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Predicting sulforaphane-induced adverse effects in colon cancer patients via in silico investigation

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ABSTRACT

Colorectal cancer (CRC) is a significant global health burden that ranks as the third most diagnosed and second most common cause of cancer related deaths worldwide. New therapeutic strategies include chemoprevention and use of molecules which could prevent, suppress or reverse CRC progression such as sulforaphane (SFN). However, evidences about its safety in CRC patients are still lacking. The aim of this in silico investigation was to predict SFN-induced adverse effects in CRC patients by computational analysis. The study showed that 334 genes were consistently dysregulated in CRC (223 downregulated and 111 upregulated), while 38 were recognized as significant and might be used as predictive biomarkers for overall survival and metastasis (TCGA, GEO, R studio). Among them, SFN interacted with 86 genes, out of which 11 were marked as significant (correlate with overall prognosis and metastasis). Sulforaphane potentiates the overexpression of *TIMP1, AURKA,* and *CEP55*, and promotes inhibition of *CRYAB, PLCE1*, and *MMP28*, that might lead to the progression of CRC (CTD). Pathway enrichment analysis revealed that SFN stimulated Transcriptional activation of RUNX2, AURKA activation by TPX2, IL-10 signaling, while inhibited Differentiation of White and Brown Adipocyte process, an underlying pathway which inactivation led to obesity (Cytoscape ClueGo + CluePedia, DAVID). Thus, genome signature of CRC patients could serve as important factor when addressing the risk-to-benefit profile of SFN. Patients with colon cancer and increased expression of *TIMP1, CCL20, SPP1, AURKA, CEP55, NEK2, SOX9* and *CDK1*, or

Abbreviations: ABHD3, abhydrolase domain containing 3, phospholipase; AOC3, amine oxidase copper containing 3; AURKA, aurora kinase A; ARNTL2, aryl hydrocarbon receptor nuclear translocator like 2; BMP2, bone morphogenetic protein 2; CA1, carbonic anhydrase 1; CCL8, C-C motif chemokine ligand 8; CCL20, C-C motif chemokine ligand 20; CDH19, cadherin 19; CDK1, cyclin dependent kinase 1; CEP55, centrosomal protein 55; CEL, carboxyl ester lipase; CHRDL1, chordin like 1; CLCA4, chloride channel accessory 4; COL10A1, collagen type X alpha 1 chain; COL1A1, collagen type I alpha 1 chain; CPA3, carboxypeptidase A3; CRYAB, crystallin alpha B; CXCL1, C-X-C motif chemokine ligand 1; CXCL3, C-X-C motif chemokine ligand 3; EDN3, endothelin 3; EDNRA, endothelin receptor type A; EDNRB, endothelin receptor type B; FGL2, fibrinogen like 2; FABP6, fatty acid binding protein 6; F13A1, coagulation factor XIII A chain; GREM2, gremlin 2, DAN family BMP antagonist; GDF15, growth differentiation factor 15; GRP, gastrin releasing peptide; GPX3, glutathione peroxidase 3; GINS1, GINS complex subunit 1; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HPGD, 15-hydroxyprostaglandin dehydrogenase; HIGD1A, HIG1 hypoxia inducible domain family member 1A; IL1R2, interleukin 1 receptor type 2; INSL5, insulin like 5; KCNMA1, potassium calcium-activated channel subfamily M alpha 1; KLF4, Kruppel like factor 4; LGI1, leucine rich glioma inactivated 1; MYH11, myosin heavy chain 11; MMP12, matrix metallopeptidase 12; MMP28, matrix metallopeptidase 28; MFAP4, microfibril associated protein 4; MFAP2, microfibril associated protein 2; NEDD4L, NEDD4 like E3 ubiquitin protein ligase; NEBL, nebulette; NEK2, NIMA related kinase 2; PPARGC1A, PPARG coactivator 1 alpha; PDZRN4, PDZ domain containing ring finger 4; PLCE1, phospholipase C epsilon 1; PCK1, phosphoenolpyruvate carboxykinase 1; PLAC8, placenta associated 8; PTX3, pentraxin 3; PRKAR2B, protein kinase cAMP-dependent type II regulatory subunit beta; PRKACB, protein kinase cAMP-activated catalytic subunit beta; PSPH, phosphoserine phosphatase; RRM2, ribonucleotide reductase regulatory subunit M2; SGK1, serum/glucocorticoid regulated kinase 1; SIM2, SIM bHLH transcription factor 2; SHMT2, serine hydroxymethyltransferase 2; SOX9, SRY-box transcription factor 9; SLCO1B3, solute carrier organic anion transporter family member 1B3; SPP1, secreted phosphoprotein 1; SMPDL3A, sphingomyelin phosphodiesterase acid like 3A; TRIB3, tribbles pseudokinase 3; TIMP1, TIMP metallopeptidase inhibitor 1; UGDH, UDP-glucose 6-dehydrogenase; ZG16, zymogen granule protein 16.

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downregulation of *CRYAB, PLCE1, MMP28, BMP2* and *PLAC8* may not be ideal candidates for SFN chemoprevention.

1. Introduction

Colorectal cancer (CRC) is a significant global health burden that ranks as the third most diagnosed and second common cause of cancer related deaths worldwide [44]. In the year of 2018, there were around 2 million new cases of CRC and 880,000 deaths according to International Agency for Research on Cancer (IARC) [46]. The occurrence of CRC is higher in western countries mainly due to the diet rich in red and processed meat, sweets, fat, and alcohol consumption [44], but the last decade also records an upward trend for CRC incidence in China [53]. Recent cancer statistics in China indicates that CRC is 4th most common cancer type in males and 5th in females causing the death in almost 50% of all CRC – diagnosed patients [8].

Age, genetic and environmental factors play a major role in the development of CRC. Some of the well-known associations with CRC include, for example: African American ethnicity, male sex, inflammatory bowel diseases, obesity, sedentary lifestyle, tobacco and alcohol use, as well as history of abdominal radiation [48]. The most common type of CRC is adenocarcinoma, followed by the rare neuroendocrine, squamous cell, adenosquamous, spindle cell and undifferentiated colorectal carcinomas [20].

While recent development of more efficacious therapy regimes significantly improved the treatment of cancer patients, finding ways for further decrease of CRC risk and mortality are still in the focus of oncology research [3]. One of the promising strategies is chemoprevention approach, defined in 1976, which includes applying natural, synthetic, or biologic chemical agents with aim to reverse, suppress, or prevent carcinogenic progression to invasive cancer [1]. For example, tamoxifen prevents the development of second primary tumors and de novo breast cancer in estrogen-positive high-risk patients [33], while chemoprevention of CRC was mostly linked to aspirin and metformin [3]. Long-term follow-up studies showed that the regular use of 75–300 mg of aspirin daily for at least 5 years could reduce the risk of colon cancer incidence. Benefit was higher for cancers of the proximal colon in comparison with the rectal colon [36]. Similarly, metformin could act as immune-promoting agent by stimulating differentiation of CD^{8+} T cells and improving their antiapoptotic function. As Zhang et al. [54] explained, metformin regulates the AMPK–miR-107–Eomes–PD-1 pathway, which enhances Eomesodermin (Eomes) expression and further inhibits the transcription of programmed cell death 1 protein (PDCD1) in metformin-treated CD^{8+} T cells and the CAR-T cell therapy model [54].

Another proposed molecule that could be used in colon cancer patients as a chemoprevention is sulforaphane (SFN), an isothiocyanate isolated from leafy vegetables [19]. It was reported that SFN could activate different proapoptotic pathways in human colon cancer cells SW620, such as p53 and caspase-2-JNK pathway [37], as well as block cells progression and angiogenesis by inhibiting HIF-1α and VEGF expression [29]. Moreover, when combined with conventional cytostatic drug, cisplatin, SFN was able to inhibit key steps of metastatic events in triple negative breast cancer cells. Authors proposed suppression of sirtuins and reversion of the epithelial–mesenchymal transition (EMT) process as potential underlying mechanism [40]. Similarly, it was reported that epigallocatechin gallate and SFN combination treatment could induce apoptosis in ovarian cancer cells resistant to paclitaxel most probably by promoting DNA damage response [7]. However, even though there is an increasing number of in vitro based research showing positive effects of SFN, very few studies explained its toxic potential. For example, when used in high doses (from 150 to 300 mg/kg), SFN caused harmful effects in mice, such as sedation, hypothermia, impairment of motor coordination, decrease in skeletal muscle strength, leucopenia, and even deaths [42]. Moreover, Mahéo et al. [35] reported that SFN could inhibit glutathione S-transferases and some enzymes of cytochromes P-450 family in intact human and rat hepatocytes [35], which could cause the impaired metabolism of different cytotoxic agents used in cancer patients' treatment. Therefore, it is rational to hypostasis that SFN-based therapy could induce adverse outcomes especially when used in immunocompromised cancer patients. However, to the best of our knowledge, its safety profile for the treatment of CRC patients has not been investigated yet. Thus, the aim of this research was to identify potential SFN-induced adverse outcomes in CRC patients by applying in silico toxicogenomics approach.

