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Identification of novel Nrf2 target genes as prognostic biomarkers in colitis-associated colorectal cancer in Nrf2-deficient mice

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ABSTRACT

Aims: Colorectal cancer (CRC) is the third most common cancer worldwide. Nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of many cytoprotective genes, plays a protective role in carcinogenesis. Recent studies have identified a specific gene-expression signature regulated by the Nrf2 pathway in lung adenocarcinoma and head-and-neck squamous cell cancer. However, the roles of Nrf2 in the development of colitis-associated colorectal cancer (CACC) have not been well characterized. Nrf2 target genes as prognostic biomarkers in CACC remain to be explored. Thus, this work aimed to identify the molecular changes that occur during mouse CACC progression to facilitate the development of diagnostic and prognostic biomarkers.

Main methods: The CACC model was established using azoxymethane (AOM) with dextran sulfate sodium salt (DSS) in BALB/c mice for 3 weeks to induce colitis-associated adenoma (CAA, early stage) and for 9 weeks to induce colitis-associated carcinoma (CAC, late stage). Using RNA-sequencing and bioinformatics analyses we examined the mRNA expression profiles of 6 groups: wild-type control (WT-C), WT-CAA, WT-CAC, Nrf2 knockout control (Nrf2KO-C), Nrf2KO-CAA, and Nrf2KO-CAC.

Key findings: In the AOM/DSS model of colitis-associated tumorigenesis, *Nrf2*$^{-/-}$ mice showed a phenotype similar to WT mice, but with significantly more tumors and a much higher percentage of adenocarcinomas. We identified 47 novel Nrf2 genes *via* gene expression profiling of tumor samples. Survival analysis showed that 23 of these genes were biomarkers of a poor prognosis in colon cancer patients.

Significance: Nrf2 target genes deserve exploration as prognostic and therapeutic targets for CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most common types of cancer worldwide [1], the third most common cancer after lung and genital cancers worldwide with > 1.2 million new cases diagnosed annually [2], and the fourth leading cause of cancer-related deaths worldwide [3]. An emerging theme in cancer biology is that metabolic regulation is intricately linked to cancer progression, so factors that promote proliferation may also directly or indirectly promote metabolic changes to support rapid proliferation and metastasis [4]. Moreover, the dysregulation of processes pertaining to cell proliferation, inflammation, and lipid metabolism is involved in the progression of colon cancer [5].

Chronic inflammation is also one of the hallmarks of cancer [6] and has been linked to the pathogenesis of CRC [7]. Colitis-associated CRC (CACC) is a subtype with a high mortality that is closely associated with inflammatory bowel disease [8]. A mouse model initiated by azoxymethane (AOM) and promoted by dextran sulfate sodium (DSS) has been widely used to simulate the pathogenesis in patients with CACC [9]. Actually, many steps in the carcinogenesis process are induced in mice by an AOM injection and DSS in the drinking water [3].

Nuclear factor erythroid 2-like factor 2 (Nrf2) is a transcription factor that controls both the basal and inducible expression of a battery of cytoprotective genes which protect cells from damage by intracellular oxidative stress and extracellular oxidizing agents, and create intracellular redox homeostasis *via* transcriptional activity and interaction with kelch-like ECH-associated protein 1 [11]. Recently, the

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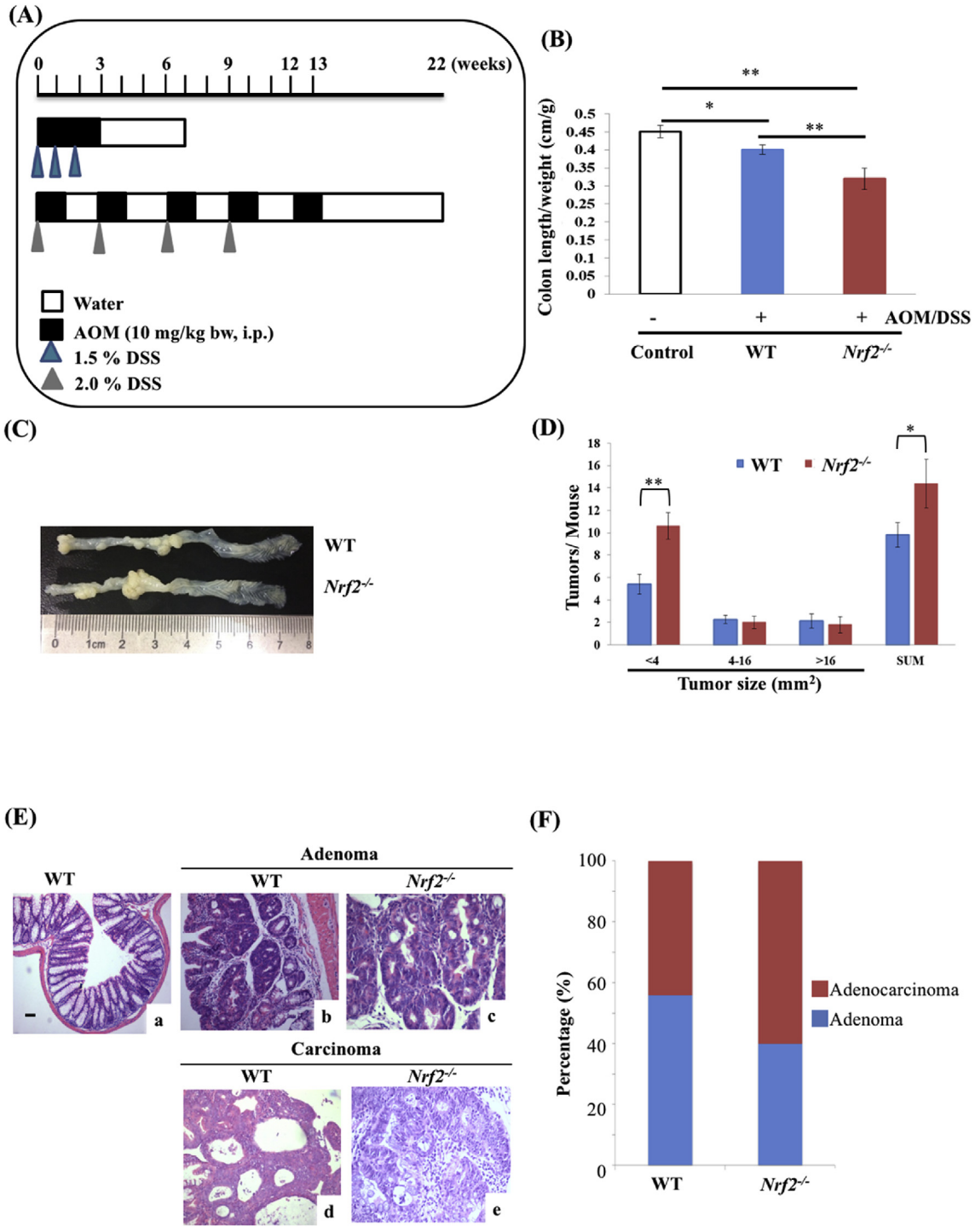

