Transcriptome Profiling Unveils GAP43 Regulates ABC Transporters and EIF2 Signaling in Colorectal Cancer Cells

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Abstract

Background

The growth- and plasticity-associated protein-43 (GAP43) is biasedly expressed in indigestive system and nervous system. Recent study has shown that GAP43 is responsible for the development of neuronal growth and axonal regeneration in normal nervous tissue, while serves as a specific biomarker of relapsed or refractory neuroblastoma. However, its expression pattern and function in digestive system cancer still remains to be clarified.

Results

In this study, we found GAP43 was downregulated in colorectal cancer (CRC) compared to the adjacent tissues. DNA methylase inhibitor 5-Aza-CdR treatment could significantly induce GAP43, indicated that the silencing of *GAP43* gene in CRC is closely related to DNA methylation and histone deacetylation. Bisulfite genomic sequencing confirmed the promoter methylation of GAP43 in CRC. To explore the transcriptional alterations by overexpressed GAP43 in CRC, we performed RNA-seq and found that upregulated genes were significantly enriched in the signaling pathways of ABC transporters and ECM-receptor interaction, while downregulated genes were significantly enriched in Ribosome signaling pathway. Further Ingenuity Pathway Analysis (IPA) showed that EIF2 signaling pathway was significantly repressed by overexpression of GAP43.

Conclusion

Our findings provide a novel mechanistic insight of GAP43 in CRC. Transcriptome profiling of overexpressed GAP43 in CRC uncovered the functional roles of GAP43 in the development of human CRC.

Background

Colorectal cancer (CRC) is one of the most common type of the digestive tract cancers and it ranks the third in the world's cancer incidence[1]. CRC is mainly caused by genetic factors, chronic inflammation, unhealthy living habits, etc.; the geographical distribution of the disease is quite different, and the incidence of CRC is higher in developed areas[2]. China is a low-incidence area for CRC, but the incidence rate is increasing[3]. As of 2016, the number of new cases of CRC in China has exceeded 270,000 and more than 130,000 deaths[4]. As the third malignancy in China, previous researches showed that CRCs are caused by various genetic and epigenetic changes, including WNT, PI3K-Akt, GPCR, FGFR, MAPK, TGFB, and TP53 signaling pathways [5–7]. To date, the exact cellular and molecular mechanisms leading to CRC have not been systematically evaluated. Identification of novel markers in CRC is critical for its detection and precision treatment.

GAP43 was reported as a ‘growth’ or ‘plasticity’ protein, which was responsible for the development of neuronal growth and axonal regeneration[8–10]. Previous studies of GAP43 thus far were focused on the neuron biology. However, the role of GAP43 in human cancers, especially CRC, has not been explored to date. Previously, with the next-generation sequencing technologies, we found that the expression level of GAP43 was significantly downregulated in three paired tissues (normal versus cancer tissues) of CRC patients (Data not shown).

Here, we investigated the expression level of GAP43 in CRC cell lines. The mechanism of the silenced GAP43 gene expression in CRC tissues was further studied with the analysis of its promoter methylation. Furthermore, the transcriptome alteration of GAP43-overexpressed versus control CRC cell line was analyzed with RNA-sEq. Taken together, we disclosed the potential functions of GAP43 in the development of human CRC.

Results

GAP43 is expressed in human colon tissues, but not in colorectal cancer cells

According to the RNA-seq data from normal tissues of 95 human individuals[11], *GAP43* gene is biasedly expressed in brain, adrenal, gall bladder, heart, placenta, prostate, salivary gland and a series of indigestive tissues, including duodenum, small intestine, colon and appendix (Fig. 1a). Considering that GAP43 serves as a specific biomarker of relapsed or refractory neuroblastoma, we wonder whether GAP43 functions in development of CRC. To investigate the expression level and mutation of GAP43 in CRC tissues, we analyzed the publicly available databases (cBioPortal, Oncomine, and TCGA). In cBioPortal database, we found that GAP43 was mutated in CRC cancer tissues with the 1% − 3% frequency (Supplementary Fig. S1a), and in Oncomine, compared with normal colorectal tissues, we found that the expression of GAP43 was significantly repressed in CRC tissues (Supplementary Fig. S1b). Further validations were performed with Quantitative real-time PCR (qRT-PCR) in five CRC cell lines HCT116, SW620, SW480, HT29, DLD-1 and two human adjacent non-cancer tissues (Fig. 1b). The results showed that GAP43 was significantly down-regulated in these five CRC cells, compared to normal tissues. Thus, these data indicated that *GAP43* gene was expressed in human normal colon tissues, but significantly down-regulated in CRC.