2. Material and methods

2.1. Microarray data

The key word 'colon cancer' was searched in the GEO database (ncbi.nlm.nih.gov/geo/), public repository for high-throughput microarray and next-generation sequence functional genomic data sets with more than 1 million samples currently available in the public domain. In addition, GEO provides tools for identification, analysis and visualization of data, such as GEO2R [2]. The research was restricted to *Homo sapiens* and the study type was confined to RNA sequencing data. Study type selected: expression profiling by array. The total of 521 results were retrieved, and 3 gene expression datasets were selected for further investigation.

Three gene expression profiles used in our study consisted of patients derived colon cancer tissues, and thus could be compared with other publicity available colon cancer data. Chosen sets were: GSE41258, GSE62932, and GSE68468. Among these, GSE41258 and GSE68468 were screened on GPL96 platform [HG-U133A] Affymetrix Human Genome U133A Array, while GSE62932 was based on GPL570 platform [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The samples were divided into two groups – primary colon cancer tissues and normal colon mucosae, as a control. Two of the sets, GSE41258 and GSE68468 contained 186 colon cancer tissues and 55 normal colon mucosae, while GSE62932 was generated from 64 colon cancer and 4 normal colon tissues. The Cancer Genome Atlas, TCGA (http://tcga-data.nci.nih.gov/tcga/) colon adenocarcinoma (COAD) dataset was downloaded from Genomic Data Commons Data Portal (GDC; https://portal.gdc.cancer.gov/), containing 285 cases of adenocarcinoma colon cancer with survival time data and 41 normal, healthy colon tissues.

2.2. Identification of differentially expressed genes (DEGs)

Differently expressed genes (DEGs) between primary colon cancer samples and normal controls in GSE sets were identified using the GEO2R online analysis tool (ncbi.nlm.nih.gov/geo/geo2r). $|\text{Log FC} = > 1.0$ and corrected $p < 0.05$ were set as the cutoff criteria. The edgeR (Empirical Analysis of Digital Gene Expression Data in R) R package (https://doi.org/10.1093/bioinformatics/btp616) was used to identify the tumor-normal DEGs from TCGA data. In the screening, the FDR value < 0.01 and $|\text{logFC}| > 1.0$ were set as the cutoff criterion. Among detected DEGs, consistent DEGs of the four gene expression datasets (GEO and TCGA) were screened using the Venn diagram drawing tool from TBtools software [5]. Consistent genes were than separated into 2 groups: downregulated and upregulated gene-sets which were analyzed separately in the next steps of this study.

2.3. Network analysis

Network analysis was performed using Cytoscape plug-in STRING, an application which helps predict interconnections between genes from the set with an evidence score of 0.4 or greater. We extracted consistently downregulated genes from analyzed expression profiles and uploaded them to the STRING protein-query tool to retrieve the genes' network. The same process was repeated with upregulated subset of DEGs.

2.4. Gene-set enrichment analysis

Functional annotation clustering was performed with the Cytoscape plug-in BINGO. Up-regulated group of genes was uploaded to the BINGO with all human genes as background and run in a Gene Ontology (GO) Biological Process analysis with p value < 0.05 set as the level of significance. Hypergeometric test with FDR multiple testing correction was chosen as the statistical test and overrepresented categories after correction were retrieved. The same process was repeated with the subset of downregulated DEGs.

Pathway analysis was performed by Cytoscape ClueGO plug-in version 2.5.7. KEGG, Reactome, and WikiPathways databases were selected to extract the list of pathways. The two-sided hypergeometric test was used for the enrichment with a Bonferroni step down correction and a κ score of 0.3 to link the terms. The ClueGo analysis was integrated with CluePedia plug-in 1.5.7 to link the examined up and downregulated colon cancer genes with molecular pathways. Moreover, we used the web-based tool DAVID (Database for Annotation, Visualization and Integrated Discovery, version 6.8, http://david.abcc.ncifcrf.gov/home.jsp) for verification of obtained results.

2.5. Survival and metastasis analysis

Patient's survival time and status, combined with tumor-DEGs, were used for the univariable Cox regression analysis, performed with "survival" R package. The cut-off criterion was set to p-value < 0.05 for screening of the survival-related gene at overall survival. The same analysis was used for predicting metastasis-related genes among tumor-DEGs, with $p < 0.05$.

2.6. Sulforaphane interacting genes

In this investigation, SFN-interacting genes were obtained from the Comparative Toxicogenomics Database (CTD; http://CTD.mdibl.org), a publicity available and scientifically useful resource that contains integrated data for the better understanding of the relationships between chemicals, genes, and diseases [15]. The analysis reported here was based on the data downloaded in September 2021. Moreover, CTD Chemical-Gene Interaction Query helped in detection of binary interactions between SFN and important genes.

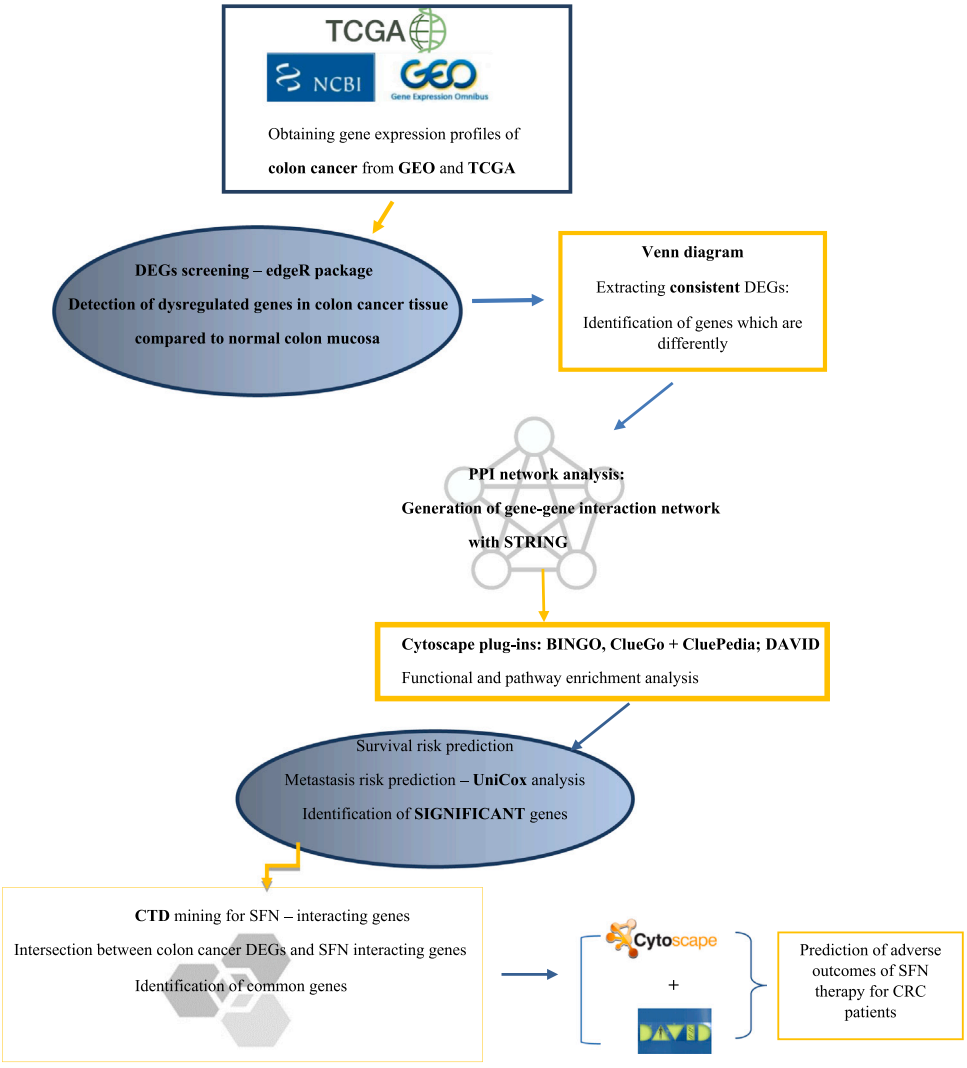


Fig. 1. Flow chart of the steps used in this in silico data-mining approach.

