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Original Article

Integrative bioinformatics analysis of ACS enzymes as candidate prognostic and diagnostic biomarkers in colon adenocarcinoma

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Abstract

Background and purpose: Acyl-CoA synthetase (ACS) enzymes play an important role in the activation of fatty acids. While many studies have found correlations between the expression levels of ACS enzymes with the progression, growth, and survival of cancer cells, their role and expression patterns in colon adenocarcinoma are still greatly unknown and demand further investigation.
Experimental approach: The expression data of colon adenocarcinoma samples were downloaded from the Cancer Genome Atlas (TCGA) database. Normalization and differential expression analysis were performed to identify differentially expressed genes (DEGs). Gene set enrichment analysis was applied to identify top enriched genes from ACS enzymes in cancer samples. Gene ontology and protein-protein interaction analyses were performed for the prediction of molecular functions and interactions. Survival analysis and receiver operating characteristic test (ROC) were performed to find potential prognostic and diagnostic biomarkers.
Findings/Results: ACSL6 and ACSM5 genes demonstrated more significant differential expression and LogFC value compared to other ACS enzymes and also achieved the highest enrichment scores. Gene ontology analysis predicted the involvement of top DEGs in fatty acids metabolism, while protein-protein interaction network analysis presented strong interactions between ACSLs, ACSSs, ACSMs, and ACSBG enzymes with each other. Survival analysis suggested ACSM3 and ACSM5 as potential prognostic biomarkers, while the ROC test predicted stronger diagnostic potential for ACSM5, ACSS2, and ACSF2 genes.
Conclusion and implications: Our findings revealed the expression patterns, prognostic, and diagnostic biomarker potential of ACS enzymes in colon adenocarcinoma. ACSM3, ACSM5, ACSS2, and ACSF2 genes are suggested as possible prognostic and diagnostic biomarkers.

Keywords: Acyl-CoA synthase; Cancer; Colon adenocarcinoma; Colon cancer; Fatty acid activation.

INTRODUCTION

Colorectal cancer (CRC) is a type of tumor that occurs in the gastrointestinal region and has been ranked as one of the top causes of cancer-related global deaths over recent decades (1,2). Colon adenocarcinoma (COAD) is one of the common forms of CRC that form due to abnormal growth of the epithelial cells covering the inner surface of the intestine (3,4). Multiple risk factors are linked to the formation of CRC, such as geographic factors, nutritional practices, and inherited genetic profiles (5). CRC develops through the gradual accumulation of mutations, epigenetic alternations, and reprogramming in different metabolic pathways (5,6).

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Metabolism of lipid molecules has been seen to get deregulated in multiple types of cancers and promote tumor development (7).

Recent studies have largely noticed the significant role of fatty acids (FAs) in the progression and development of different forms of cancers (8-11). The expression levels of different enzymes involved in the metabolism of FAs, such as FA synthase and acyl-CoA synthetase long-chain-FA-CoA ligase 1 (ACSL1), have been suggested to influence the survival, invasion, and metastasis of CRC cells and play a significant role in carcinogenesis of colorectal cancer (12-15). CRC patients with higher tumor stages have shown higher serum levels of FA synthase enzyme (12). FAs metabolism includes different stages, such as FA uptake, *de novo* synthesis of FAs, FA activation, and their incorporation into the synthesis of other lipid molecules, such as phosphor lipids and triglycerides (10,16).

While a great majority of research has focused on the importance of FA synthesis and oxidation in a variety of cancers, less attention has been paid to clarifying the role of FA activation in the biological pathways of tumor cells (16,17). FAs need to get activated before entering other metabolic reactions, such as fatty acid oxidation (18). Activation of FAs occurs by ligation of FAs with CoA molecules through an ATP-dependent mechanism catalyzed by ACS enzymes (19,20). ACS enzymes get classified into different subtypes based on the carbon length of their FA substrate, such as ACSL, ACS medium-chain (ACSM), and ACS short-chain (ACSS) enzymes that activate long-chain FAs (LCFAs), medium-chain FAs (MCFAs), and short chain FAs (SCFAs), respectively (21,22). Multiple studies have shown that altered expression levels of ACS enzymes were associated with the growth, survival, and progression of cancer cells (23-26). Therefore, ACS enzymes could be considered important therapeutic targets in the treatment of different cancers, such as ACSS2 and ACSS3 enzymes their functions have been associated with the regulation of tumor metabolism under metabolic stress and hypoxic conditions (23,26,27).

In this study, we have employed differential gene expression and gene set enrichment analysis (GSEA) techniques to clarify the expression patterns of 17 different members of ACS enzymes in the expression data of TCGA COAD samples. To better understand the biological functions and different interactions of ACS enzymes with other proteins, the gene ontology (GO) and protein-protein interaction (PPI) network of ACS enzymes with top differentially expressed genes (DEGs) were analyzed. Finally, to specify the best diagnostic and prognostic biomarkers among ACS enzymes in COAD, receiver operating characteristic and survival analyses were performed.

MATERIALS AND METHODS

Identification of DEGs

The gene expression data (RNAseq) of the COAD project was downloaded from the TCGA dataset (https://portal.gdc.cancer.gov/) with the help of the "TCGAbiolinks "package and as previously described by us (28). This data was analyzed according to the Declaration of Helsinki and TCGA guidance for data access and utilization. The expression data were analyzed after data preparation by the Limma package using the R program. Normalization of count data was performed using the Voom normalization method. Normalized count data of all samples were converted into Logarithmic form (Log2 ratio) and differential expression analysis was performed using TCGAbiolinks, and edgeR packages to identify DEGs in COAD cancer samples compared to normal samples.