GAP43 level is repressed in CRC due to aberrant promoter methylation

To investigate the mechanisms involved in the silencing of *GAP43* gene in CRC, we investigated the DNA methylation and mRNA expression data of GAP43 from CRC tissue of the TCGA database. The results showed that the downregulated expression of GAP43 may due to the hyper DNA methylation of its promoter regions in CRC tissues (Supplementary Fig. S2).

To demonstrate that silencing of GAP43 expression in CRC cells is caused by methylation of its promoter, we treated SW620 cells with the DNA demethylating agent 5-Aza-CdR. As shown in Fig. 2a, mRNA levels of GAP43 were significantly increased after the treatment of SW620 cells with high concentrations of 5-Aza-CdR for 72 hours. When SW620 cells were treated with 10 μM 5-Aza-CdR, the mRNA level of GAP43 was significantly increased with the increasing treatment time (Fig. 2b).To further confirm the DNA methylation in GAP43 promoter of CRC cells, we performed bisulfite genomic sequencing to analyze the methylation status of the GAP43 promoter in CRC cell lines SW620 and HCT116. The results showed that in the CRC, multiple CpG islands in the promoter region of the *GAP43* gene were methylated (Fig. 2c). These results show that the downregulation or silencing of GAP43 in CRC is associated with its promoter methylation.

Identification of transcriptome alterations in GAP43 overexpressed CRC cells through RNA-seq

As GAP43 expression is silenced in CRC cells, in order to investigate the transcriptome alterations, we performed RNA-seq on GAP43-overexpressed SW620 cells and control SW620 cells. For transcriptome analysis, we acquired valid RNA-seq data from stable transfected GAP43 (29,592,934 bp and 25,180,042 bp) and control (28,506,301 bp and 27,436,701 bp) SW620 cells with Q30 > 90%, respectively (Supplementary Table S1). For more accurate results, the RNA-seq data was filtered with estimated FPKM values less than 1.0. Ultimately, with statistical analysis (Volcano plot), we found that the transcriptome of stable transfected GAP43 were significantly different from control SW620 cells (Fig. 3a). Totally 1,056 differentially expressed genes (DEGs) were identified in stable transfected GAP43 compared with control SW620 cells. Among these genes, 474 genes were upregulated and 582 genes were downregulated, including upregulated FILP1L (WNT suppressor)[12], downregulated KRT6A (wound healing and epithelial migration)[13] and S100A14 (cell proliferation and cell migration)[14–17], et al. (Supplementary Table S2) (p-value < 0.05). The heatmap for some significantly altered genes by overexpressed GAP43 was shown in Fig. 3b. According to cellular functional categorization, significantly upregulated genes were classified as follows: transport (p-value = 2.60*E-04); glucose homeostasis (p-value = 5.51*E-04); retinoid metabolic process (p-value = 8.78*E-04); peripheral nervous system development (p-value = 8.93*E-04); extracellular matrix organization (p-value = 1.05*E-03). The significantly downregulated genes were classified as follows: translation (p-value = 8.02*E-56); SRP-dependent cotranslational protein, targeting to membrane (p-value = 2.98*E-54); nuclear-transcribed mRNA catabolic process, nonsense-mediated decay (p-value = 3.62*E-50); rRNA processing (p-value = 1.92*E-49); translational initiation (p-value = 7.24*E-49) (Supplementary Table S3).

Transcriptome analyses of GAP43-overexpressed CRC cells through DAVID and IPA

After bioinformatics analysis of RNA-seq data, the upregulated or downregulated genes in GAP43 stable transfected SW620 cells were summarized. We submitted these genes to DAVID (a web-based high-

throughput functional genomics analysis tool) for systematically clustering these genes. In GOTERM_BP_FAT category, we identified significantly enriched GO terms that characterized GAP43 stable transfected SW620 cells. In total, 24 subcategories (p-value < 0.01) were found among upregulated genes and 38 subcategories (p-value < 0.01) among downregulated genes (Supplementary Table S3). Among the up-regulated genes, we found that cell membrane proteins were significantly enriched, such as transport (p-value = 2.60*E-04) and extracellular matrix organization (p-value = 0.001); while among down-regulated genes, protein translation related genes were significantly enriched. In KEGG_PATHWAY category, we found that the upregulated genes were clustered into 6 subcategories (p-value < 0.05, 1 subcategories with p-value < 0.01), including ABC transporters (p-value = 0.001) and ECM-receptor interaction (p-value = 0.019) (Fig. 4a). While the down-regulated genes were clustered into 12 subcategories (p-value < 0.05, 10 subcategories with p-value < 0.01) (Fig. 4b), including ribosome (p-value = 2.98*E-62); biosynthesis of antibiotics (p-value = 6.87*E-08); biosynthesis of amino acids (p-value = 3.11*E-05); arginine and proline metabolism (p-value = 1.35*E-04); metabolic pathways (p-value = 1.61*E-04) (Supplementary Table S4). These results showed that overexpressed GAP43 may influence the membrane transporters, and then repress the cancer cells' metabolism and cause the repression of proliferation, invasion and migration effects in CRC cells.