2.6.1. Sulforaphane – interacting genes in colon cancer

To verify if SFN could change the expression of genes previously defined as important, in human colon cancer cells, GEO dataset GSE174444 was analyzed with GEO2R online tool [2]. Three biological replicates of the colon cancer cell line, SW480, were treated with 25 μmol/l SFN, and three with a control, double-distilled water, for 48 h. RNA was pooled, processed and hybridized to the GN-GeneChip Clariom S Array, human (Thermo Fisher Scientific; cat. no. 902926) [21]. All the results were downloaded and saved in text format. The genes that met the cut-off criteria of $p < 0.05$ and $|\text{Log FC} = > 1.0$ were considered DEGs. Among them, important genes were extracted.

An overview of the methodology applied in this study is shown sequentially in the Fig. 1.

3. Results

3.1. Identification of differentially expressed genes (DEGs)

The GEO gene expression profiles from 3 different datasets (GSE41258, GSE62932, and GSE68468) were selected for identification of DEGs among the colon cancer and normal colon mucosa. These genes were compared with the TCGA datasets containing gene expression profiles of primary colon adenocarcinoma, as well as the samples from healthy control patients' tissues. The total of 721 colon cancer tissues was compared to 155 normal colon mucosa samples to identify genes which had changed expression in the primary tumor tissues. In the GSE41258, 813 genes were differently expressed, while GSE62932 and GSE68468 contained 941 and 1206 DEGs, respectively. From TCGA dataset 4471 genes were extracted as differently expressed, 1865 downregulated and 2067 upregulated.

The Fig. 2 shows Venn diagram of DEGs among all the investigated gene expression profiles. The total of 334 genes was consistently dysregulated. For our further analyses we generated two sets: first containing 223 underexpressed and the second 111 overexpressed genes in the primary colon cancer tissue versus normal colon mucosa (supp. Table 1).

3.2. Network analysis

Protein-protein interaction (PPI) network analysis was conducted separately for down and upregulated genes. Firstly, we uploaded genes which expression was decreased in tumor tissue and, based on the STRING prediction results, a network with 222 nodes and 370 edges was constructed (Fig. 3a). The number of segments connected to each gene (node) represents its degree. As seen in the Fig. 3a, the majority of the genes are clustered together, while 39 stand alone. The highest connectivity score (0.998) was generated between *PRKAR2B* and *PRKACB*, followed by *EDN3 – EDNRB* (0.995), *SGK1 – NEDD4L* (0.993), and *GREM2 – BMP2* (0.992).

The same study was repeated for the set of upregulated genes. The network with the total of 111 nodes and 427 edges was constructed where genes clustered in 2 main groups, mutually connected via *TRIB3-GDF15* edge. Five gene pairs (*ENDRA-GRP; FABP6-CEL; PSPH-SHMT2; SLC22AB-SLCO1B3; ARNTL2-SIM2*) were formed with high connectivity score (> 0.4), while 13 genes stand-alone (Fig. 3b).

3.3. Gene-set enrichment analysis

In the next step, GO enrichment analysis of the up and downregulated DEGs were performed separately, by using Cytoscape plug-in BiNGO to better understand their function. Among the underexpressed genes, the total of 48 enriched biological processes (BP) were identified, where primary BP regulated circulatory system process, blood circulation, chemical homeostasis, blood pressure, cellular chemical homeostasis, small molecule metabolic process, response to nutrient levels, ion homeostasis, lipid metabolic process, and signaling. Additionally, downregulated genes were enriched for response to wounding, response to chemical stimulus, cell differentiation, positive regulation of leukocyte chemotaxis, cell surface receptor linked signaling pathway, and positive regulation of leukocyte migration (Fig. 4).

Pathway enrichment analysis performed by ClueGo + CluePedia version 2.5.7 and 1.5.7 revealed that colon cancer downregulated genes can be grouped into 14 clusters among which are: Aldosterone-regulated sodium reabsorption, Complement and coagulation cascades, Adipocytokine signaling pathway, Mineral absorption, Glycolysis/Gluconeogenesis, Sulfur metabolism (Fig. 5). Moreover, colon cancer downregulated genes were enriched for immune-related processes such as regulation of leukocyte chemotaxis, negative regulation of leukocyte migration, regulation of cellular extravasation, and myeloid leukocyte cytokine production.

On the other hand, upregulated set of genes were enriched for 83 BP, among which multicellular organismal metabolic process, multicellular organismal catabolic process, collagen metabolic process, multicellular organismal macromolecule metabolic process, collagen catabolic process, and regulation of cell proliferation were at the top of the BP list (Fig. 6).

Fig. 7 shows the most significantly enriched KEGG, REACTOME and WikiPathways for the set of over expressed genes in the colon cancer. These pathways were grouped in 13 different clusters, among which are Collagen degradation, Senescence and autophagy in cancer, Extracellular matrix organization, Pathways regulating Hippo signaling, etc (Fig. 7). Moreover, immune-related pathways were also enriched; they

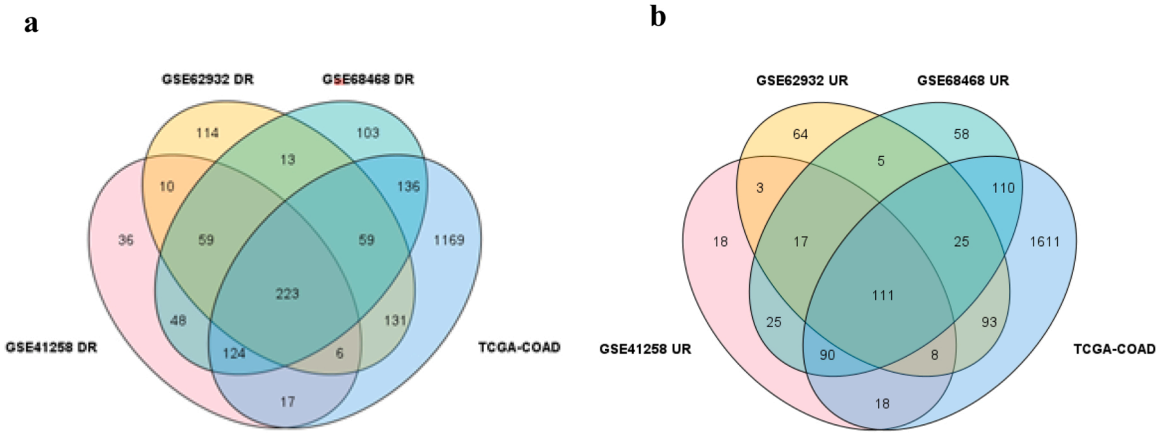


Fig. 2. Venn diagram of DEG in primary colon cancer versus normal cancer mucosa for (a) Downregulated genes and (b) Upregulated genes (Venn diagram drawing tool from TBtools software).