GSEA

GSEA analysis is a useful method that enables users to measure the degree of correlation of the expression levels of an interesting set of genes that are all involved in a specific biological pathway in an interesting disease. Gene set analysis (GSA) is also known as pathway analysis as it considers a group of genes involved in a specific pathway together. In this study, a list of genes that are predicted to be involved in the activation and synthesis of Acyl-CoA molecules were selected from the HGNC database (http://www.genenames.org) for GSEA analysis (Liberzon *et al*. 2011;

Bruford *et al*. 2007; Subramanian *et al*. 2005). Normalized expression data of COAD TCGA samples were selected as an expression matrix. Two "cancer" and "normal" phenotypes were defined for each sample based on the histopathological and clinical data provided in the TCGA database. The GSEA software (version 4.0.3) from BOARD institute was used for this analysis and the t-test method was chosen for the ranking method of the gene list and the rest of the parameters were left as the default setting of the software. GSEA analysis measures the correlation between the expression level of each gene within the gene set in all samples regard to the defined phenotypes and reports a ranking metric score based on the degree of correlation, which represents the degree of enrichment of each gene within the gene set.

GO and PPI analysis

To better understand the biological functions and PPI of selected members of ACS enzymes along the top 100 significantly differentially expressed genes in COAD, gene ontology (GO) function enrichment analysis was performed using the online DAVID database (https://david.ncifcrf.gov/), as well as KEGG pathway enrichment analysis to highlight important pathways that are most predicted to be related with COAD. To identify the interacting proteins, the STRING database (http://www.string-db.org/) was used first, which gives a protein interaction score for each gene in the set. Then, Cytoscape software (version 3.9.1) was used to build a PPI network based on the predicted interaction scores and the basic features and properties of the PPI network were estimated using the network analyzer option in the software. GO analysis for biological processes, molecular functions, and cellular components was also performed for the ACS enzymes using the "ClusterProfiler"," AnnotationDbi", and "org.Hs.eg.db" packages in the R program.

Survival analysis

The Kaplan-Meier method was used for survival analysis using the survival package in the R program (version 3.6.3) and Kaplan-Meier plots were generated for all selected members of ACS enzymes and the t-test was performed to measure the significance between the expression level of each gene among cancer samples and the survival period of each patient. Samples were divided into two 'high' and 'low' groups based on a cut-off value, which was defined based on the median expression level of each gene among the samples. Genes with a two-sided *P*-value smaller than 0.05 were considered statistically significant.

Receiver operating characteristic test

One of the common tests used for the prediction of the diagnostic potential and performance of specific genes based on their expression level in cancer and normal samples is the receiver operating characteristic (ROC) test, which was performed for 17 different members of ACS-enzymes based on normalized gene expression data of COAD cancer and normal samples. Genes with a higher area under the curve (AUC) and smaller *P*-values are predicted to perform better as diagnostic biomarkers.

Statistical analysis

The expression data (RNAseq data in raw count format) of TCGA COAD samples were normalized employing the Voom function in the Limma package using the RStudio program (version 4.1.0) and were used for differential gene expression analysis with the help of TCGAbiolinks and edgeR packages. Survival analysis was performed using the survival package in the R program. GraphPad Prism software (version 8.4) was used for ROC analysis and generation of ROC graphs.

RESULTS

Top DEGs in COAD

Expression data of COAD and normal tissue samples were normalized and subjected to differential expression analysis to clarify the difference in expression patterns of different members of ACS enzymes. The expression levels of all ACS enzymes in tumor samples were significantly different from normal samples (Fig.1). Among different ACS enzymes, 7 genes were upregulated in tumor samples including ACSL1 (Log2 fold change

(FC) = 0.30, *P*-value = 0.04), ACSL3 (Log2 FC = 0.15, *P*-value = 0.09), ACSL4 (Log2 FC = 0.85, *P*-value = $6.26e^{-10}$), ACSL6 (Log2 FC = 2.72, *P*-value = $2.01e^{-08}$), ACSS1 (Log2 FC = 0.26, *P*-value = 0.06), ACS bubblegum family member 1 (ACSBG2; Log2 FC = 0.58, *P*-value = 0.005), and acetoacetyl-CoA synthetase (AACS; Log2 FC = 0.14, *P*-value = 0.08), while 10 genes were down regulated in tumor samples compared to normal samples, including ACSL5 (Log2 FC = -0.41, *P*-value = 0.001), ACSM1 (Log2 FC = -1.21, *P*-value = 2.66e-08), ACSM3 (Log2FC= -1.04, *P*-value= 1.54e-08), ACSM4 (Log2FC= -1.30, *P*-value= $7.54e^{-08}$), ACSM5 (Log2 FC = -3.13, *P*-value= $9.26e^{-32}$), ACSS2 (Log2 FC = -1.106, *P*-value = $2.15e^{-20}$), ACSS3 (Log2 FC = -1.02, *P*-value= $3.16e^{-06}$), ACSBG1 (Log2 FC = -0.92, *P*-value= $2.16e^{-09}$), ACS family member 2 (ACSF2; Log2 FC = -1.65, *P*-value= $5.49e^{-26}$), ACSF3 (Log2 FC = -0.16, *P*-value = 0.05) enzymes (Fig. 2A-C). ACSL6 and ACSM5 genes had the highest and lowest Log2 FC values, respectively among the rest of the ACS enzymes. Among different members of ACS enzymes that were analyzed, the differential expression levels of ACSL3, ACSS1, and AACS genes were not significant in COAD samples compared to normal samples.