The expression levels of the most significant DEGs in RNA-seq data were confirmed by qRT-PCR in GAP43-overexpressed and control SW620 cells (Fig. 5). Furthermore, we applied these DEGs into IPA analysis, and we found that the most significant is the EIF2 signaling pathway (-log(p-value) = 25.5 and z-score=-4.802) (Fig. 6a) [18, 19]. In EIF2 signaling pathway, the translation initiation factor (eIF1) and transcriptional factor (ATF4 and ATF5) were significantly downregulated (Fig. 6b). These results suggested that except the function on membrane, GAP43 may also involve in the regulation of cellular translation through the EIF2 signaling pathway.

Discussion

In previous studies, the functional studies of GAP43 were focused on the neuronal development, and the protein was considered to play crucial roles in effective regenerative responses, and learning and memory processes of the nervous system[20–22]. GAP43 is highly expressed in nervous system, and also biasedly expressed in indigestive tissues, including duodenum, small intestine, colon and appendix. Recent study has shown that GAP43 serves as a specific biomarker of relapsed or refractory neuroblastoma[23, 24]. However, the relationship between GAP43 and CRC development has been uncovered. In this study, we confirmed that GAP43 is expressed in human colon tissues, but is downregulated in CRC cells and tissues due to the methylation of its promoter region.

Further transcriptome analysis indicated multiple potential biological functions of GAP43 in CRC. The most significant different expression gene is upregulated FILIP1L, downregulated KRT6A and S100A14. While FILIP1L is a WNT pathway inhibitor[12], and the reduction of FILIP1L in ovarian cancer is associated with poor survival, progression and chemoresistance[25]. Previous studies showed that KRT6A and S100A14 were involved in cell migration and cell proliferation[13, 14, 17]. These results

remind us that the poor prognosis of reduced GAP43 in CRC may due to its transcriptional alterations and the regulation of some potential signaling pathways, such as WNT signaling.

In KEGG_PATHWAY category, we found that the upregulated genes were clustered into 6 subcategories including ATP-binding cassette (ABC) transporters ($p = 0.001$). ABC transporters constitute a superfamily of membrane proteins that couple the hydrolysis of adenosine triphosphate (ATP) to vectorial transport of substrates across biological membranes[26]. Previous studies have showed that ABC superfamily utilizes the free-energy from ATP hydrolysis to shuttle many different substrates across various biological membranes, and consequently, are involved in both normal and abnormal physiology[27].In this study, we found that with the overexpression of GAP43 in CRC cells, the expression of ABC transporters is also upregulated. However, the expression of bothGAP43 and ABC transporters is down-regulated in CRC tissues and cell lines, which indicates that ABC transporters might be related with the development of CRC.

In addition, we analyzed the transcriptome of GAP43 overexpressed CRC cells through DAVID and IPA. The IPA analysis showed that EIF2 signaling pathway is the most significant psychological phenomena (-log(*p*-value) = 25.5 and z-score=-4.802) (Fig. 6a), and three important members(eIF1, ATF4, ATF5) of EIF2 signaling pathway were significantly downregulated (Fig. 6b). Incidentally, we also found that GAP43 is differentially expressed, elevated or decreased in gastric cancer. Survival analysis showed that there was no significant change in survival of gastric cancer patients with low or high expression of GAP43 (Fig. S3). However, we found that patients with gastric cancer who were treated with the 5-FU anticancer drug had higher survival rates in patients with high GAP43 expression than those with low expression (Fig. S3). This suggests that GAP43 is likely to be associated with drug resistance to cancer chemotherapy drugs. Considering that the tolerance of chemotherapy drugs is related with ABC transporters, and GAP43 level is associated with the expression of ABC transporters. Therefore, GAP43 might affect the chemotherapy drug resistance in cancer patients through ABC transporter-mediated signaling pathway.