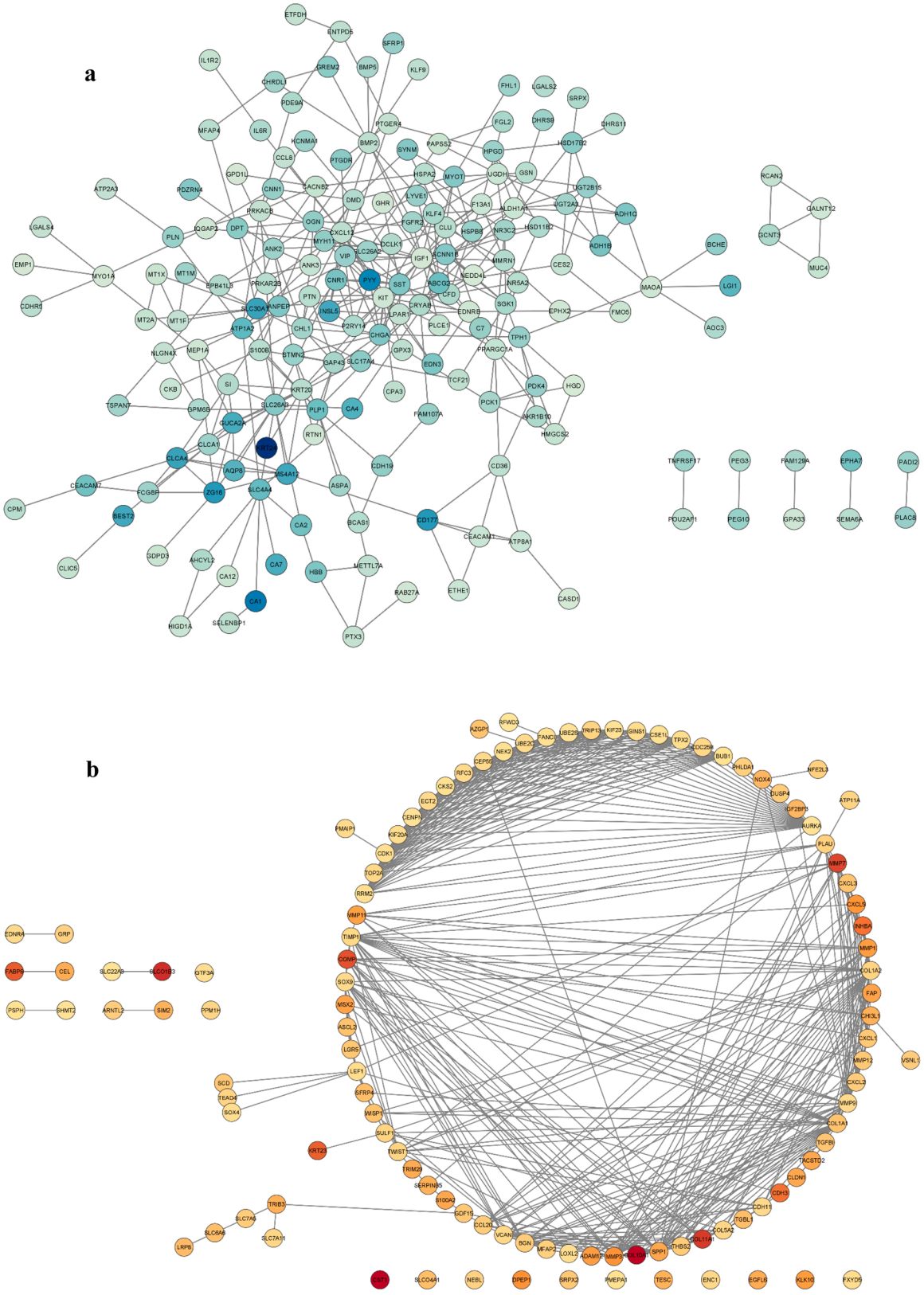


Fig. 3. a) Downregulated colon cancer genes interaction network; b) Upregulated colon cancer genes interaction network. Nodes represent genes where the highest intensity of the color indicates the highest down(up)regulation of genes' expression. The edge thickness was proportional to the combined score of the genes (STRING).

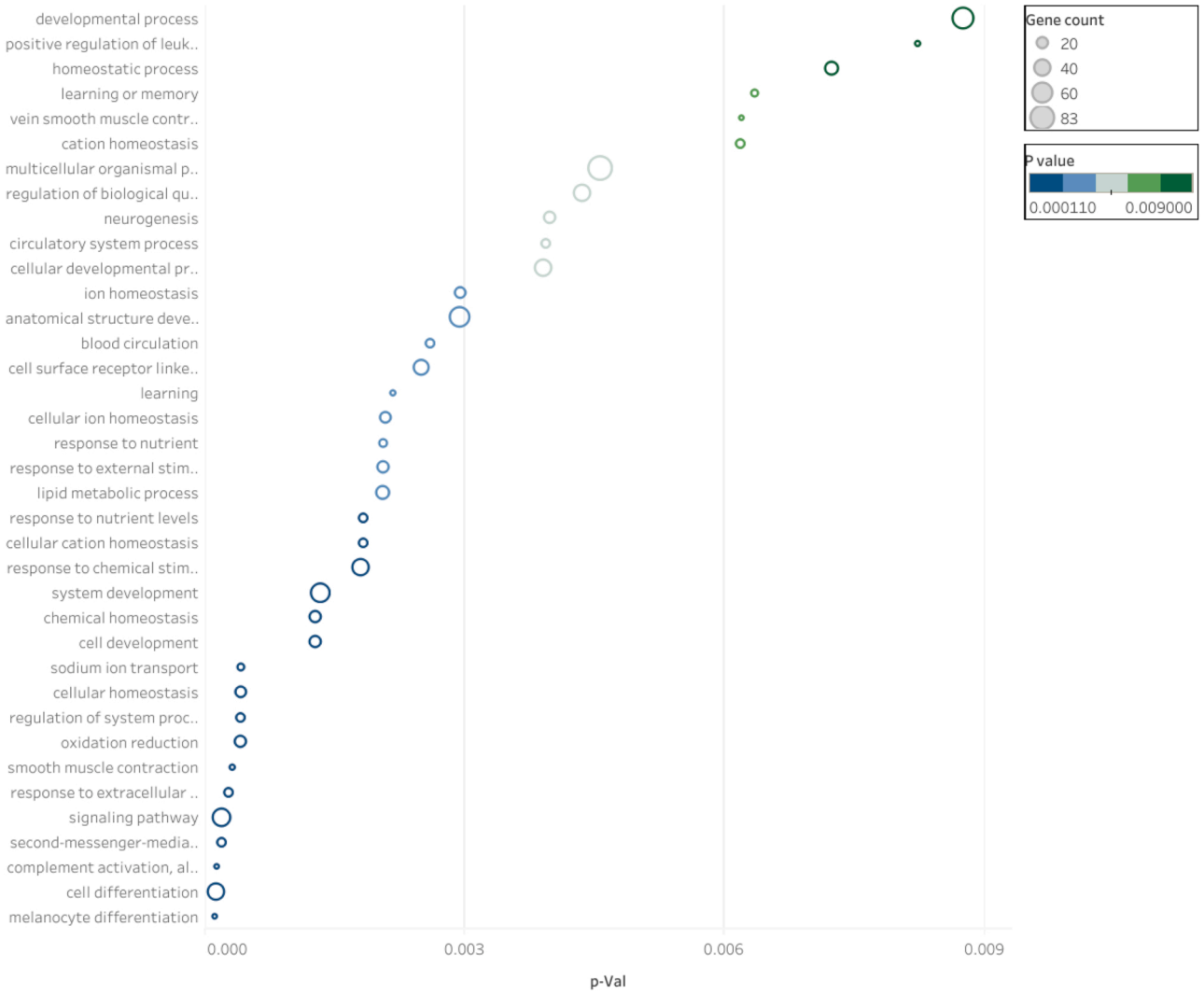


Fig. 4. Enriched biological pathways (BP) among colon adenocarcinoma (COAD) downregulated set of genes with associated p-values (Cytoscape BINGO, p value < 0.05; Hypergeometric test with FDR multiple testing correction); the size of the dots represents the number of DEGSs associated with the GO term and the color of the dots represents the p-values.

clustered in 2 groups with p value < 0.05, positive regulation of gamma delta T cell differentiation and Neutrophil chemotaxis containing 8 and 9 immune-processes, respectively.

3.4. Survival and metastasis analysis

The univariable Cox regression analysis, performed with "survival" R package was used for screening of the survival- and metastatic-related genes among tumor-DEGs, and the total of 38 genes was retrieved.