Fig. 1. Nrf2 deficiency promotes colon carcinogenesis. (A) Experimental protocols for induction of colitis-associated tumorigenesis in WT and *Nrf2*$^{-/-}$ mice. BALB/c WT and *Nrf2*$^{-/-}$ mice received AOM/DSS for 6 weeks to induce CAA, and for 22 weeks to induce CAC. **(B)** Colon length in mice with CAC, represented as a ratio (cm/g) *vs* the starting weight of mice prior to AOM/DSS administration (n = 4 for control, 13 for WT, and 5 for *Nrf2*$^{-/-}$). Values are the mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* Control WT mice without any treatment. **(C)** Representative images of colon carcinomas from mice with AOM/DSS induced CAC. **(D)** Size distribution (left) and total number (SUM, right) of colonic tumors per mouse in mice with CAC (n = 15 for WT and 5 for *Nrf2*$^{-/-}$). Values are the mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* WT mice. **(E)** Representative images of H&E staining of colonic tumor sections in the WT (a) and at the end of AOM/DSS treatment in the adenoma (b, c) and carcinoma models (d, e). WT Control, normal sections from WT mice without any treatment. Scale bar, 100 μm; original magnification × 200. **(F)** Percentages of CAA and CAC at the end of AOM/DSS treatments in WT and *Nrf2*$^{-/-}$ mice with CAC.

role of Nrf2 in cancer has been the subject of numerous interesting studies. Activation of Nrf2 has traditionally been considered beneficial for the prevention of cancer since Nrf2 is the main cellular defense mechanism against carcinogens, reactive oxygen species (ROS), and other DNA-damaging factors. Accumulation of DNA damage can also result in Nrf2 hyperactivity, which in turn enhances the rate of survival of cancer cells when exposed to high levels of endogenous ROS and their escape from apoptosis [10]. Persistent Nrf2 activation leads to elevation of the expression of downstream genes which consequently induce metabolic reprogramming and promote cell proliferation [11].

High Nrf2 levels in various cancer cells are associated with a poor prognosis, radio- and chemo-resistance, and the aggressive proliferation of cancer cells [12]. Furthermore, it contributes to the survival and chemoresistance of CRC cells *via* the overexpression of cytoprotective and multidrug resistance genes [2]. More recently, Ngo et al. reported that the induction of hepatocarcinogenesis is inhibited in Nrf2-deficient mice [13]. Although that study suggested that the role of Nrf2 may be model- and context-dependent, the authors state that inhibition of Nrf2 transcriptional activity could be beneficial in treating advanced cancers.

Previous studies have shown that Nrf2 is involved in carcinogenesis by impacting various signaling pathways and molecules involved in inflammation, tumor initiation, and tumor progression. However, its roles in the development of CACC remains relatively understudied. RNA sequencing (RNA-Seq) is rapidly emerging as a powerful tool for transcriptome-oriented studies [14]. The aim of this work was to use RNA-Seq to identify the molecular and signaling changes that occur during mouse CACC progression to facilitate the development of diagnostic and prognostic biomarkers as well as novel treatments.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals were from Sigma-Aldrich Co., Ltd (St. Louis, MO, USA). Antibodies against mouse Nrf2 (H300; sc-13032) were from Santa Cruz Biotechnology. DSS (36–50 kD) was from MP Biomedicals (Aurora, OH, USA).

2.2. Animals and ethics statement

BALB/c background WT mice were purchased from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). C57BL/6 background *Nrf2*$^{-/-}$ mice were kindly provided by Prof. Masayuki Yamamoto (University of Tsukuba, Japan) [15]. BALB/c background *Nrf2*$^{-/-}$ mice were produced by 8 back-crossings of C57BL/6 background *Nrf2*$^{-/-}$ with BALB/c WT mice. The genotypes of *Nrf2*$^{-/-}$mice were routinely determined by RT-PCR and confirmed by Western immunoblotting. All animal procedures were performed with the approval of the Laboratory Animal Ethics Committee of Zhejiang University.

2.3. Induction of colitis-associated colon cancer (CACC)

Two colitis-associated mouse tumor models of adenoma and adenocarcinoma (CACC), which represent the initial and later stages of CRC, respectively, were induced following AOM/DSS treatment protocols modified from that of Suzuki et al. [16]. Briefly, to induce CAA, 5–6-week-old male BALB/c mice were given a single injection of AOM (10 mg/kg i.p.) once per week for the first three weeks, and also exposed to drinking water containing 1.5% DSS for the first three weeks. This was followed by normal drinking water for 3 weeks. To induce CAC, 5–6-week-old male mice were first given AOM (10 mg/kg i.p.), and exposed to drinking water containing 2% DSS for one week. This was followed by normal drinking water for 2 weeks. This treatment was repeated for three additional cycles. The mice were further exposed to drinking water containing 2% DSS for 7 days. During the final 9 weeks, mice were on normal drinking water (Fig. 1A). Throughout, the mice were monitored for body weight, diarrhea, and hematochezia. At the end of the experiments, the mice were sacrificed by cervical dislocation, and the distal colon was removed. The tumors and the adjacent normal colon tissues were harvested separately as previously described [17]. Tissues were immersed in TRIzol reagent (Invitrogen, Carlsbad, CA) for total RNA extraction, and stored frozen in liquid nitrogen at −80 °C. Tissues were fixed in buffered formalin for histological analysis [18]. Tissue sections (3 μm) were stained with hematoxylin and eosin (H&E) as previously described [19].