Conclusions

GAP43 is expressed in human indigestive tissues, including colon tissues, but is downregulated in CRC due to the methylation of its promoter region. The RNA-Seq transcriptome data analysis showed the gene expression profile between GAP43-overexpressed and control CRC cells. The analysis of GAP43-overexpressed versus control CRC cell line data gives insight into potential genes and pathways that may uncover the functional roles of GAP43 in the development of human CRC and expand the knowledge of the CRC progression.

Methods

Cell Culture and Transfection

All cell lines used in this study were originally purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The CRC cell lines HCT116, SW620, SW480, HT29, DLD-1 were cultured in RPM1640 medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were transiently transfected with the indicated plasmids using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-Time PCR

Quantification of mRNA levels was performed in 20 ml of a mixture consisting of 10 μl of 2 × SYBR Green Mix, 0.2 ml of RT product, 1 ml of a primer set mixture, each primer having a concentration of 5 pmol/ml, and 8.8 ml of sterile water. As an internal control, in addition to the primers, GAPDH mRNA was carried out under the same conditions. The experiment was performed on an ABI 7500 real-time PCR system (Foster City, CA, USA), and the PCR procedure was recommended according to the protocol (1 cycle at 10 °C for 95 minutes, 10 seconds at 95 °C, 34 seconds at 60 °C and 40 cycles at 72 °C). 30 seconds, 1 cycle for 10 minutes at 72 °C). Primers for qRT-PCR are described in Supplementary Table S6.

Bisulfite DNA Sequencing

Bisulfite DNA sequencing was performed as previously described[28]. Briefly, the bisulfite modification of DNA was performed according to the protocol of the Applied Biosystems methylated SEQr bisulfite conversion kit. The bisulfite-treated DNA was amplified using nested PCR using the primers described in Table S1. The amplified bisulfite-treated DNA was sequenced and compared to the original sequence of the GAP43 gene.

RNA-seq Library Preparation and Sequencing

Total RNA was isolated from 4×10^6 GAP43 overexpressed and control SW620 cells using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA integrity was checked using an Agilent Technologies 2100 Bioanalyzer with an RNA integrity number (RIN) value greater than 8. The mRNA was purified and fragmented from total RNA (2 μg) using poly-T oligo-attached magnetic beads with two rounds of purification. Cleaved RNA fragments primed with random hexamers were reverse transcribed into first-strand cDNA using reverse transcriptase and random primers. The RNA template was removed and synthesized a replacement strand to generate double-strand cDNA. End repair, A-tailing, adaptor ligation, cDNA template purification and enrichment of the purified cDNA templates using PCR were then performed. Constructed libraries were 150 bp paired-end sequenced by an Illumina X10 sequencer, following manufacturer’s instructions.

DAVID and IPA

Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov), Functional Annotation Bioinformatics Microarray Analysis was used to identify significantly enriched gene ontology(GO) and Kyoto Encyclopedia of Genes and Genomes(KEGG) terms among the given list of genes that are differentially expressed in response to curcumin. Statistically overrepresented GO and KEGG categories with p-value ≤ 0.05 were considered significant. For Ingenuity Pathway Analysis (IPA),

DEGs were submitted to IPA software (Qiagen bioinformatics, Ingenuity Pathway Analysis), the canonical pathways were considered significant with p-value ≤ 0.05 and z-score ≥ 1 or z-score≤-1.

Statistical Analysis

Statistical data were represented as mean ± standard deviation from at least three independent experiments performed in triplicate. Gene expression were statistically analyzed using repeated measures two-way ANOVA.