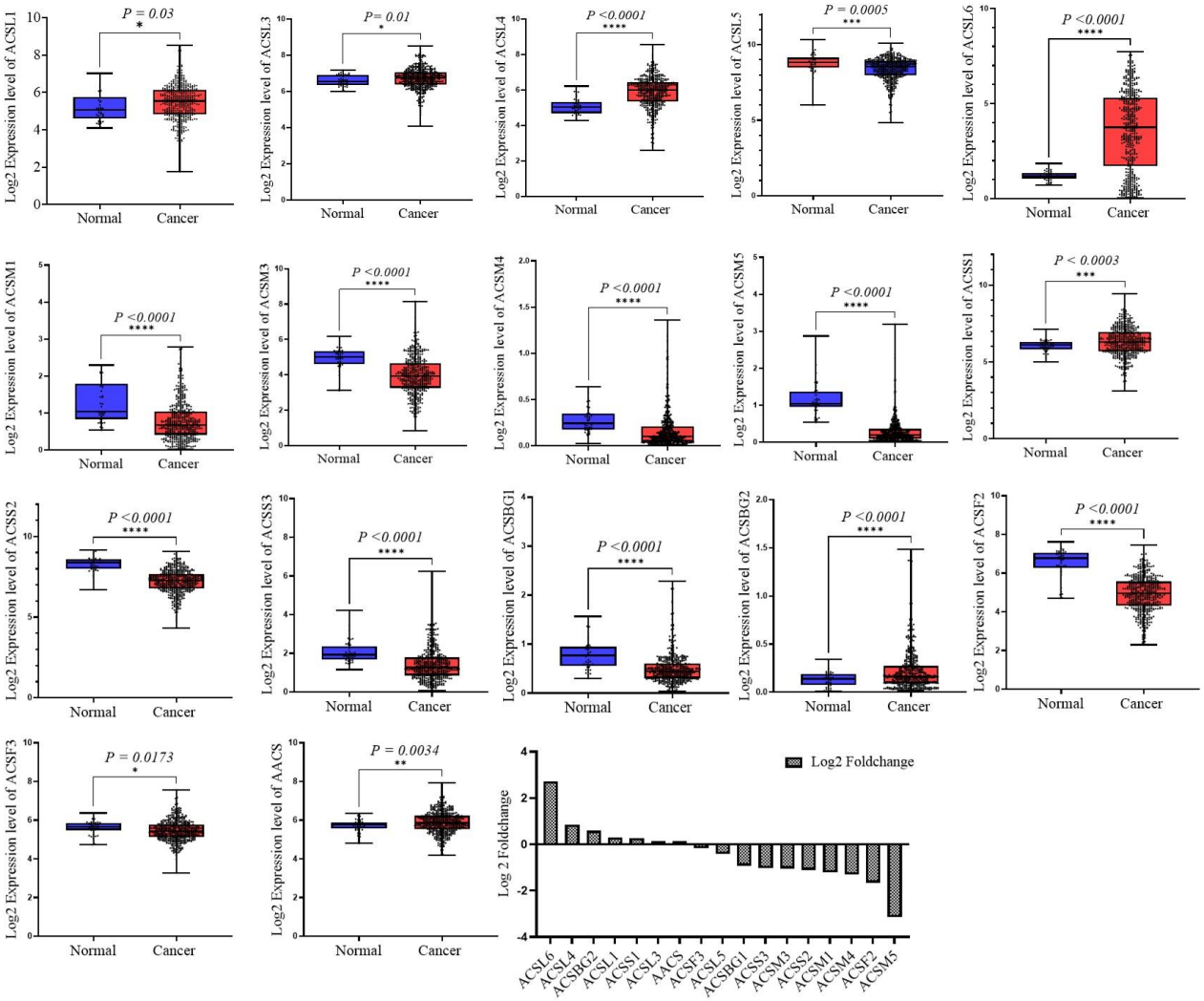

Fig. 1. The Log2 of expression level and Log2 foldchange of 17 members of ACS enzymes in colon adenocarcinoma cancer tissues. Most of the ACS enzymes demonstrated significant differences in their expression levels in cancer tissues compared with control tissue samples. ACSL6 and ACSM5 enzymes revealed the highest and lowest Log2 foldchange values respectively. P-values ≤0.05 were considered statistically significant. ACS, Acyl-CoA synthetase; ACSS, ACS short-chain-fatty acid-CoA ligase; ACSM, ACS medium-chain-fatty acid-CoA ligase 1; ACSL, ACS long-chain-fatty acid-CoA ligase.