2.4. Animals groups

Animals were randomly divided into six groups: wild-type control (WT-C), WT-CAA, WT-CAC, Nrf2 knockout control (Nrf2KO-C), Nrf2KO-CAA, and Nrf2KO-CAC. Each group contains three animals. For control groups of WT-C and Nrf2KO-C, total RNA extract was a pooled sample from three animals. For the tumor groups of WT-CAA, WT-CAC, Nrf2KO-CAA and Nrf2KO-CAC, total RNA extract was a pooled sample from multiple tumors, which were harvested from three animals at biopsy.

2.5. RNA-Seq

Total RNA was extracted using TRIzol reagent by following the manufacture's instruction. RNA quality was checked using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Illuminam RNA-Seq libraries were prepared with 2 μg of total RNA using the SMARTer Stranded RNA-Seq Kit (Clontech, Mountain View, CA) according to the manufacturer's instructions, and index codes were added to attribute the sequences to each sample.

Briefly, mRNA was purified from total RNA using magnetic beads with Oligo (dT), followed by fragmentation into short fragments of ~200 bp. The strand-specific cDNA was synthesized, and mRNA was used as a template to synthesize a strand of cDNA by random primers. When the double-stranded cDNA was synthesized, deoxyuridine triphosphate was replaced by deoxythymidine triphosphate. End Repair Mix end, the A tail, and the adaptor were added, and ligated to prepare the cDNA for hybridization and the double-stranded cDNA was purified with the AMPure XP system (Beckman Coulter, Beverly, MA, USA). The double-stranded cDNA was digested with USER enzyme so the library contained only single-stranded cDNA, then PCR enrichment and PCR amplification for 15 cycles were used to obtain the final cDNA library. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an IlluminaHiSeq 2000 platform and reads were generated.

2.6. Quality control and gene expression

Raw data (raw reads) in the FASTQ format were first processed using in-house Perl scripts and the Q20, Q30, and GC contents of the clean data were calculated. For mapping, an index of the reference genome was built using Bowtie v2.0.6, and paired end clean reads were aligned to the reference genome using TopHat v2.0.9.

The counts of the read numbers mapped to each gene were processed by HTSeq v0.6.1, and the FPKM (expected number of fragments per kilobase sequence per million base pairs sequenced) of each gene was calculated based on the length of the gene and the reads count-mapped to that gene [20]. The FPKM considers the effect of both sequencing depth and gene length on the read count and is currently the most commonly used method for estimating gene expression levels [21].

2.7. Identification of differentially-expressed genes (DEGs)

The DEGs among the WT-C, WT-CAA, WT-CAC, Nrf2KO-C, Nrf2KO-CAA, and Nrf2KO-CAC sets were identified using the R statistical software package (www.r-project.org). Significant DEGs were defined as genes with fold-changes of 1.5 and with a cutoff threshold of p values < 0.05 [22]. The DEGs with fold-changes between 1.5 and −1.5 were removed from subsequent analysis. The significant DEGs between WT-C and WT-CAA, WT-C and WT-CAC, Nrf2KO-C and Nrf2KO-CAA, and Nrf2KO-C and Nrf2KO-CAC were identified. In these comparisons,

the genes that were differentially expressed in all of the pairs of comparisons were identified as persistent DEGs.

2.8. Enrichment analysis of Gene Ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathways

The gene annotation enrichment analysis using GO (http://www.geneontology.org/) and KEGG (http://www.genome.jp/kegg/) data for gene sets was performed using The NIH Database for Annotation, Visualization and Integrated Discovery (DAVID) software [23]. This software can provide a functional interpretation of large gene lists derived from genomic studies. A Benjamini p-value of < 0.05 was used in the analysis.

2.9. Cluster analysis

Unsupervised hierarchical clustering of the mouse expression profiles of the DEGs was performed using Heatmapper [24].

2.10. In silico analysis

To identify the Nrf2 binding sites within the promoter regions of genes, we used the transcription factor-binding site finding tool LASAGNA-Search 2.0 with cutoff p-values ≤ 0.001. The search was limited to the -2 kb upstream promoter region relative to the transcription start site [25].

2.11. Survival analysis

Cox proportional hazard regression was performed using the online survival analysis and biomarker validation tool SurvExpress [26]. SurvExpress separated the patient samples into two groups, high- and low-risk, based on the average values of gene expression, and we analyzed the survival probability of the two groups using the log-rank method. SurvExpress used the log-rank test to generate Kaplan-Meir plots based on the 'Survival' package of the R platform, which is integrated into its website. Log-rank test p-values < 0.05 were considered to be statistically significant.

2.12. Statistical analysis

Statistical analysis was carried out using Stata7 for Windows (StataCorp LLC, College Station, TX, USA). Student's t-test was used to compare two groups. Groups of more than two were compared by one-way ANOVA followed by Bartlett's test. Spearman's correlation was used to analyze two ranked variables. p values < 0.05 were considered statistically significant.

3. Results

3.1. Colon carcinogenesis is exacerbated in *Nrf2*$^{-/-}$ mice challenged with AOM/DSS

Previously we reported that *Nrf2* deficiency increases susceptibility to CAA; the number of tumors per mouse is significantly higher in *Nrf2*$^{-/-}$ mice than in their WT counterparts [19]. In the present study, two colitis-associated mouse tumor models of CACC were induced following the protocols shown in Fig. 1A. *Nrf2* deficiency increased the severity of the colitis developed in the AOM/DSS-treated mice with CACC, which had a significantly shorter colon (Fig. 1, B – C). Although the number of smaller tumors (< 4 mm^3) per mouse was significantly higher in *Nrf2*$^{-/-}$ mice (11 ± 2, $p < 0.01$) than in the WT (5 ± 1.4), the numbers of medium-sized (4–16 mm^3) and large (> 16 mm^3) tumors per mouse were similar in the *Nrf2*$^{-/-}$ (2 ± 0.5) and WT (2 ± 0.5) mice (Fig. 1D). Normal WT colon stains with H&E served as a control (Fig. 1E, a). Histological evaluation after 6 weeks of AOM/DSS treatment revealed that the majority of tumors from WT and *Nrf2*$^{-/-}$ mice were CAA, representing the initial stage of tumorigenesis (Fig. 1E, b and c). However, after 22 weeks of AOM/DSS treatment some of the tumors from WT and *Nrf2*$^{-/-}$ mice were CAC, representing the later stage of tumorigenesis (Fig. 1E, d and e). *Nrf2*$^{-/-}$ mice had significantly more CAC tumors than WT mice (Fig. 1F).