Declarations

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figures

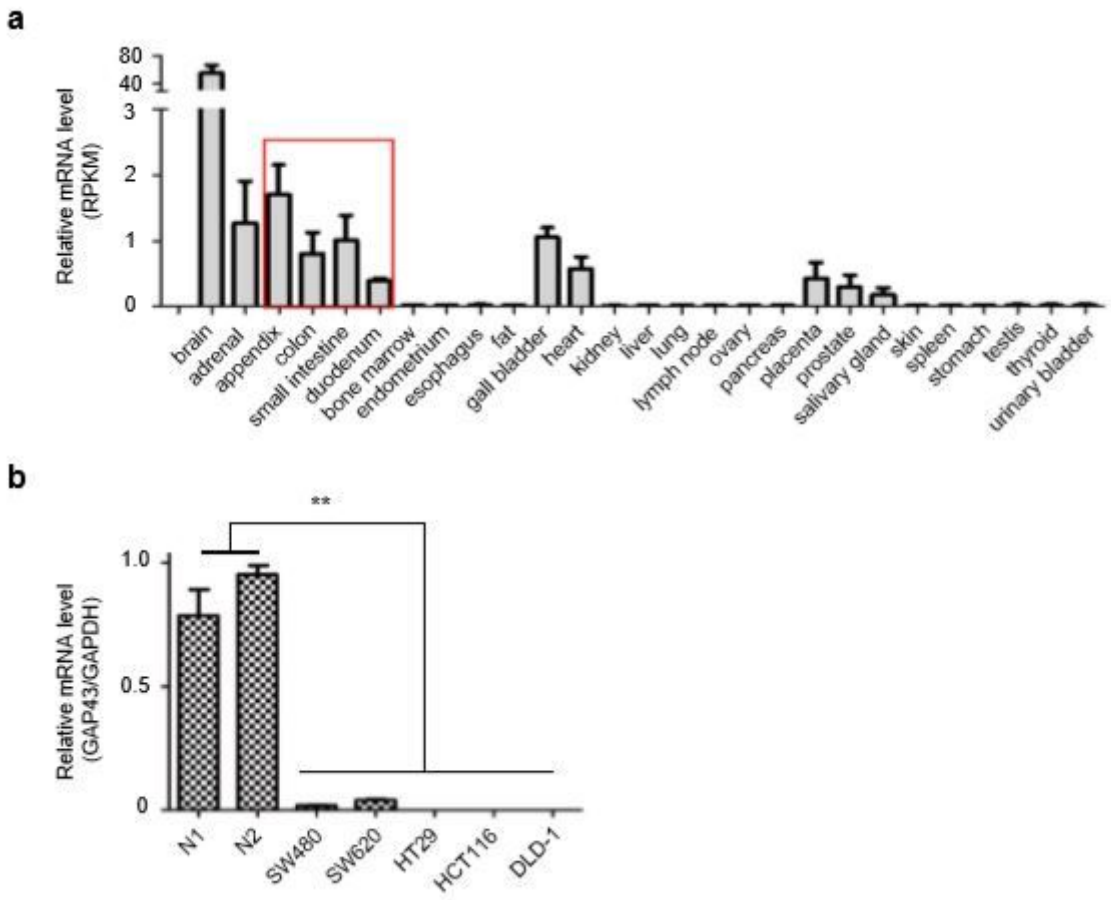

Figure 1

GAP43 expression levels are detected in normal human tissues and colorectal cancer cell lines. a Relative mRNA levels are analyzed, according to the RNA-seq data from normal tissues of 95 human individuals. b qRT-PCR was performed in five CRC cell lines HCT116, SW620, SW480, HT29, DLD-1and 2 human

adjacent non-cancer tissues. The relative mRNA levels are analyzed. The results represent at least three independent experiments and are reported as mean ± SEM. *p<0.05.