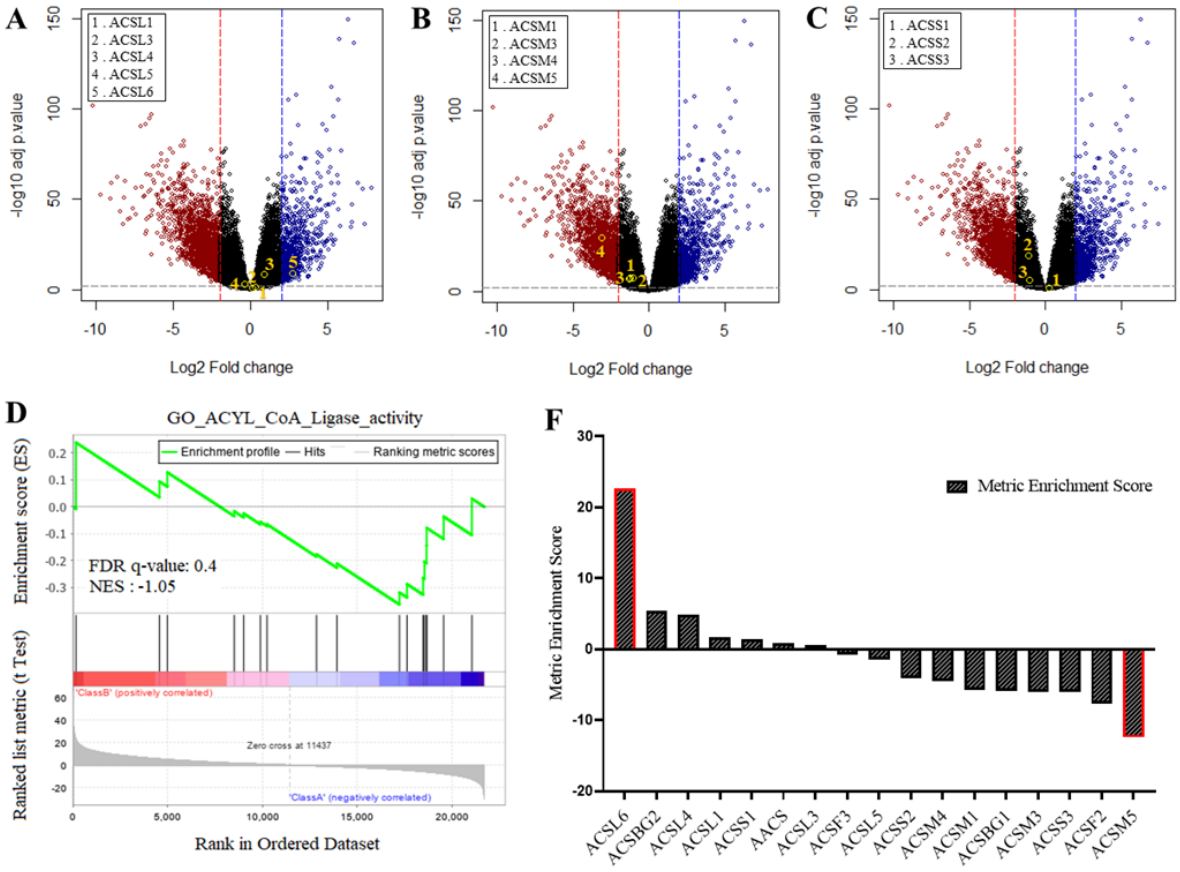

Fig. 2. Differential gene expression and GSEA analyses of ACS enzymes in COAD cancer. (A-C) scatter plots show the log2 foldchange of differentially expressed genes in COAD cancer, and the members of ACSL, ACSM, and ACSS genes are marked with yellow circles. (D-F) demonstrate the GSEA enrichment results of the ACS gene. GSEA, gene set enrichment analysis; COAD, colon adenocarcinoma; ACS, Acyl-CoA synthetase; ACSS, ACS short-chain-fatty acid-CoA ligase; ACSM, ACS medium-chain-fatty acid-CoA ligase 1; ACSL, ACS long-chain-fatty acid-CoA ligase.

GSEA of ACS enzymes

GSEA analysis is based on the Kolmogorov Smirnov statistical test, which can detect general differences in the form of cumulative distribution. The enrichment score predicted by this test is based on the different expression levels of each member of the gene set in the two defined phenotypes, which considers an adjusted *P*-value during its calculations and reports a false discovery rate at the same time. Most of the permutation analytical methods lack an appropriate adjustment concerning the number of gene sets being tested and a standard multiple-hypothesis test would be needed to make the GSEA results more reliable. Therefore, a false discovery rate is also reported along the enrichment score to correct the false positive predictions. GSEA analysis was performed using a gene set containing 17 different members of ACS enzymes on the expression matrix of COAD and normal tissue samples. The gene set was predicted to be upregulated in samples with normal phenotype compared to tumor samples with an enrichment score of -0.362, normalized enrichment score of -1.057, and false discovery rate q-value of 0.4 (Fig. 2D). Metric enrichment scores of ACS enzymes revealed that ACSL6 (rank metric score = 22.63) and ACSM5 (rank metric score = -12.43) had the highest positive and negative enrichment scores, respectively. The enrichment score is used to rank each gene within the selected gene set based on their expression levels with regard to defined phenotypes for each sample. Positive and negative metric enrichment scores correlate well with the Log2 FC values of the genes obtained from DEG analysis results, and it can be concluded that ACSL6 and ACSM5 genes are positively and negatively enriched in tumor samples, respectively (Fig. 2F).

GO and PPI analysis of ACS enzymes

Function annotation and GO analysis were performed by the DAVID database for ACS enzymes and the top 100 DEGs in COAD tumor samples compared to normal samples. Analysis of biological processes showed that most of the genes were predicted to be involved in LCFA biosynthesis and acyl-CoA metabolic processes (Fig. 3A). GO analysis of molecular function revealed that the two LCFA-CoA ligase and butyrate-CoA ligase activities were most common among the genes (Fig .3B). GO analysis of the cell component predicted that most genes are located in the mitochondrial matrix and peroxisomal membrane (Fig. 3C). KEGG pathway analysis was also performed, which showed that most of the genes were linked to butyrate metabolism and FA biosynthesis pathways (Fig. 3D). The results of functional enrichment and GO analysis by another method performed using the R program is shown in Fig. 4 as well. It can be understood that the ACS enzymes are more enriched biological pathways related to the metabolism of FAs and molecular functions that are related to CoA-ligase and FA-ligase activities (Fig. 4A and B). Also, most of the ACS genes were predicted to be localized in mitochondrial space (Fig. 4C). For a better understanding of the protein interactions between ACS enzymes and the top 100 DEGs, a PPI network was constructed using the STRING database, and Cytoscape software was used for the visualization of the network. As shown in Fig. 5, the PPI network contained 50 nodes with 154 edges. Based on DEG analysis results, genes that were upregulated and downregulated in tumor samples were colored blue and orange colors, respectively in the PPI network. According to the network, ACSS enzymes, ACSBG1, and ACSBG2 enzymes are predicted to have significant PPI with ACSL enzymes.