3.2. Nrf2 deficiency significantly diminishes the diversity of gene expression in colon cancer

RNA-Seq is a powerful tool to identify the molecular and signaling changes that occur during mouse CACC development, so we analyzed the profiles of DEGs between diseased and normal control groups using two criteria: fold-change LogFC > 1.5 and p-value < 0.05 from ANOVA tests. A library of size-normalized counts for samples was generated, and volcano plots for the DEGs are shown in sFig. 1.

Comparisons of DEGs of the two stages in the WT CACC groups to the WT-C groups (sFig. 1 and sTable 1) revealed a total of 553 DEGs in the WT-CAA group, composed of 213 (38.52%) up-regulated and 340 (61.48%) down-regulated DEGs. In the WT-CAC group, of the total 421 DEGs detected, 210 (49.88%) were up-regulated and 211 (50.12%) down-regulated. These findings showed that the number of DEGs in adenocarcinoma was significantly greater than in carcinoma (553 *vs* 421). In addition, 221 DEGs were persistent (commonly found in the WT groups at both stages), including 92 (41.63%) up-regulated and 129 (58.37%) down-regulated genes (Fig. 2). All the gene IDs and fold-changes are listed in sTable 2.

Deletion of *Nrf2* significantly diminished the diversity of gene expression in colon cancer; the total numbers of DEGs were lower than in the WT (sFig. 1 and sTable 1). Compared to the Nrf2KO-C group, the total numbers of DEGs were 269 in the Nrf2KO-CAA and 447 in the Nrf2KO-CAC group. One hundred nine genes (40.52%) were up-regulated and 160 (59.48%) were down-regulated in the Nrf2KO-CAA group, while 167 (37.36%) were up-regulated and 280 (62.64%) were down-regulated in the Nrf2KO-CAC group. The total number of persistent DEGs in common to the two stages of cancer in *Nrf2*$^{-/-}$ mice was 81, which included 38 (46.91%) up-regulated and 44 (54.32%) down-regulated genes, with *Hbb-bs* down-regulated in adenoma and up-regulated in adenocarcinoma (Fig. 2 and sTable 3, a – b). All the comparisons of DEGs in the *Nrf2*$^{-/-}$ groups are listed in sTable 4 (including all the gene IDs and fold-changes).

To determine the role of Nrf2 in colon cancer using RNA-Seq data, we compared the functions and metabolic pathways between persistent DEGs in WT and *Nrf2*$^{-/-}$ mice generated by GO and KEGG pathways analysis (sTable 5–8).

To gain new insights into the dynamic molecular and signaling changes that occur during mouse CACC development in addition to the differences between WT and *Nrf2*$^{-/-}$ mice, we used GO analysis of the persistent DEGs (common DEGs in different stages in WT and *Nrf2*$^{-/-}$ mice). Our analysis of persistent DEGs in WT mice showed that 25 categories were significantly enriched in DAVID and included genes associated with functions including positive regulation of endothelial cell proliferation, fatty acid metabolic process, cell-matrix adhesion, lipid metabolic process, positive regulation of transforming growth factor beta receptor signaling pathway, cell adhesion, and response to hypoxia (sTable 7). We also found that genes that regulate drug metabolism, metabolism of xenobiotics by cytochrome P450, and response to drug, whose associated enzymes oxidize small foreign organic molecules such as toxins or drugs, were down-regulated in tumors (sFig. 2). For persistent DEGs in altered groups, 10 categories were significantly enriched: neural tube closure, establishment of planar polarity, cochlea morphogenesis, Wnt signaling pathway, inflammatory response, visual perception, elastic fiber assembly, cellular response to drug, establishment of epithelial cell apical/basal polarity, wound healing, and regulation of embryonic development (sTable 8).