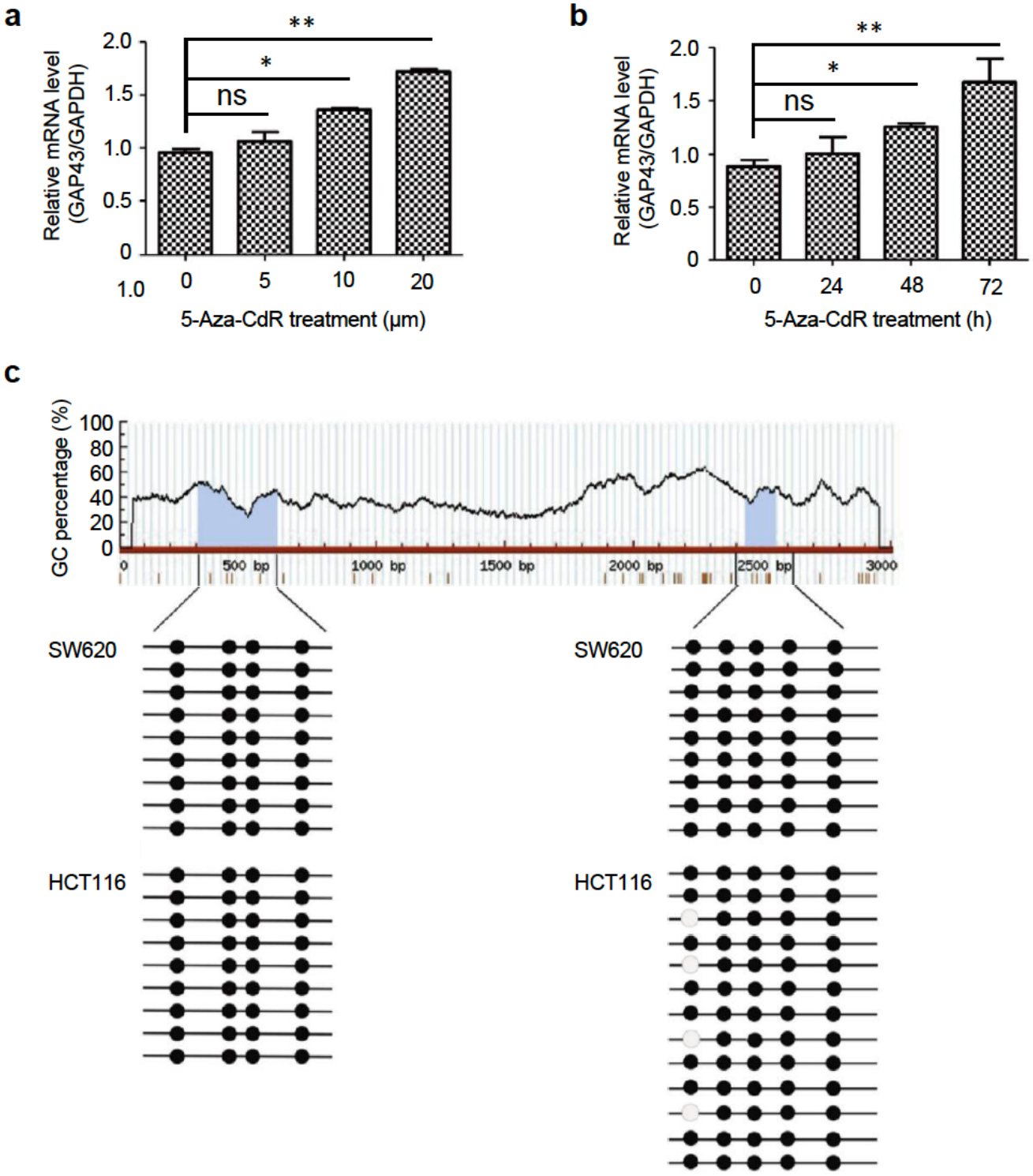

Figure 2

Silencing of GAP43 gene is due to aberrant promoter methylation. a SW620 cells were treated with the indicated concentration of DNA demethylating agent 5-Aza-CdRfor 72 hours, or b SW620 cells were treated with 10 μM 5-Aza-CdR for indicated treatment time (0, 24, 48, 72 h), and then mRNA levels of

GAP43 were analyzed by qRT-PCR. c Bisulfite genomic sequencing was performed to analyze the methylation status of the GAP43 promoter in CRC cell lines SW620 and HCT116. Multiple CpG islands in the promoter region of the GAP43 gene were analyzed. The results represent at least three independent experiments and are reported as mean ± SEM. *p<0.05, **p<0.001.