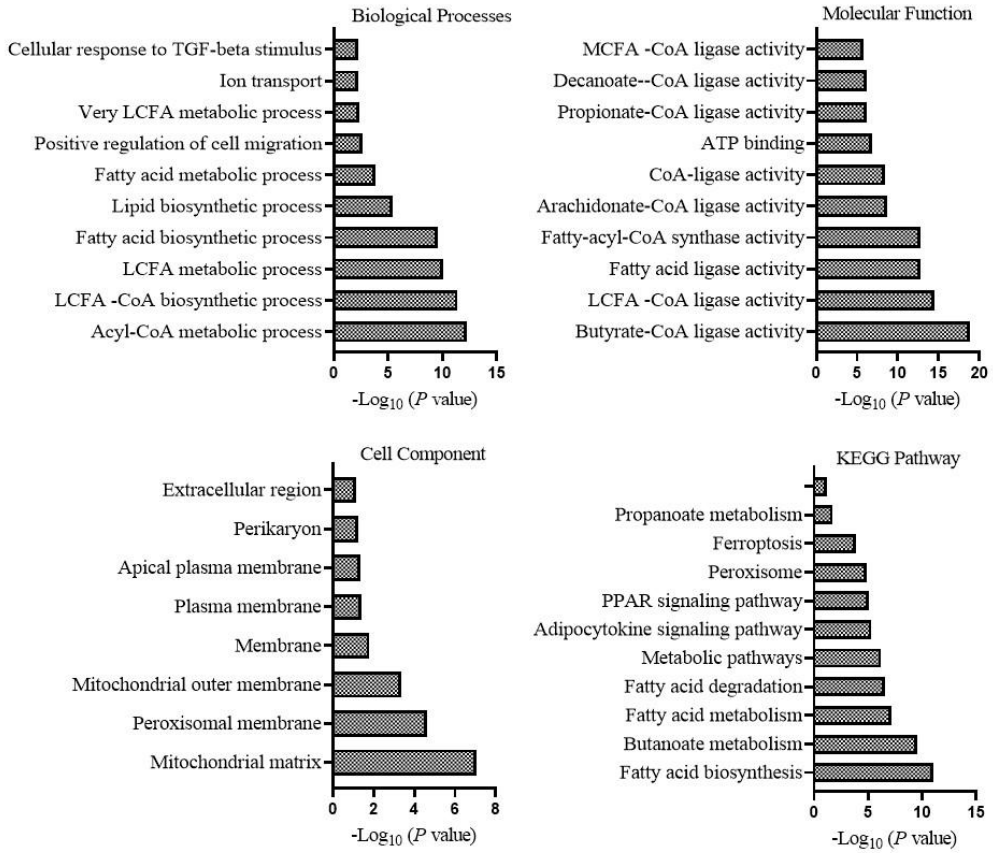

Fig. 3. GO and KEGG analysis result of top DEG and ACS genes in colon adenocarcinoma TCGA cancer. (A) GO analysis of biological processes shows that the acyl-CoA metabolic process was more common among the DEG and ACS genes and (B) the molecular function of most of the selected genes was butyrate-CoA and long-chain-fatty-acyl-CoA ligase activities; (C) cell component of most of the genes were the matrix of mitochondria and peroxisomal membrane. (D) KEGG pathway analysis demonstrates that DEG and ACS genes were mostly associated with the fatty acid biosynthesis pathway. GO, Gene ontology; DEG, differentially expressed genes; ACS, Acyl-CoA synthetase; LCFA, long-chain fatty acid.

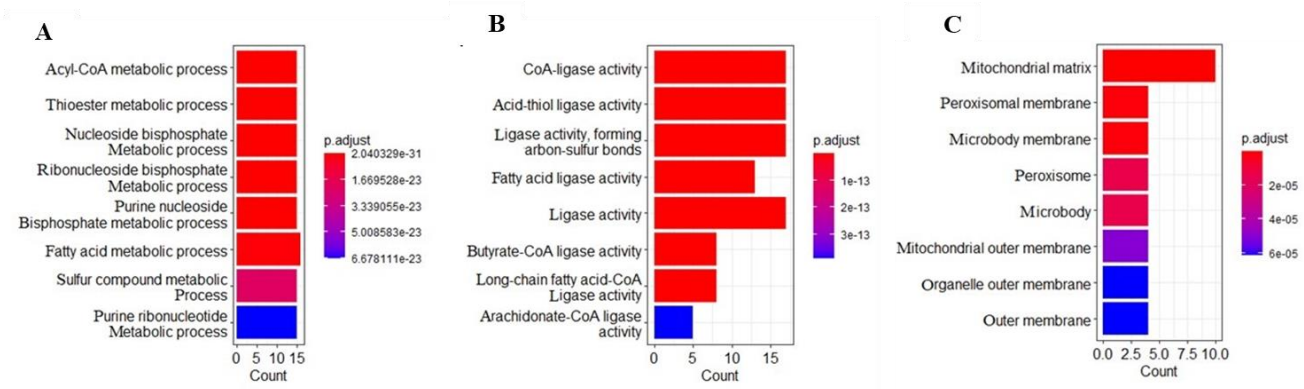

Fig. 4. Functional enrichment and gene ontology analysis of acyl-CoA synthetase; enzymes. (A) Biological processes; (B) molecular functions; and (C,) cellular components.