KEGG analysis of persistent DEGs in WT mice showed that 8

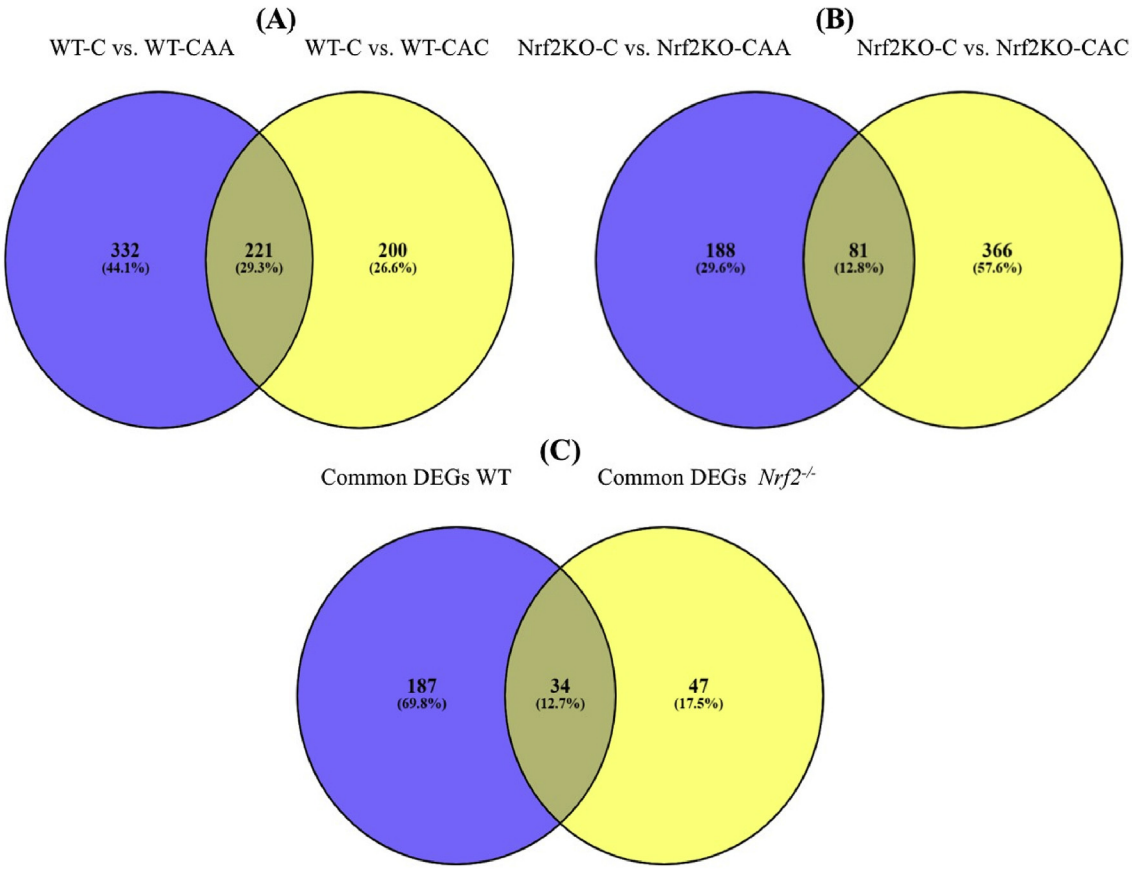

Fig. 2. Nrf2 deficiency alters global transcription in tumors in the colon of mice challenged with AOM/DSS. Venn diagrams showing common and distinct DEGs induced by AOM/DSS in WT and *Nrf2*$^{-/-}$ mice. (A) WT-C *vs* WT-CAA/CAC data sets, (B) Nrf2KO-C *vs* Nrf2KO-CAA/CAC data sets, and (C) common DEGs in WT *vs* Nrf2KO data sets.

pathways were significantly enriched in DAVID: metabolic pathways, focal adhesion, regulation of actin cytoskeleton, drug metabolism – other enzymes, ECM-receptor interaction, retinol metabolism, pyrimidine metabolism, and drug metabolism-cytochrome P450 (sFig. 2).

Function and pathway differences between the persistent DEGs in WT and *Nrf2*$^{-/-}$ mice support the hypothesis that Nrf2 is involved in regulating the functions that appeared in WT mice and disappeared in *Nrf2*$^{-/-}$ mice, such as positive regulation of endothelial cell proliferation, fatty acid metabolic process, cell-matrix adhesion, lipid metabolic process, positive regulation of transforming growth factor beta receptor signaling pathway, cell adhesion, and response to hypoxia.

3.3. Novel Nrf2-related transcriptome (NNRT) involves 48 genes in CACC

In comparing the DEGs common to WT and *Nrf2*$^{-/-}$ at both cancer stages, we considered that only those genes differentially-expressed in *Nrf2*$^{-/-}$ and not in common with the WT were Nrf2-related (NNRTs). Among these DEGs, 18 were up-regulated while 30 were down-regulated, including the Nrf2 gene and Hbb-bs that was down-regulated in adenoma and up-regulated in adenocarcinoma (sTable 3, a – b). All the common genes at both cancer stages in *Nrf2*$^{-/-}$ are listed in sTable 4 and sTable 8 (including all gene IDs and fold-changes). Functional annotation analysis revealed that 28 genes, neglecting Nrf2 and Hbb-bs, were significantly enriched in several biological processes that induce tumor progression (Table 1), and hierarchical clustering illustrated the expression profiles of these genes (Fig. 3).

In silico analysis validation by identification of Nrf2-binding sites within the promoter regions of NNRTs was done using the transcription factor-binding site-finding tool LASAGNA-Search 2.0 with cutoff *p*-values ≤ 0.001. The search was limited to the −2 kb upstream promoter region relative to the transcription start site. We identified the Nrf2-ARE (antioxidant response element) sequences within the −2000-bp upstream promoter regions of 22 genes. However, we did not find an ARE sequence in the promoter regions of 6 of the 28 genes (Fig. 4, sTable 9). Together, our results suggest that Nrf2 binds directly with the promoter regions of 22 of the genes in the signature and triggers their over expression; the 6 genes are exceptions.

3.4. Novel Nrf2 target genes as prognostic biomarkers in colitis-associated colorectal cancer

To verify the prognostic performance of the identified NNRTs, we first used the SurvExpress online tool which provides survival analysis and risk assessment. Survival analysis showed that 23 of the 28 NNRTs were able to significantly differentiate low-from high-risk groups in the 3 data sets (GSE17536 [27,28], GSE41258 [29], and GSE14333 [30]) (Fig. 5 and sFig. 3). Concordance index values approaching 0.5 mean putatively "random" while higher values mean better prediction. In addition, the *p*-value of log-rank tests for the differences between the two groups was < 0.05. An estimate of the hazard ratio by the Cox proportional hazards model represents the relative risk between two risk groups. Thus, our analysis strongly suggests that these genes are regulated by Nrf2 and are powerful predictors of a poor prognosis in colon cancer patients.

4. Discussion

CRC is the third leading cause of cancer-related deaths worldwide [31]. An epidemiological study showed that the number of colon cancer patients has increased remarkably in China [32], but the reasons for

Table 1
KEGG pathway and GO functional enrichment analysis of up- and down-regulated NNRTs.

Term	*p*-value	Genes
GO_Biological Process		
GO:0008285 – negative regulation of cell proliferation	0.013073771	SLC9A3R1
GO:0098779 – mitophagy in response to mitochondrial depolarization	0.014371909	REP15, MYH11
GO:0042493 – response to drug	0.010490815	GGH
GO:0045600 – positive regulation of fat cell differentiation	0.021714902	NOCT
GO:0055114 – oxidation-reduction process	0.00857917	STEAP3, UGDH
GO:0008152 – metabolic process	0.002222411	UGDH
GO:0007275 – multicellular organism development	0.004105271	ATOH1
GO:0045944 – positive regulation of transcription from RNA polymerase II promoter	0.035731116	LUM
GO:0030855 – epithelial cell differentiation	0.048236708	TAGLN
GO:0006810 – transport	1.59E-04	STEAP3, SLCO2B1
GO:0048251 – elastic fiber assembly	0.048519932	MYH11
GO:0006874 – cellular calcium ion homeostasis	0.008562603	ANK2
GO:0098910 – regulation of atrial cardiac muscle cell action potential	0.03719297	ANK2
KEGG Pathway		
mmu01100: Metabolic pathways	0.000886	NAGS, UGDH, GPT
mmu04612: Antigen processing and presentation	0.04119	H2-Q1
mmu04145: Phagosome	0.03832	H2-Q1
mmu05410: Hypertrophic cardiomyopathy	0.002182	DES
mmu05205: Proteoglycans in cancer	0.01839	ANK2, LUM

GO, Gene Ontology; mmu, *Mus musculus* KEGG pathway.

this increase are not fully understood. Known risk factors, such as obesity, alcohol, smoking, and a sedentary lifestyle, are likely to underlie early-onset cancers [33]. Sometimes, traditional therapies such as surgery, neoadjuvant chemotherapy, and radiotherapy are ineffective in the late stages of CRC [34].