The expression of 6 consistently upregulated genes (*COL10A1, CXCL3, NEBL, RRM2, TIMP1,* and *TPX2*) and decreased expression of 16 genes: *SMPDL3A, IL1R2, HIGD1A, PPARGC1A, AOC3, CHRDL1, GPX3, CLCA4, CRYAB, MYH11, UGDH, KCNMA1, ABHD3, PDZRN4, ZG16*, and *HMGCS2* were in correlation with lower overall survival in COAD patients. Moreover, among upregulated genes, 4 (*GINS1, AURKA, MMP12*, and *CEP55*) were related to the colon cancer metastasis, together with 12 genes, which suppressed expression was seen in metastatic colon cancer patients. These genes were: *CPA3, MYH11, CA1, INSL5, SMPDL3A, CDH19, FGL2, CCL8, LGI1, PLCE1, MMP28,* and *PRKACB*. All of the mentioned genes (N = 38) are considered significant and were used in further analysis.

3.5. SFN-interacting genes

SFN interacted with 1896 genes in total (source: CTD), among which were 86 genes differently expressed in colon cancer patients.

Pathway enrichment analysis for the set of 86 genes was done as previously explained (ClueGo + CluePedia version 2.5.7 and 1.5.7). Obtained results showed the implication of 21 genes from the set in FOXO-mediated transcription, Differentiation of white and brown adipocytes, Prostaglandin synthesis and regulation, Transcriptional regulation by RUNX2, AURKA activation by TPX2, Interleukin 10 signaling, Human complement system, Molecules associated with elastic fibers, and Oligodendrocyte specification and differentiation (including remyelination) leading to myelin components for CNS (Fig. 8). Moreover, among detected SFN-interacting genes, 11 were identified as significant and correlated with overall survival or metastasis (*ABHD3, AURKA, CEP55, CRYAB, FGL2, MMP28, NEBL, PLCE1, PPARGC1A, TIMP1,* and *UGDH*). Three genes were in common between 2 sets: *AURKA*, *PPARGC1A, TIMP1*.

Furthermore, the CTD Chemical-Gene Query was used for identification of binary reactions between SFN and 21 genes from the set associated with the predicted pathways (Fig. 8). Sulforaphane-induced or decreased mRNA expression was compared with the expression of selected genes in colon cancer tissue. Moreover, the same method helped

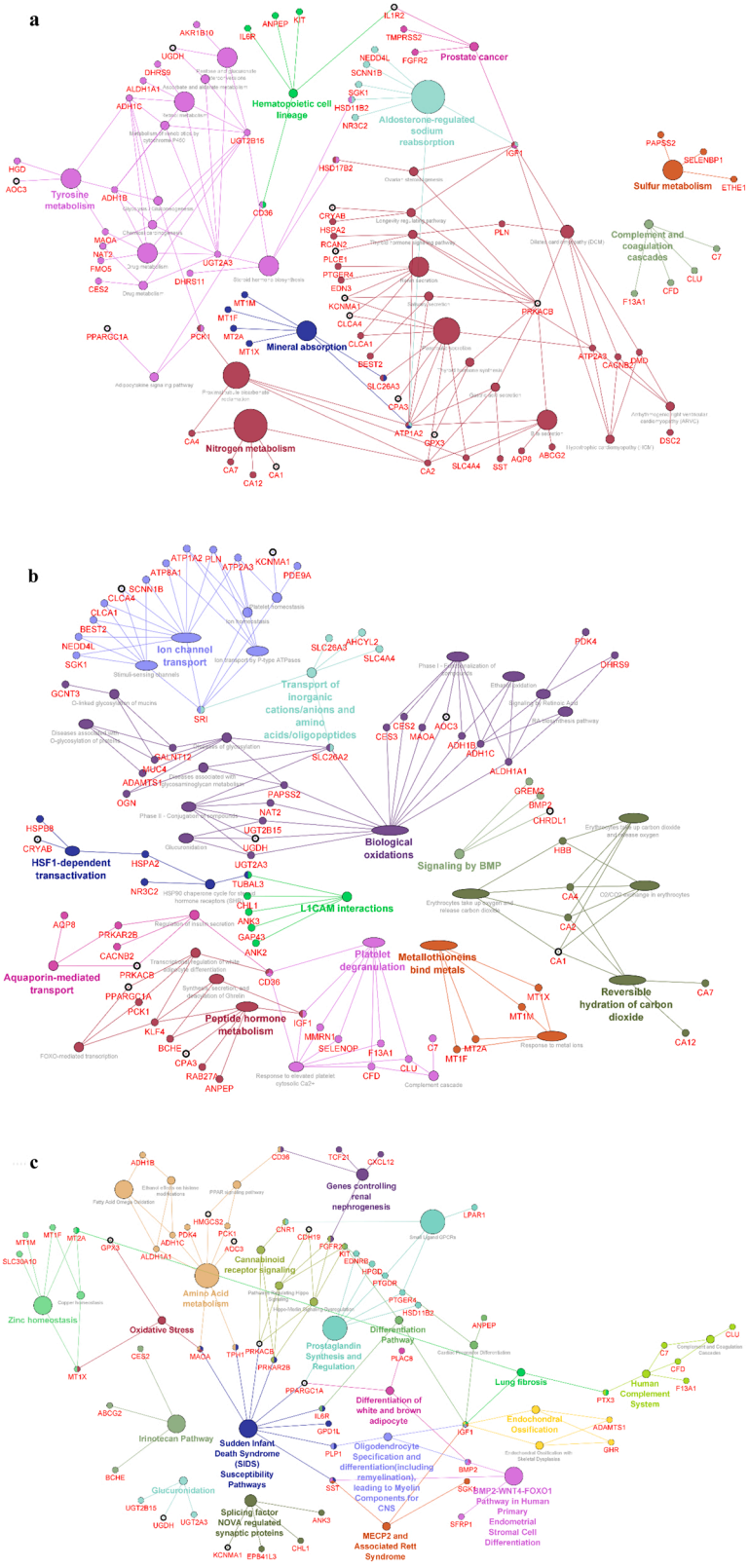
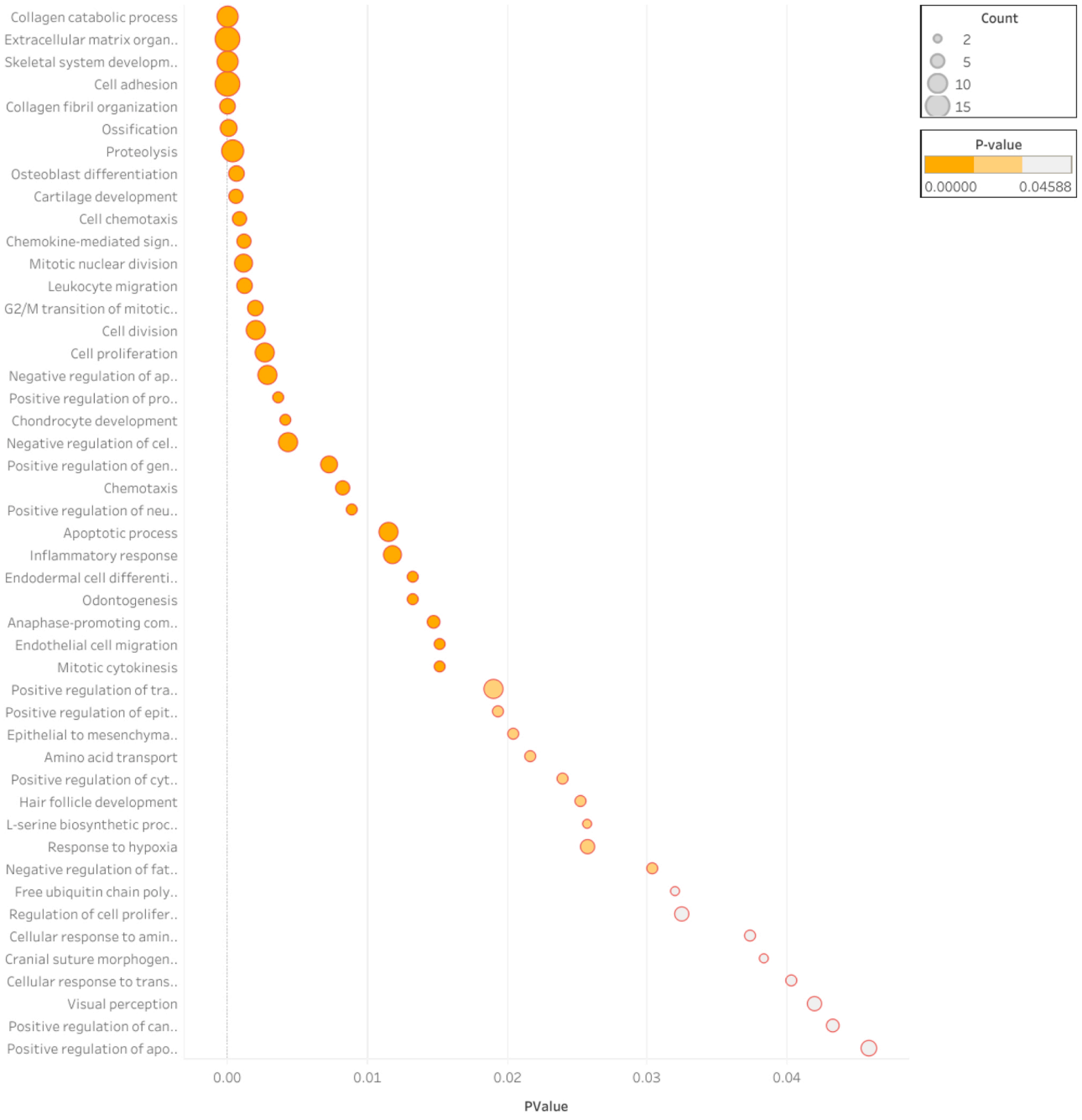