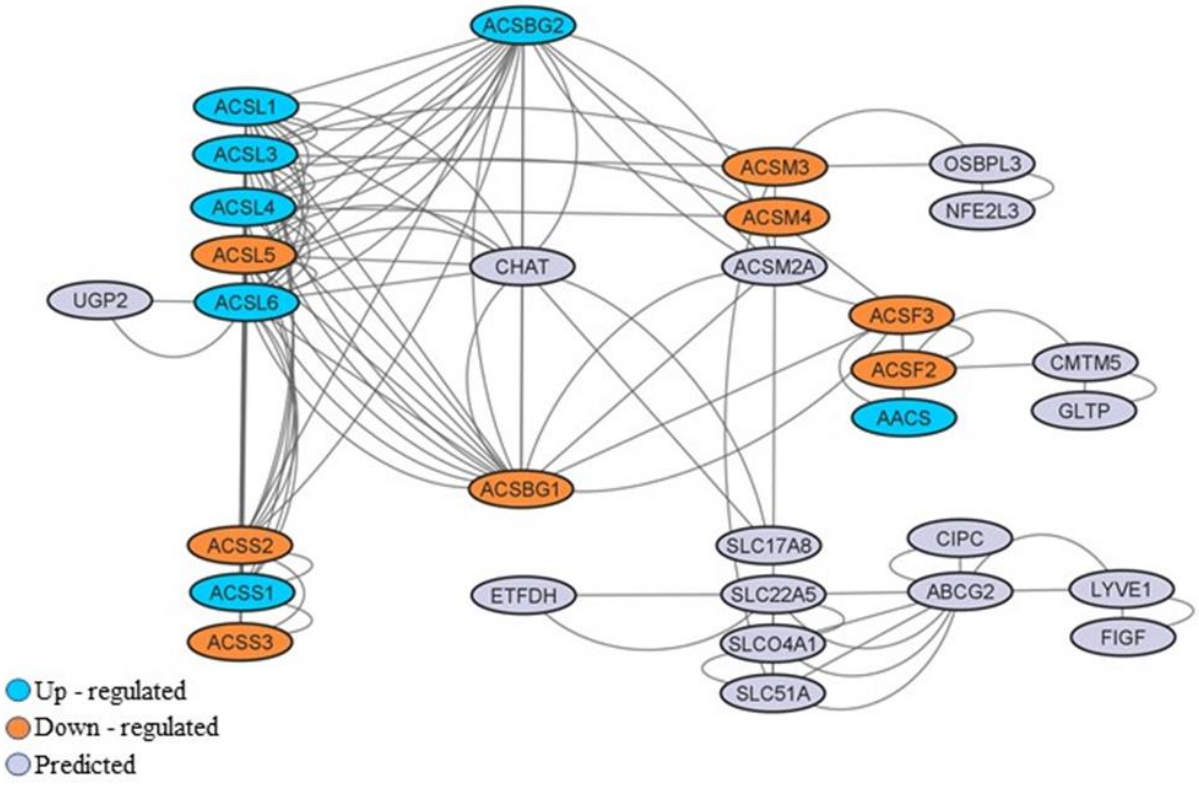

Fig. 5. Differentially expressed genes-mediated protein-protein interaction network in colon adenocarcinoma cancer. Up-regulated and down-regulated genes in colon adenocarcinoma cancer are shown with blue and orange colors, respectively. The rest of the predicted genes are shown with grey color. ACS, Acyl-CoA synthetase; ACSS, ACS short-chain-fatty acid-CoA ligase; ACSM, ACS medium-chain-fatty acid-CoA ligase 1; ACSL, ACS long-chain-fatty acid-CoA ligase; ACSBG, ACS bubblegum family member.

ACSM3 and ACSM5 as prognostic markers in COAD

Kaplan-Meier plots were generated for 17 different members of ACS enzymes using the survival package in the R program based on their expression level and clinical data in patients with *COAD*. The t-test statistical method was performed to compare the survival period between patients with "high" or "low" expression levels of each member of ACS enzymes. The difference in expression levels of most of the ACS enzymes did not significantly correlate with the survival of the patients, except for ACSM3 (*P*-value = 0.03) and

ACSM5 (*P*-value = 0.05) enzymes, which their expression significantly correlated with the survival period of the patients (Fig. 6). Patients with higher expression levels of ACSM3 and ACSM5 were shown to survive significantly longer compared to those who had lower expression levels of these two enzymes. Therefore, ACSM3 and ACSM5 are predicted to have better potential to be used as prognostic markers in patients with COAD compared to other members of ACS enzymes.

Diagnostic potential of ACS enzymes

The ROC test is a practical method for analyzing the accuracy of the diagnostic potential of candidate biomarkers in the detection and differentiation of tumor samples from control tissue samples. The diagnostic potential of selected members of ACS enzymes in COAD samples was assessed using GraphPad Prism software (version 9.1.0). All ACS members demonstrated relatively significant diagnostic potential (Fig. 7), especially ACSM5 (AUC = 0.96, *P*-value < 0.0001), ACSS2 (AUC = 0.90, *P*-value < 0.0001), and ACSF2 (AUC = 0.90, *P*-value < 0.0001) enzymes, which presented the highest AUC values and can be suggested as candidate diagnostic biomarkers in early detection of COAD.