Several recent studies have revealed that Nrf2 is associated with a poor prognosis and worse cancer-specific survival in different kinds of cancer such as those of the lung [35], and liver [36]. The physiological and pathological functions of Nrf2 protein are similar to those of an oncogene, due to its ability to up-regulate the expression of enzymes and antioxidant proteins, which in turn change the redox state and maintain the balance in cells [37]. But the available data on the roles of Nrf2 in colon cancer tumorigenesis are not clear and the molecular changes induced by Nrf2 in all stages of colon cancer have not been fully identified.

In this study, we examined the mRNA expression profiles of colonic

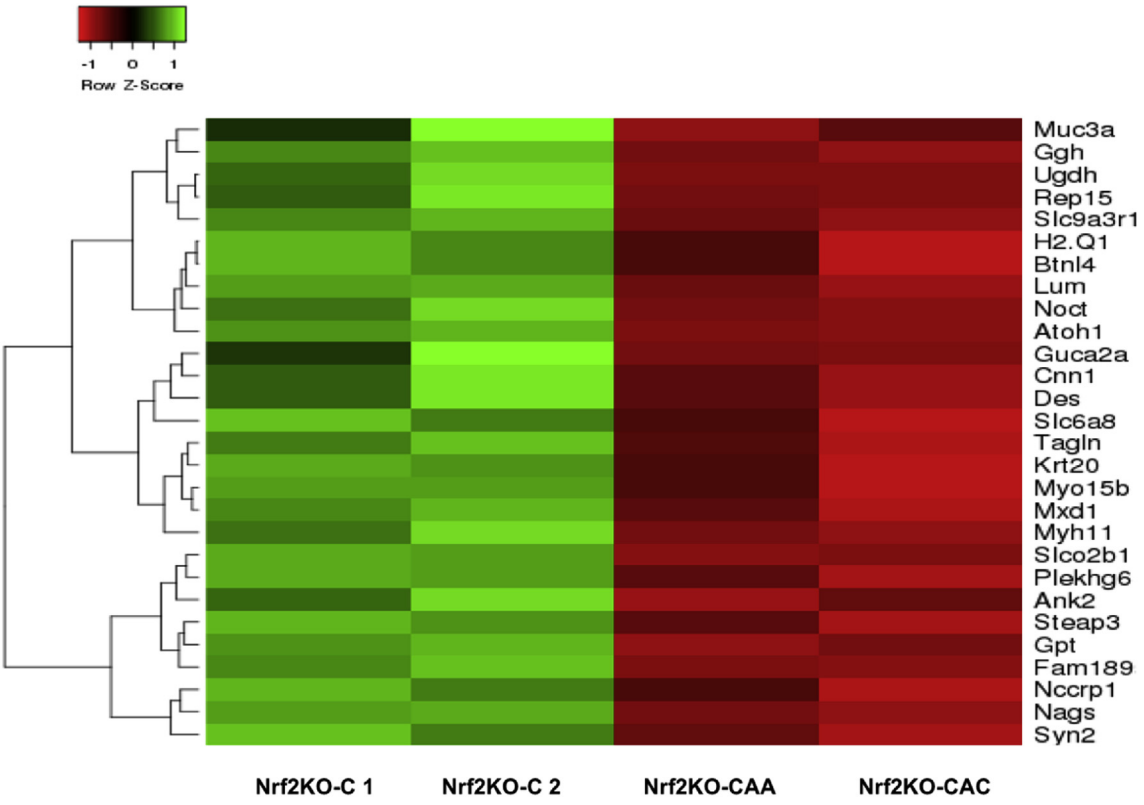

Fig. 3. Heatmap displaying profiles and hierarchical clustering for the expression of 28 genes regulated by Nrf2 in CAA and CAC. Green, relatively higher expression; red, relatively lower expression; scale bar, normalized expression values. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