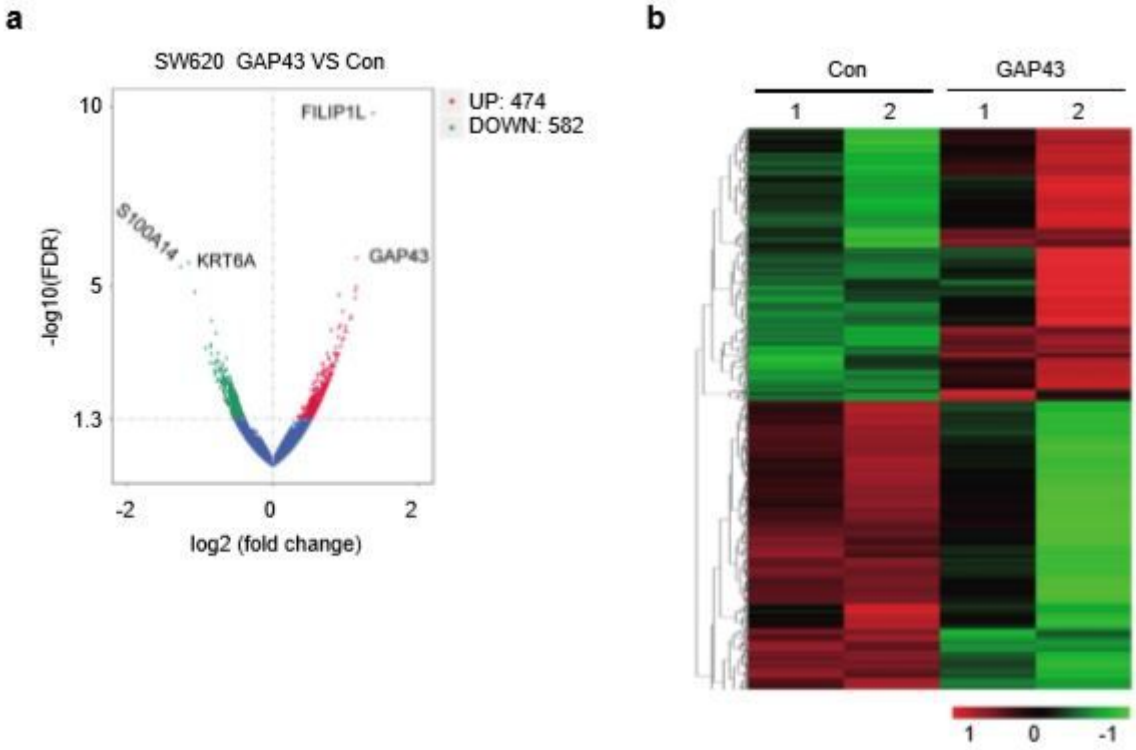

Figure 3

Heatmap and volcano plot. a The volcano plot was constructed by plotting the negative log of the log10 FDR value on the y-axis. These results in data points with low log10 FDR values (highly significant) appearing toward the top of the plot. The x-axis is the log(fold change) between GAP43-overexpressed and control SW620 cells. b The heatmap of up- and down-regulated genes in red and green, respectively.

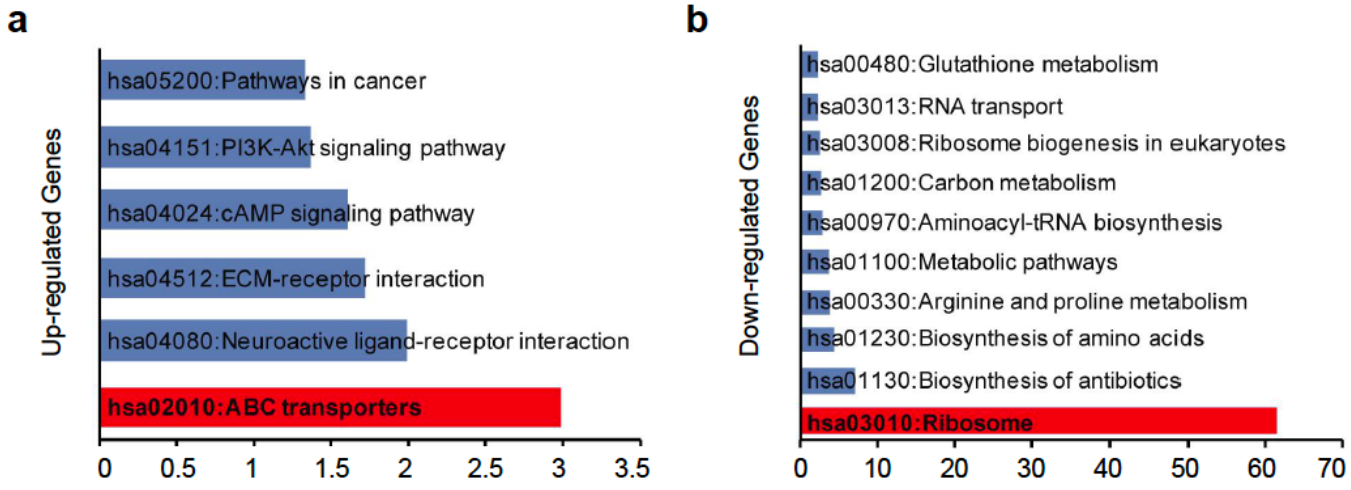

Figure 4

Functional pathway enrichment analysis. a The upregulated DEGs or b down-regulated DEGs are involved in various KEGG biological pathways.

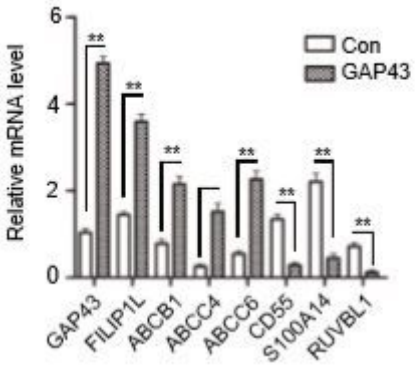

Figure 5

The expression levels of the most significant DEGs in RNA-seq data were confirmed by qRT-PCR in GAP43-overexpressed and control SW620 cells.