Fig. 5. Pathways analysis of COAD downregulated genes a) KEGG identified enriched pathways; b) WikiPathways identified enriched pathways; c) Reactome identified enriched pathways. Size of nodes implies the percentage of genes from the set involved in the regulation of each molecular pathway; color indicates genes' association to a particular molecular pathway; black circled genes are identified as significant for CRC survival and metastasis (Cytoscape ClueGo + CluePedia: KEGG, Reactome, and WikiPathways databases were selected to extract the list of pathways; two-sided hypergeometric test with a Bonferroni step down correction and a κ score of 0.3).

in detection of interactions reported between SFN and significant genes which correlated with overall survival or metastasis. It was noticed that SFN further upregulated *TIMP1, AURKA*, and *CEP55*, while stimulated inhibition of *CRYAB, PLCE1,* and *MMP28*. Interestingly, SFN also reversed CRC expression of 5 significant genes (*PPARGC1A, ABHD3, FGL2, NEBL*, and *UGDH*) (Table 1).

3.6. SFN-interacting genes in colon cancer cells

To verify the presented results, gene expression profiles from GSE174444 dataset was used and analyzed with GEO2R online tool. After being treated with 25 μmol/l of SFN for 48 h, SW480 colon cancer cells showed increased the expression of *CDK1, NEK2, SPP1, ABHD3,*



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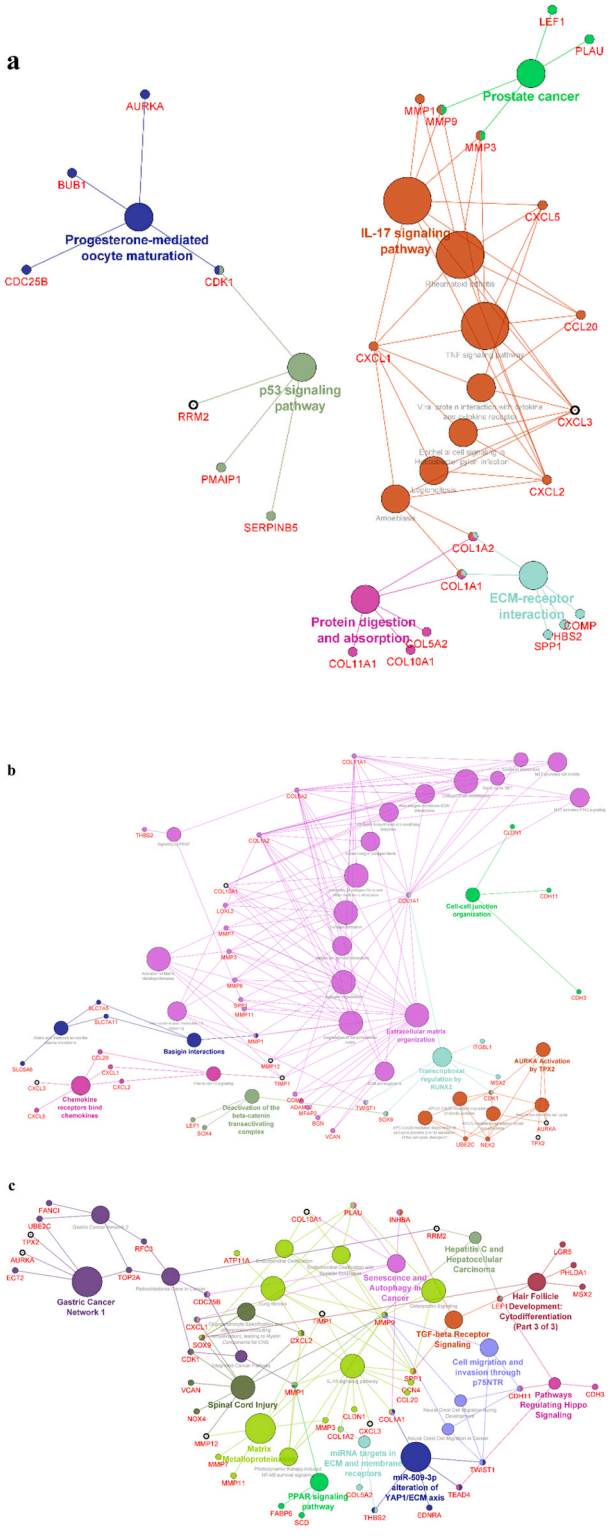
CEP55, and *UGDH*, while the expression of *CFD* was significantly downregulated in comparison to the control (untreated cells). This was in accordance with the previously obtained results, showing the potential of SFN to impact mentioned genes and further induce positive or negative effects in COAD patients. On the contrary, when used in the mentioned dose, SFN promoted the expression of *PLCE1* in colon cancer cells.

4. Discussion

The risk-to-benefit profile of SFN has not been fully explained yet, even though it is a well-known chemoprotective agent suggested as a potent therapeutic molecule for different diseases such as: autism spectrum disorder [39], neurodegenerative diseases [25,32], as well as cancer [11]. Cancer patients are particularly vulnerable to substances used as an addition to a given therapy due to the known compromission of their immune system, as well as numerous side effects caused by cytostatic treatments [18]. Thus, when choosing chemoprevention for cancer patients, it is of great importance to discover which harmful effects could be caused by the selected molecule. The adverse effects could be related to the cancer type or stage, among the other factors.

The increasing number of colon cancer cases, the gradually developed radio- and chemotherapeutic resistance and the expected adverse outcomes of conventional treatments pointed out the need for combination therapy in CRC patients [27]. Sulforaphane, as one of the proposed adjuvant molecules, should be thoroughly investigated before its



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application, bearing in mind pharmaco-toxicological profile of this molecule. Therefore, this in silico investigation aimed to predict the possible SFN-triggered molecular mechanisms which could cause adverse outcomes when used as an accompanying treatment for patients with CRC.

4.1. Colon-cancer dysregulated genes

We firstly identified consistently dysregulated genes in colon cancer tissue when compared to normal colon mucosa by using available TCGA and GEO datasets. The total of 111 genes were overexpressed and 223 underexpressed in colon cancer tissues (334 in total), among which increased expression of *COL10A1, CXCL3, NEBL, RRM2, TIMP1,* and *TPX2* (n = 6), and decreased expression of *SMPDL3A, IL1R2, HIGD1A, PPARGC1A, AOC3, CHRDL1, GPX3, CLCA4, CRYAB, MYH11, UGDH, KCNMA1, ABHD3, PDZRN4, ZG1,* and *HMGCS2* (n = 16) were in correlation with the lower overall survival in COAD patients. Similarly, Liu et al. [34] suggested that *SERPINE1, SPP1,* and *TIMP1* could serve as biomarkers closely related to the outcome in CRC patients [34]. Moreover, survival and correlation analyses revealed that *COL1A1, CXCL5, GNG4, TIMP1, SPP1*, and *LPAR1* could be considered as hub genes, important for diagnosis and therapeutic strategies guiding in colon cancer [24]. In addition, *GINS1, AURKA, MMP12*, and *CEP55* (n = 4) were noted as upregulated genes related to the colon cancer metastasis, together *CPA3, MYH11, CA1, INSL5, SMPDL3A, CDH19, FGL2, CCL8, LGI1, PLCE1, MMP28,* and *PRKACB* (n = 12), which suppressed expression was seen in metastatic colon cancer patients. Overexpression of *AURKA, MMP12* and other metalloproteinases were already recognized as markers of poor prognosis in different tumor types including colon carcinoma [22,30]. On the other hand, high *PLCE1* expression significantly inhibited the proliferation of colon cancer cells and degraded its malignant degree [50], which was in accordance to our results suggesting that inhibition of the *PLCE1* activated synthesis of phospholipase C epsilon which could stimulated CRC growth. Yao et al. [52] showed that the low expressions of *PRKACB* was linked to the worsening of overall survival in CRC patients, making it a potential therapeutic target [52].

