, helping in expression induction or activation of ETHE1 and SQOR proteins would hinder CRC tumor growth. TST thiosulfate sulfurtransferase encodes a protein that is localized to the mitochondria and catalyzes the conversion of thiosulfate and cyanide to thiocyanate and sulfite. Therefore, like the previous two mitochondrial enzymes, it acts in Hydrogen sulfide metabolism [120].

ABAT upregulation in component 6, is one of the hallmarks of estrogen positive breast cancer. Tamoxifen is the main element in treatment of estrogen receptor (ER)-positive breast cancer as it is an agonist of ER. In addition, It is a novel biomarker for tamoxifen

resistance in advanced breast cancer [121]. This gene showed a mixed expression trend in MvsP analysis. SORD is another element of component 6 upregulated in MvsN and PvsN analyses. The connection of this gene with cancer has not efficiently investigated. Since it exhibited a totally ascending trend in two different analyses, it might be a potential target and biomarker for CRC treatment. TCF25 in component 7 witnessed a mixed expression trend in MvsP analysis. This gene is a basic helix-loop-helix transcription factor that is vital in embryonic development. LGALS4 is implicated in regulating cell-cell and cell-matrix interactions. This gene is mostly expressed in small intestine, colon, and rectum, which is suppressed in CRC [122]. It was downregulated in MvsN and PvsN analyses. It is also a blood marker of CRC [123].

Summary

Suppression of Pirh2 expression might inhibits p53 degradation and focal adhesion formation. Suppression of HLA-F gene might be a CRC prognostic marker. CTSH expression suppression might disturb focal adhesion formation and inhibits EMT. Induction of GPX3 has anti-proliferative effects by reducing the amount of cellular H_2O_2 and induction of EHTE1 and SQOR prevents cancer cells to fabricate energy from H_2S. Inhibition of TMPO-AS1 attenuates activity of mitotic pathways. RFC3 inhibition hinders the migratory behavior of cancer cells. Upregulation of RFC3 and TOP2A together might be a prognostic marker of CRC. GINS1 is a known biomarker of CRC and its expression inhibition might disturb DNA replication machinery and tumor growth. Down-regulation of CDC6 might be a CRC specific biomarker. Expression inhibition of CSK2 would halt the effects of Akt/mTOR oncogenic pathway. PSMA7 inhibition could be a therapeutic treatment for CRC. Augmentation of active GSK-3β together with EIF2B would hinder the EMT process in CRC. TWF1 could be biomarker of EMT in CRC and its suppression could bolster the sensitivity of CRC cells to chemotherapy drugs. Amplifying NDRG1 signaling as a therapeutic strategy might have a positive effect on the primary tumors but it could have reverse effects on the secondary metastatic sites. Down-regulation of PNMA1 and Sgk1 genes might be prognostic markers for primary tumors in colon. Targeting P4HA1 and P4HA2 expressions would inhibit the progression of metastasis via HIF1-Collagen pathway. Expression suppression of ATF5

would prevent liver and lung metastasis in CRC. TRIB3 could be pertinent target for inhibition of angiogenesis in CRC. LGALS4 downregulation and its present in blood is are prognostic markers of CRC and as they are implicated in cell-cell interaction, its induction might have positive curative impacts on CRC cells. TMEM131, DARS and SORD genes implicated in CRC and have specific expression trends as cells go from normal to metastatic. These genes are reported for the first time in this study so more investigation is required to find the role of these genes in CRC.

Conclusion

In this study we illustrated some therapeutic targets and biomarkers for CRC. A combination of these targets would beneficially disturb progression of colorectal cancer. A combination that inhibits angiogenesis (TRIB3 suppression), aberrant proliferation (GINS1 and CSK2 suppression), EMT (suppression of Pirh2, CTSH and TWF1 and induction of GSK-3β together with EIF2B), migratory behavior (suppression of RFC3, P4HA1), energy production under hypoxic conditions (induction of ETHE1 and SQOR) and ROS generation (induction of GPX3).

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