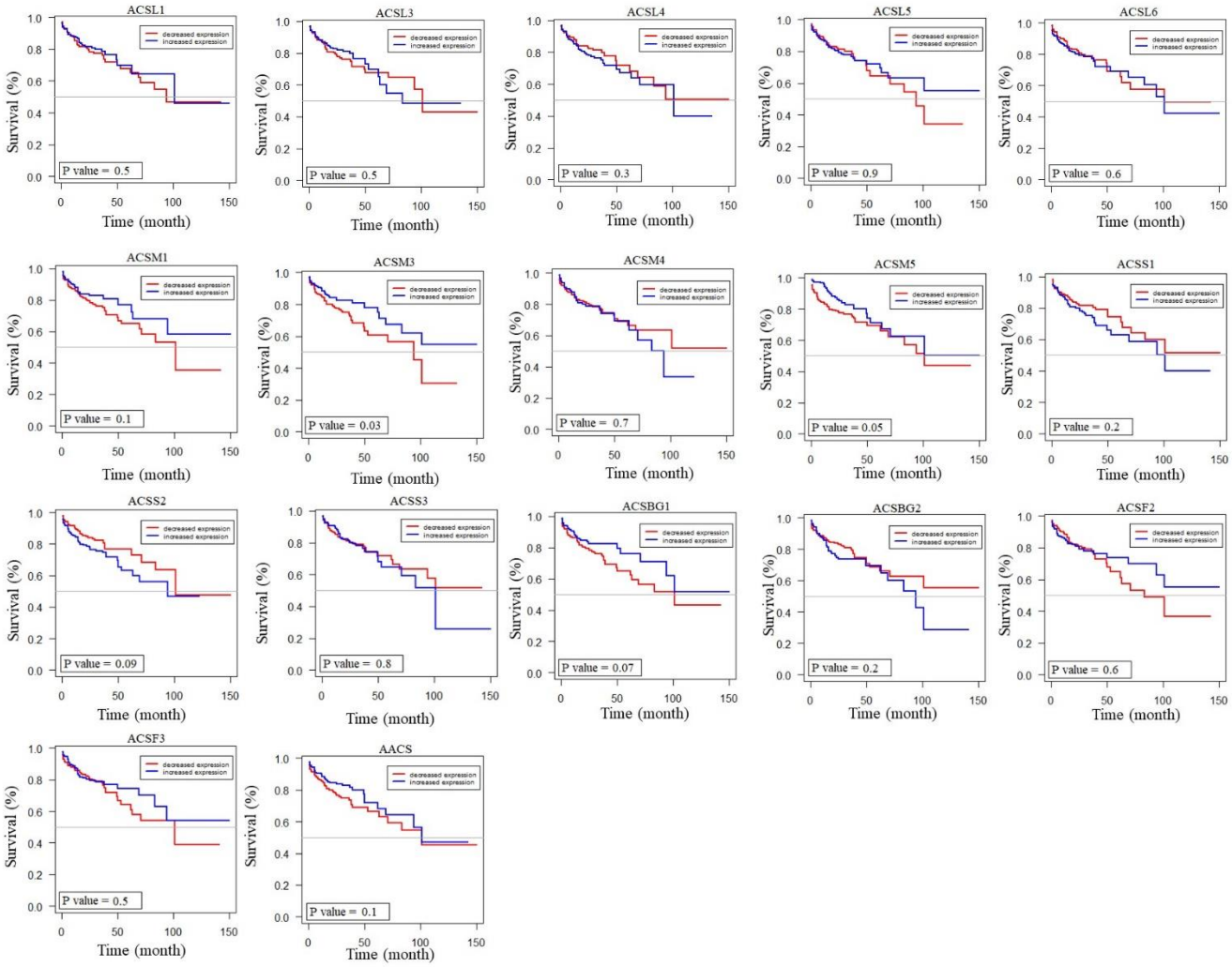

Fig. 6. Analysis of the prognostic capability of ACS enzymes in colon adenocarcinoma cancer. Kaplan Meier plots of 17 members of ACS enzymes are shown, which demonstrate the prognostic potential of each gene in the survival of patients with colon adenocarcinoma cancer. P-values smaller than 0.05 were considered statistically significant. ACS, Acyl-CoA synthetase; ACSS, ACS short-chain-fatty acid-CoA ligase; ACSM, ACS medium-chain-fatty acid-CoA ligase 1; ACSL, ACS long-chain-fatty acid-CoA ligase; ACSBG, ACS bubblegum family member.

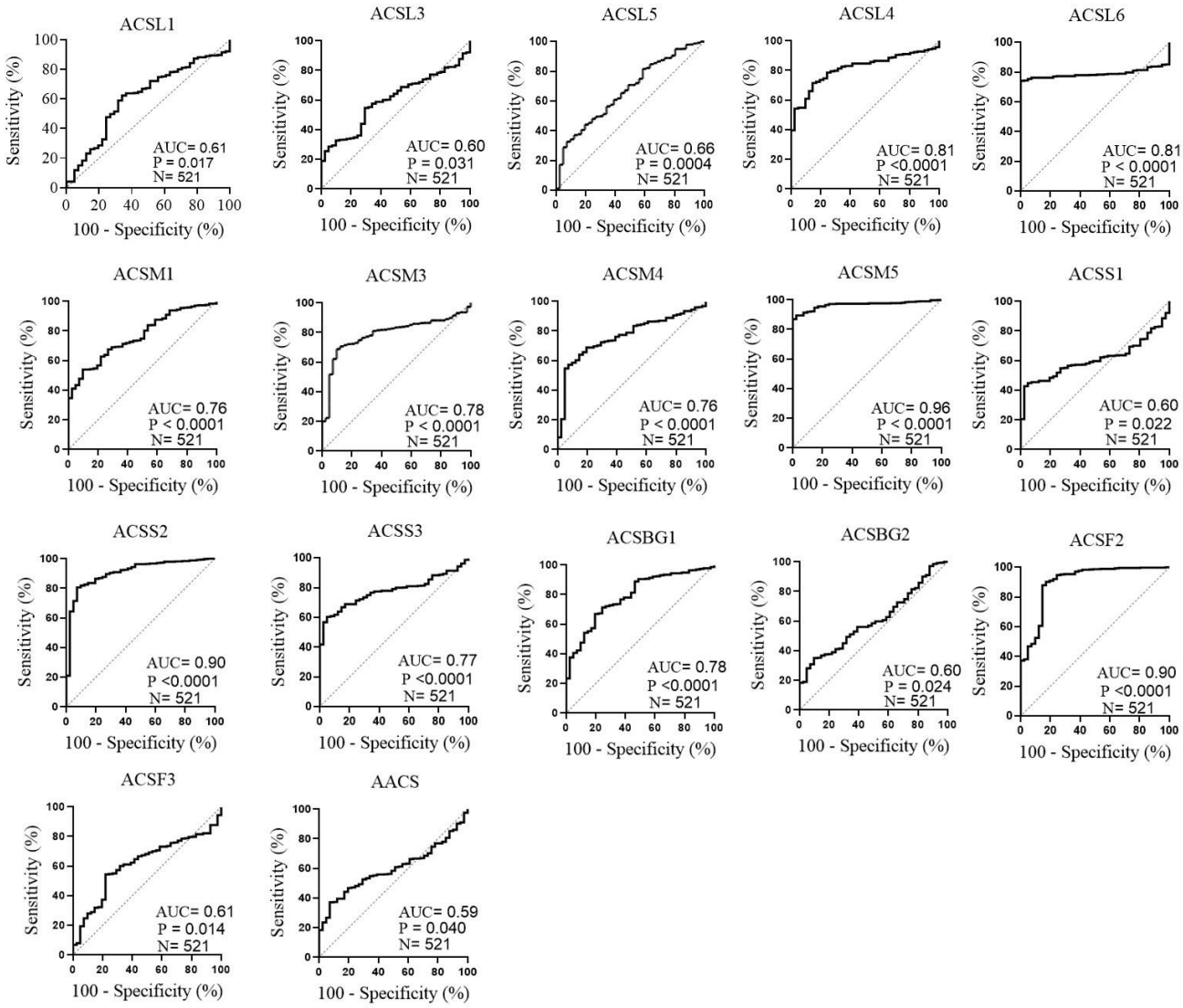

Fig. 7. Receiver operating characteristic (ROC) curves of 17 members of ACS enzymes in COAD cancer. The diagnostic potential of each ACS enzyme in the detection of COAD cancer samples has been compared from the healthy control tissues. P-values smaller than 0.05 were considered statistically significant.

DISCUSSION

The metabolism of FA plays a notable role in the progression and survival of cancer cells. While a significant amount of evidence supports the role of different enzymes involved in the uptake, synthesis, and oxidation of FAs in the progression, invasion, and survival of colorectal cancer cells., fewer experiments have been taken to clarify the role and expression patterns of enzymes involved in the activation of FAs in CRC (16, 29-32). FAs need to get activated before entering beta-oxidation pathways or other modification reactions such as desaturation and chain elongation of fatty acids (19). FA activation occurs by ligation of acetyl-CoA molecules with FAs in the presence of ATP molecules.