Furthermore, in this research BP and pathways regulated by CRC DEGs were identified. As expected, they were mostly involved in the gastro-intestinal and metabolic processes such as: Aldosterone-regulated sodium reabsorption, Complement and coagulation cascades, Adipocytokine signaling pathway, Mineral absorption, Glycolysis/Gluconeogenesis, Sulfur metabolism, but also cancer progression (Collagen degradation, Senescence and autophagy in cancer, Extracellular matrix organization, etc). Moreover, colon cancer dysregulated genes were enriched for immune-related processes, such as regulation of leukocyte chemotaxis, negative regulation of leukocyte migration, positive regulation of gamma delta T cell differentiation and Neutrophil chemotaxis, to name a few. These findings were previously explained in the study performed by Ding et al. [16]. They identified CRC DEGs which were mainly enriched in BPs involved in collagen catabolic process, extracellular matrix organization, collagen fibril organization, cell division, and G1/S transition of the mitotic cell cycle for the upregulated genes; and bicarbonate transport, muscle contraction, regulation of intracellular pH, chloride transmembrane transport, and one-carbon metabolic process for the downregulated genes [16].

4.2. Sulforaphane – interacting genes

Sulforaphane – interacting genes were downloaded from the CTD. The total of 1896 genes were retrieved and compared to colon cancer DEGs, aiming to identify the set of common genes, or in other words, genes that can be affected by SFN and are consistently dysregulated in CRC patients. We obtained 86 genes, which were further used for pathway enrichment analysis that helped in detection of molecular pathways that could drive adverse outcomes in CRC patients treated

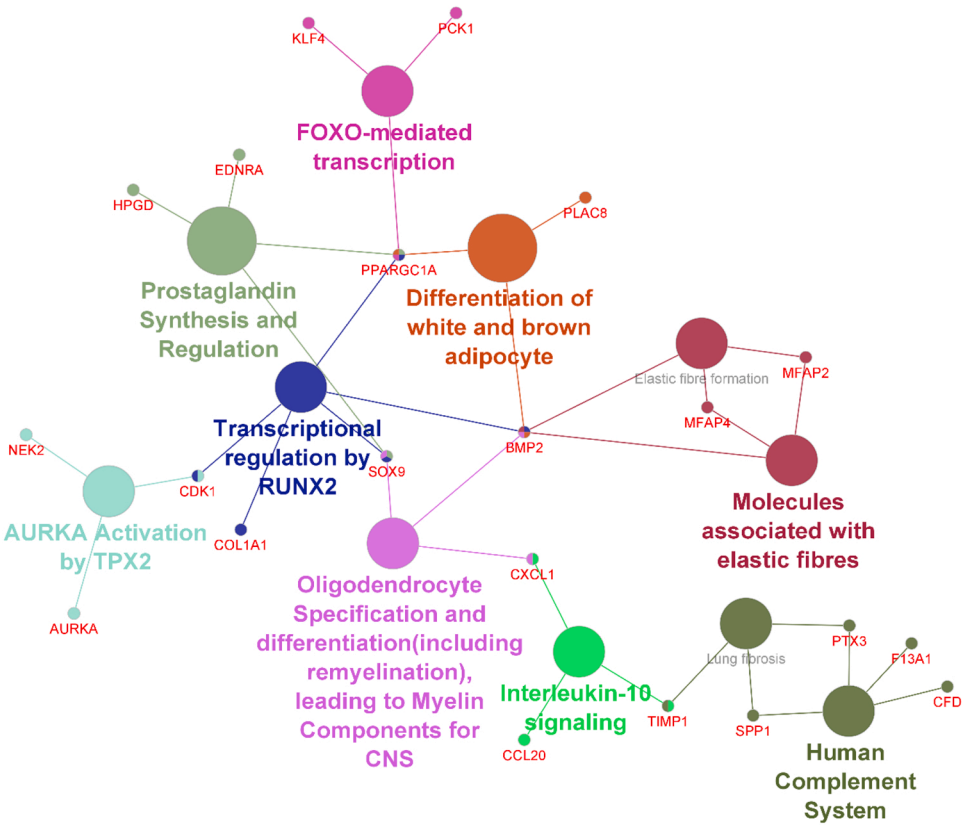

Fig. 8. Pathways analysis of SFN-interacting COAD disregulated genes: size of nodes implies the percentage of genes from the set involved in the regulation of each molecular pathway; color indicates genes' association to a particular molecular pathway; significant genes: *PPARGC1A, AURKA, TIMP1* (Cytoscape ClueGo + CluePedia: KEGG, Reactome, and WikiPathways databases were selected to extract the list of pathways; two-sided hypergeometric test with a Bonferroni step down correction and a κ score of 0.3).

with SFN. Out of them, 21 genes were reported as important for pathways regulation (Fig. 8), among which 3 were significant (*PPARGC1A, AURKA*, and *TIMP1*). Furthermore, CRC expression of each gene from the set was compared with the type of interaction described between SFN and the selected genes (source: CTD, Table 1). Moreover, SFN-interacted with 11 significant genes which correlated with overall survival or metastasis in CRC patients. Additionally, this in silico research helped us to obtain SFN-significant genes binary connections. It was noted that SFN potentiates the overexpression of *TIMP1, AURKA*, and *CEP55*, and promotes inhibition of *CRYAB, PLCE1,* and *MMP28* what might lead to the progression of CRC. Likewise, SFN further increased/decreased expression of 11 genes from the set (Table 1-blue lines). However, when used in a dose as low as 25 μmol/l, SFN promoted the expression of *PLCE1* in SW480 colon cancer cells. Thus, as expected, the dose might be the critical factor determining the effect of SFN on colon cancer growth and progression.

Interestingly, SFN also mediated reversion of CRC expression of 5 significant genes, namely: *PPARGC1A, ABHD3, FGL2, NEBL*, and *UGDH* what could explain its anti-tumor effects. In their recent research, Zhu et al. [56] explained that *FGL2* played a role in suppression of intestinal inflammation and CRC development via stimulating macrophages polarization [56]. As seen in Table 1, SFN was reported to stimulate mRNA expression of FGL2. Moreover, anticancerogenic potential of SFN could be mediated by upregulation of *PPARGC1A* gene. Accordingly, it was previously reported that when the level of PPARGC1A expression rises, the survival rate of COAD patients improves [55].

Tissue inhibitor matrix metalloproteinase 1 (TIMP1) is required for the regulation of the matrix remodeling during degradation of extracellular matrix, while its inhibition of matrix metalloproteinases is thought to be critical for the metastatic progression of cancer [43]. Hansson et al. [23] suggested that serum level of TIMP1 could be related to left ventricular hypertrophy and heart failure. They observed 1016 individuals without history of heart diseases and concluded that increased TIMP1 serum levels might be an early sign of cardiovascular extracellular matrix remodeling [23]. Similarly, in a male cohort undergoing coronary angiography, plasma level of TIMP1 was recognized as predictive biomarker of the subsequent risk of death or myocardial infraction [4,45]. According to the conducted study, SFN stimulated expression of *CCL20* as well as *SPP1* simultaneously with TIMP1 that further activated IL-10 signaling pathway and Lung fibrosis, respectively. IL-10 signaling is known immune-suppressive process which might limit host immune response to pathogens, including tumor – derived antigens [13].