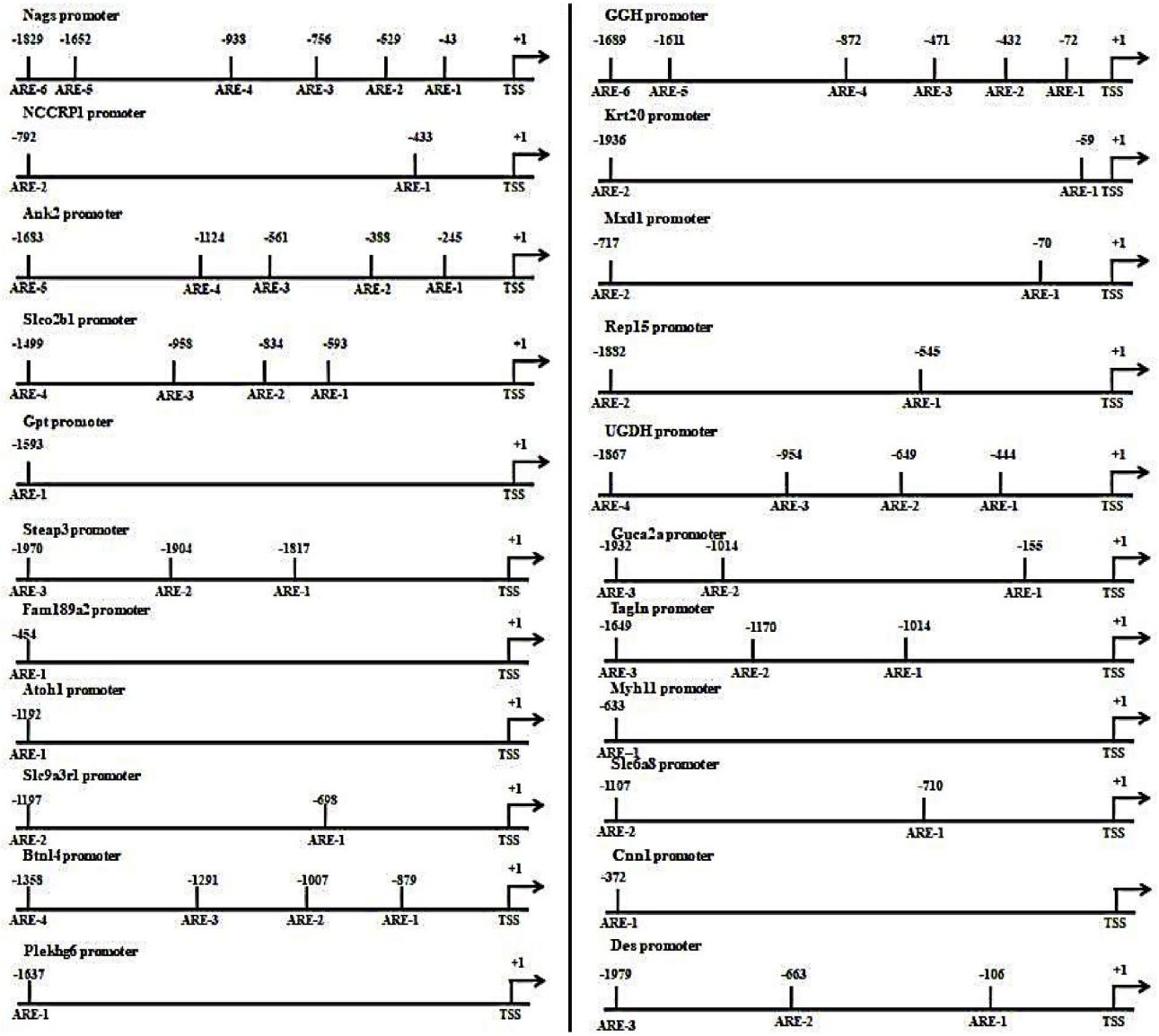

Fig. 4. *In silico* analysis of Nrf2 binding sites. Schematic representations showing the positions of predicted Nrf2 binding sites (AREs) in the promoter regions of 22 genes.

tissues at different tumor stages. To identify the role of Nrf2 in tumorigenesis, we compared the persistent DEGs in wild and *Nrf2*$^{-/-}$ mice. Our results demonstrated that CRC exhibits malignant features such as cell proliferation, tissue remodeling, cytokine-related inflammation, and several cancer-related pathways [38], while losing the physiological functions of normal colorectal tissue such as metabolism of toxins, drug metabolism, and lipid metabolism [5]. Further, our results support the notion that inflammation is a major feature of the tumor microenvironment in CRC [39–41]. We also found that genes that regulate drug metabolism, metabolism of xenobiotics by cytochrome P450, and response to drug, whose associated enzymes oxidize small foreign organic molecules such as toxins or drugs [42], were down-regulated in tumors. These results showed that CRC cells lose the key physiological function of detoxification which could be the result of epithelial cell dedifferentiation [43]. These findings are in keeping with reports that colonic dedifferentiation may initiate tumorigenesis [44].

Metastasis, the most common cause of cancer-associated mortality, is a multi-step phenomenon through which tumors spread from their primary site and form secondary growths at a distance [45]; it's among the hallmarks of cancer, and is a major cause of CRC-associated mortality [6,46]. Dysregulation of cell adhesion and cell migration pathways, such as ECM-receptor interaction, cell-matrix adhesion, and focal adhesion in persistent WT DEGs induce tumor cell migration and metastasis and our results are consistent with previous studies that showed the role of cell adhesion molecules in the progression of various cancer types, including colon, lung, and skin cancers [47,48]. Furthermore, increased ECM remodeling is necessary for tumor expansion, metastasis, and the epithelial-to-mesenchymal transition [49,50]. The absence of these molecular pathways in *Nrf2*$^{-/-}$ mice is evidence that Nrf2 participates in the regulation of these function and these coincide with the previous work in different types of cancer that reported the role of Nrf2 in cancer progression and metastasis [51]. In the *Nrf2*$^{-/-}$ mouse model, most of the common DEGs were annotated in a few GO functions related to inflammatory responses. These data showed that the absence of Nrf2 increases inflammation in the tumor microenvironment and are consistent with previous results from our lab and others on different types of tumor, showing that the elevation of Nrf2 activity or expression protects cancer cells from oxidative stress and inflammation [17,52–54].

It has been reported that Nrf2 target genes are prognostic and diagnostic markers in cancers such as those of the lung, liver, and head and neck [35,52–54], but unfortunately few studies have discussed this