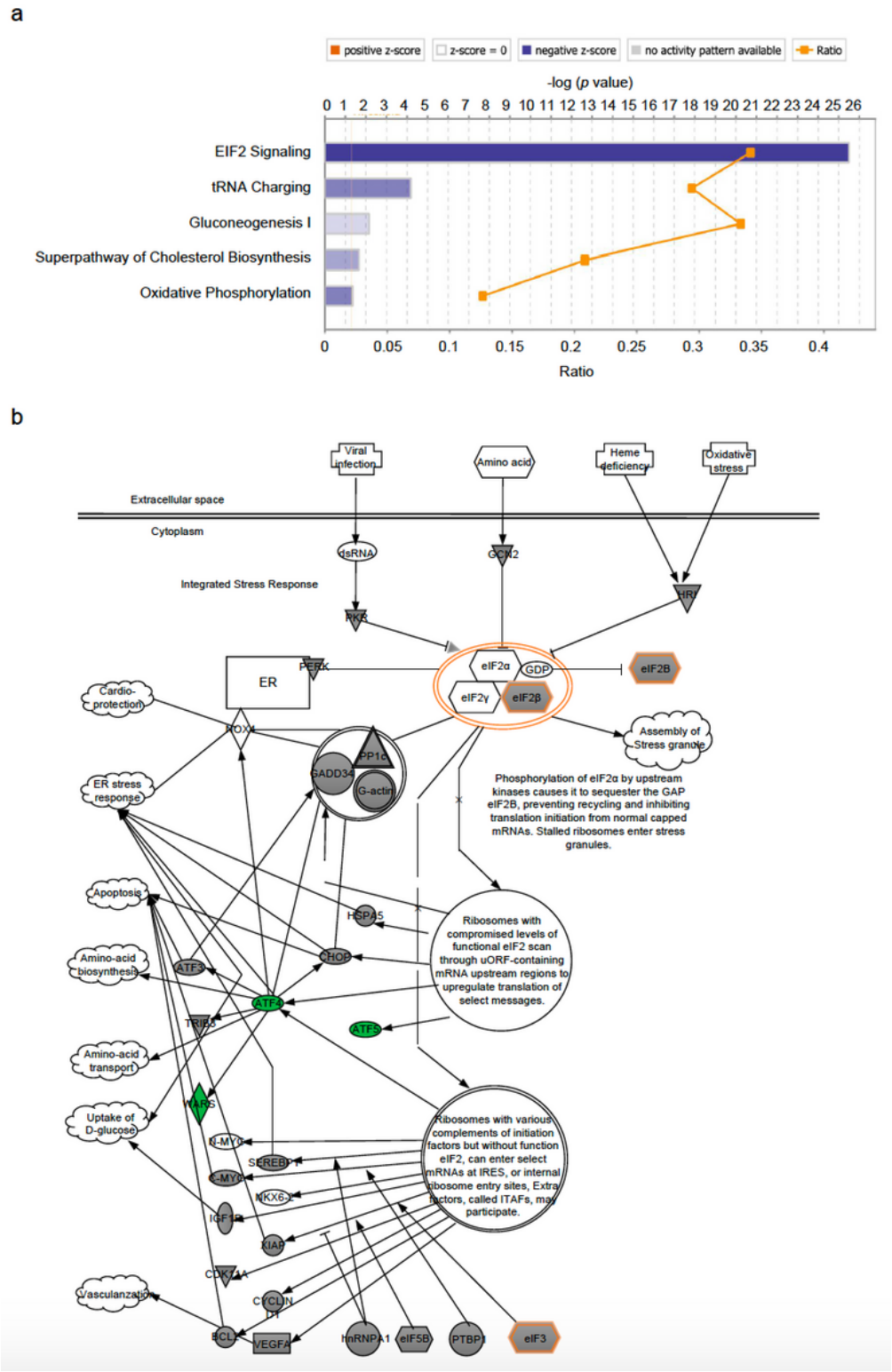

Figure 6

IPA analysis and EIF2 signaling pathway. a IPA analysis. b EIF2 signaling pathway

Supplementary Files

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- FigureS3.pdf
- SupplementaryTableS1.xlsx
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- SupplementaryTableS4.xlsx
- SupplementaryTableS5.xlsx
- SupplementaryTableS6.xlsx