Aurora Kinase A (AURKA) is a serine/threonine kinase family member involved in mitotic entry, bipolar spindle formation, centrosome maturation control, and segregation during mitosis, and as previously mentioned, it may serve as poor prognostic market for CRC patients [22], or contribute to colorectal adenoma to carcinoma progression [12]. Moreover, Jiang et al. [26] noted that overexpression of *AURKA* in renal fibroblasts promoted fibrotic response and thus, aggravated renal fibrosis in chronic kidney disease [26]. Together with SFN-induced expression of *NEK2* and *CDK1* in colon cancer cells, activation of AURKA by TPX2 could be promoted.

Moreover, it was suggested that SFN could stimulate *SOX9,* gene reported to play a role in regulation of RUNX2 transcription together with *CDK1*. Runt-Related Transcription Factor 2 (RUNX2) is an osteogenic transcription factor crucial for bone formation. However, in the cardiovascular system, it has emerged as an important factor that promotes osteogenic trans differentiation of vascular smooth muscle cells and expression of inflammatory cytokines, which might lead to atherosclerosis and other vascular complications [10]. The leucocytes from patients with symptomatic arteriosclerotic disease demonstrated the augmented mRNA level of RUNX2 and osteopontin, while osteocalcin was decreased [49]. Thus, we may suggest that SFN should be carefully applied in CRC patients with the history of cardiovascular diseases.

Genes involved in Differentiation of White and Brown Adipocyte process, namely *BMP2* and *PLAC8* were downregulated in CRC tissue

Table 1
Comparison between the CRC mRNA expression of genes involved in identified pathways (21) + significant genes (11) with CTD detected binary interactions between SFN and the same group of genes. Three genes were in common for the two sets (*AURKA*, *PPARGC1A, TIMP1*).

Gene	mRNA expression in CRC	SFN-mRNA interaction
KLF4	↓	↓
PCK1	↓	↓↑
PPARGC1A	↓	↑
EDNRA	↑	↓
HPGD	↓	↓
PLAC8	↓	↓
BMP2	↓	↓
MFAP4	↓	↓
MFAP2	↑	↓
SOX9	↑	↑
CDK1	↑	↑
COL1A1	↑	↓
NEK2	↑	↑
AURKA	↑	↑
CXCL1	↑	↓
CCL20	↑	↑
TIMP1	↑	↑
SPP1	↑	↑
PTX3	↓	↑
F13A1	↓	↓
CFD	↓	↑
ABHD3	↓	↑
CEP55	↑	↑
CRYAB	↓	↓
FGL2	↓	↑
MMP28	↓	↓
NEBL	↑	↓
PLCE1	↓	↓
UGDH	↓	↑

*Blue lines: SFN promotes the expression of genes in the same way, as it was seen in colon cancer tissue; red lines: SFN regulates the expression of significant genes' in the way opposite to colon cancer tissue expression; bold genes – recognized as significant (SFN-interacting genes that were in correlation with lower overall survival or metastasis).

and could be further inhibited via SFN, which might contribute to the development of obesity. In placenta-specific gene 8 protein (PLAC8) deficient mice the increment in fat storage was evident. Proposed mechanism relies on inhibition of thermogenesis due to inhibition of *C/EBPβ* transcription [28]. In another study, authors observed that PLAC8-knockout mice where more promoted to develop type 2 diabetes associated with obesity [38]. Moreover, *PLAC8* expression was important for establishing an optimal CD8 T cell response against influenza A virus via Th1 T cell-driven inflammation [41]. Similarly, the protein expressions of BPM-2 and phosphorylated-Smad1/5 were significantly lower in diet-induced obese mice when compared to the control [9].

4.3. Strengths of the bioinformatic analysis in toxicology

The advantage of the large-scale multi-omics datasets, such as those presented in TCGA, have largely helped in the understanding of the characteristics of a wide variety of tumors. With these cancer datasets characterization of human cancers at the DNA, RNA, protein and/or epigenetic levels can be identified, which subsequently eases the development of the better, targeted therapies. To date, TCGA is the most prominent cancer genomics program which offers more than 10,000 primary tumor and matched normal samples across the 33 cancer types [47]. Moreover, these data can help in faster development of personalized cancer therapy strategies and prediction of the possible side effects of the given treatment in different patients.

The 3R principle in toxicological research forced the development of data repositories which can be used for preliminary analysis and identification of the chemical-gene interactions and disease relationships [31]. One of such databases is the CTD, which captures public data including: date of curation, ID of the curator, PubMed ID, interaction, species, chemical, gene/protein, associated diseases and author contact information [51]. Moreover, prior to the public release on the CTD website, all the curated data goes through quality control review, a well-designed process in which each curated interaction is captured using the controlled vocabularies/ontologies with aim to ensure consistency [14]. Comparative Toxicogenomics Database uses the official gene symbols and names from the National Center for Biotechnology Information's (NCBI) Entrez-Gene database, while CTD disease vocabulary uses terms are from both MeSH (Medical Subject Headings) and OMIM (Online Mendelian Inheritance of Man) [15].

The combination of all the mentioned approaches allows preliminary toxicological investigations, which are able to give the predictions of potentially harmful effects of the investigated chemical and shape further in vitro and in vivo analysis.

4.4. Limitations of the used in silico approach

Knowing that analyzing "omics" data at the individual level is critical for the success of precision medicine, we combined toxicogenomic data mining approach with DEGs analysis aiming to explore and gain better understanding of the pharmaco-toxicological profile of SFN, as well as to predict risk factors for the onset of adverse outcomes, such as comorbidities, biomarkers etc.

However, having in mind that the data in such computational methods and processes is extracted by drawing statistical associations between genes and drug of interest, it is not possible to directly address the dose-response relationship. Moreover, it should also be mentioned that not only dose, but many other factors might influence the manifestation of substance-induced toxic effects, such as route of administration, duration, individual drug metabolism rates, various colon cancer types, etc. [6,15], which should be explored in our further in vitro and in vivo investigations.

5. Conclusion

Sulforaphane is recognized as effective chemotherapeutic molecule which could be used in various colon cancer types, even though, its safety profile for the treatment of CRC patients has not been defined. This study showed that 334 genes (223 down and 111 upregulated) were consistently dysregulated in CRC, and SFN interacted with 86. Among them SFN interacted with 86 genes and 21 of them were identified as significant. Interestingly, SFN potentiates the overexpression of *TIMP1, AURKA*, and *CEP55*, and promotes inhibition of *CRYAB, PLCE1,* and *MMP28,* that might lead to the progression of CRC, while SFN-mediated regulation of *PPARGC1A, ABHD3, FGL2, NEBL*, and *UGDH* could contribute to its anti-tumor effects. Moreover, SFN stimulated Transcriptional activation of RUNX2, and AURKA activation by TPX2, molecular pathways, which drive tumor progression and aggressiveness. Their promotion could also contribute to atherosclerosis and renal fibrosis in chronic kidney disease, respectively. In addition, SFN-contributes to the activation of immune-suppressive process, IL-10 signaling, which might limit host immune response to pathogens, including tumor – derived antigens while inhibiting Differentiation of White and Brown Adipocyte process, an underlying pathway which inactivation leads to obesity. Thus, genome signature of CRC patients could serve as important factor when addressing the risk-to-benefit profile of SFN therapy. Patients with colon cancer and increased expression of *TIMP1, CCL20*, *SPP1, AURKA*, *CEP55*, *NEK2, SOX9* and *CDK1,* or downregulation of *CRYAB, PLCE1, MMP28, BMP2* and *PLAC8* may not be ideal candidates for SFN chemoprevention.

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CRediT authorship contribution statement

Dragica Bozic: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Software, Writing – original draft, Visualization. **Katarina Baralić:** Methodology, Supervision. **Katarina Živančević:** Methodology, Supervision. **Evica Antonijević Miljaković:** Supervision. **Marijana Ćurčić:** Writing – review & editing. **Biljana Antonijević:** Writing – review & editing, Supervision. **Aleksandra Buha Đorđević:** Supervision. **Zorica Bulat:** Writing – review & editing. **Yi Zhang:** Writing – review & editing, Supervision. **Li Yang:** Writing – review & editing, Supervision. **Danijela Đukić-Ćosić:** Conceptualization, Visualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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The results reported here are based upon the data maintained by the National Cancer Institute's Genomic Data Commons: https://gdc.cancer.gov/.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112598.

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