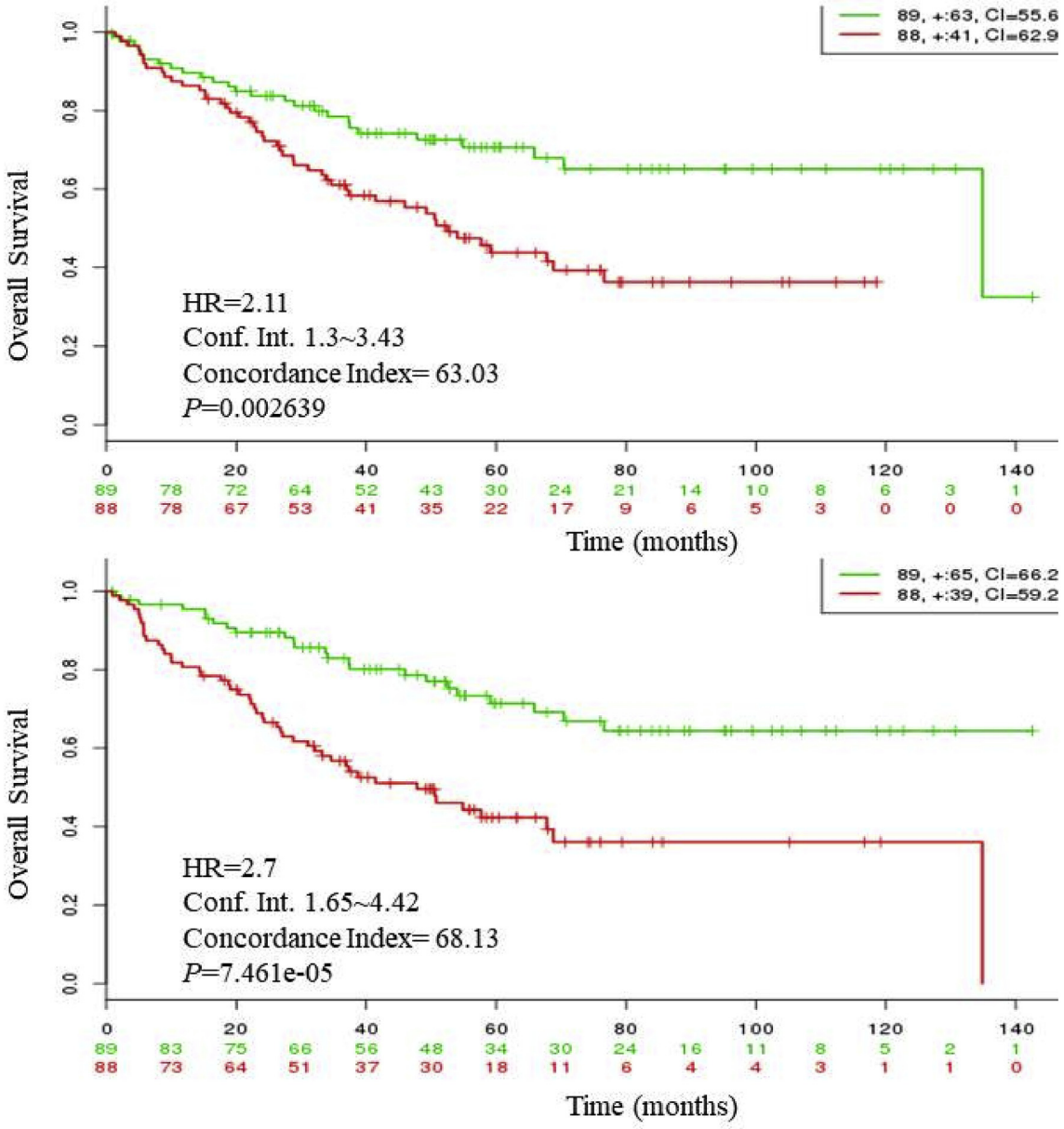

Fig. 5. Correlations between NNRT expression levels and survival in CRC patients. Kaplan-Meier survival plots for the GSE17536 dataset in the SurvExpress database. Red, high-risk curves; green, low-risk curves; insets (upper right), numbers of high- and low-risk samples, number of censored samples (+), and concordance index (CI) of each risk group. X-axis, time (days or months); y-axis, overall survival probability (HR, hazard ratio; Conf. int., confidence interval). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

phenomenon in CRC. Therefore, to extend these findings, here we found that 28 genes regulated by Nrf2 were down-regulated common DEGs in a transgenic mouse model after removing DEGs overlapping with WT mice. We also found that some of these genes have been used as diagnostic biomarkers for CRC and other kinds of tumor. For instance, overexpression of *TAGLN* (transgelin) is associated with the progression of colon cancer and may serve as a new biomarker for predicting the progression and prognosis of CRC [55,56] and lung adenocarcinoma [57]. *Keratin 20*, often abbreviated CK20, is a protein that in humans is encoded by the *KRT20* gene; the *KRT20* expression profile could be a factor in weighing treatment options for CRC patients. In cases where several treatment options are possible, patients with positive postoperative CK20 expression could be candidates for more aggressive treatment [58]. *NCCRP1* (non-specific cytotoxic cell receptor protein 1), abundantly expressed in human squamous epithelium, is involved in cell proliferation and may serve as a promising biomarker to predict the prognosis in squamous cell carcinoma of the esophagus [59]. *SLCO2B1* (solute carrier organic anion transporter family member 2B1) predicts worse disease-free survival in prostate cancer [60]. *STEAP* (six-transmembrane epithelial antigen of prostate 3), a metallo-reductase vital for cellular iron uptake and homeostasis, functions as an oncogenic mediator in the progression of brain tumors and is thus a potential therapeutic target for the treatment of this disease [61]. Moreover, the *CNN1* (calponin 1) and *TAGLN* genes function as potential molecular markers in bladder cancer patients [62]. Besides these facts, we noted that the *UGDH* gene is regulated by Nrf2 and UDP-glucose dehydrogenase (UGDH) is an enzyme that synthesizes UDP-glucuronic acid from glucose; UGDH mRNA was significantly lower in $Nrf2^{-/-}$ mice and higher in Keap1-knockdown mice and this is consistent with previous study [63].

Functional pathways associated with these genes involved cell proliferation and differentiation, metabolic process and pathways,

cancer-related pathways, and oxidation-reduction process. Most of these pathways suggested that Nrf2 induces cancer progression and these genes may be used as diagnostic and prognostic biomarkers. Our results coincide with the previous studies in that some of the 28 genes are already used as diagnostic biomarker [55,58]. We validated these genes in 3 independent cohorts to evaluate the prognostic value in the survival of colon cancer patients and found that higher expression of 23 genes leads to poor survival in CRC patients.

5. Conclusions

Our study demonstrated that CRC initiation and progression is induced by dysregulation of lipid metabolism, inflammation, and immune response pathways. In addition, Nrf2 plays a crucial role in CRC tumorigenesis by remodeling inflammatory, metastatic, metabolic, and drug metabolism components. Moreover, taking the 3 cohorts data into account, we suggest that, of the 28 NNRTs, 22 that have ARE sequences in their promoters are potentially regulated by Nrf2 and can be used in the prognosis of CRC. Functional investigations of the mechanisms associated with these genes are needed.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Abbreviations

CRC	colorectal cancer
Nrf2	nuclear factor erythroid 2-related factor 2
CACC	colitis-associated colorectal cancer
AOM	Azoxymethane
DSS	dextran sulfate sodium salt
CAA	colitis-associated adenoma
CAC	colitis-associated carcinoma
WT	wild-type
KO	Knockout
ROS	Reactive oxygen species
RNA-Seq	RNA sequencing
H&E	hematoxylin and eosin
FPKM	fragments per kilobase sequence per million DEGs differentially-expressed genes
DEGs	differentially-expressed genes
GO	gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
DAVID	The NIH Database for Annotation, Visualization and Integrated Discovery
ECM	extracellular matrix
NNRTs	novel Nrf2-related transcriptomes
ARE	antioxidant response element

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116968.

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