FAs are activated by ACS enzymes based on the length of their carbon chain (22). While many members of ACS enzymes have been reported to play significant roles in the growth, progression, and survival of different cancer cells, no study has compared the expression levels, diagnostic and prognostic capabilities of all ACS members with each other in COAD (24,25,33,34).

In this study, we performed differential expression and GSEA on 17 different members of ACS enzymes using the expression data of COAD samples from the TCGA database (35). Differential expression analysis showed that 7 and 10 members of ACS enzymes were up and down-regulated significantly in COAD samples, respectively compared to normal samples. ACSL1, ACSL3, ACSL4, and ACSL6 enzymes were upregulated while ACSL5 was downregulated in tumor samples. ACSL6 had the highest LogFC value among other ACSL members and achieved the highest positive enrichment ranking scores among different members of ACS enzymes. Another study has also reported that the ACSL6 expression level was low in many types of cancer, but its expression level was high in CRC, and overexpression of ACSL6 in CRC cells correlated with promoted cellular proliferation and increased levels of glycolytic products (36-38). Therefore, the higher expression level and activity of the ACSL6 enzyme might be associated with the progression of COAD cells.

As reviewed in Table 1, down-regulated levels of the ACSL5 enzyme were also observed in two other studies in pancreatic and colorectal tissue samples (39,40). Other studies have also reported high expression levels of ACSL1 enzyme in thyroid, breast, and ovarian cancer tissue samples (41-43). The high expression level of ACSL3 was detected in non-small cell lung cancer and pancreatic ductal adenocarcinoma samples (44,45). Also, up-regulated levels of the ACSL4 enzyme were reported in tissue samples of prostate, ovarian, and CRC (46-48). As among the members of ACSL enzymes, only the ACSL5 enzyme was shown to be downregulated in cancer samples, it could be assumed that the ACSL5 enzyme might have tumor-suppressor activities in COAD cells that interfere with the normal progression rate of cancer. However, this theory would demand further investigations in order to activity of the ACSL6 enzyme might be get clarified and validated.

Table 1. Review of ACSL enzymes expression and mechanism in a variety of cancer tissues.

Enzyme	Type of cancer	Expression level	Mechanism	Reference
ACSL1	Thyroid cancer	High	Downregulation of SNHG7 inhibits the expression of ACSL1.	(44)
ACSL1	Breast cancer	High	Overexpression of HBXIP and Sp1 transcription factor correlated with high levels of ACSL1	(42)
ACSL1	Ovarian cancer	High	high activity of ACSL1 correlated with activation of AMPK, Src proteins, beta-oxidation of fatty acids, and rate of metastasis.	(43)
ACSL3	Pancreatic ductal adenocarcinoma	High	Knockdown of ACSL3 enzyme correlated with decreased secretion level of plasminogen activator inhibitor-1 and altered progression of tumor cells.	(45)
ACSL3	Breast cancer	High	Breast cancer patients with higher protein levels of ACSL3 had shorter overall survival.	(46)
ACSL4	Prostate cancer	High	Knockdown of ACSL4 correlated with reduced cell proliferation, migration, and invasion.	(47)
ACSL4	Ovarian cancer	High	ACSL4 is inhibited by the upregulation of miR-424-5p. Upregulation of ACSL4 increases cellular sensitivity to ferroptosis.	(48)
ACSL4	Colorectal cancer	High	ELOVL6/ACSL4-targeted by apatinib enhances ferroptosis in colorectal cancer cells.	(49)
ACSL5	Colorectal cancer	Low	The expression level of ACSL5 did not differ with regard to the metastatic phenotype.	(41)
ACSL5	Pancreatic cancer	Low	Patients with the higher expression levels of ACSL5 had better survival and ACSL5 has been suggested as a potential prognostic biomarker.	(40)
ACSL6	Colorectal cancer	High	The expression level of ACSL6 enzyme was low in brain, cervical, kidney, and leukemia cancers except in colorectal cancer.	(38)

ACSL, Acyl-CoA synthetase long-chain-fatty acid-CoA ligase 1.

ACSM enzymes are responsible for the activation of MCFA. Based on differential expression analysis, ACSM1, ACSM3, ACSM4, and ACSM5 enzymes were significantly downregulated in COAD samples. ACSM5 had the smallest LogFC value among other ACS enzymes. It also achieved the highest negative enrichment score in the GSEA analysis. Previously, we and another group reported that ACSM5 expression was very low in breast cancer tissue samples and the human liver (49,50). As summarized in Table 2, ACSM1 was reported to have a higher expression level in prostate cancer compared to normal tissue samples (51). The ACSM3 expression level was reported as low in hepatocellular carcinoma and ovarian cancer tissue samples (34,52,53). ACSM4 differential expression is still unknown in many types of cancers but a study has reported a correlation between ACSM4 expression level with the survival of patients with triple-negative breast cancer (54,55). Differential expression of the ACSM6 enzyme is also unknown in many forms of cancers, but it has been suggested as a potential prognostic biomarker in bladder cancer (54,56,57). In this study, we showed that the expression levels of the most commonly known ACSM enzymes were lower in CPAD samples. Therefore, it can be hypnotized that the activation of MCFAs by ACSM enzymes, specially ACSM5 enzyme, might have an important role in the prevention of the progression of COAD cells. But extensive cellular and knock-down experiments are demanded to add further validation to this theory.

Table 2. Review of ACSM enzymes expression and mechanism in a variety of cancer tissues.

Enzyme	Type of cancer	Expression level	Mechanism	Reference
ACSM1	Prostate cancer	High	The role of ACSM1 is suggested to interact with proteins involved in receptor-extracellular matrix interactions and metabolic pathways.	(52)
ACSM3	Hepatocellular carcinoma	Low	Overexpression of ACSM3 enzyme correlated with cellular migration, invasion, and decreased phosphorylation of WNK1 and AKT kinases	(35)
ACSM3	Ovarian cancer	Low	ACSM3 is suggested as a tumor suppressor gene, as its overexpression reduced the integrin b1/AKT pathway and resulted in the inhibition of cellular migration, proliferation, and invasion.	(53)
ACSM3	Hepatocellular carcinoma	Low	The low expression level of ACSM3 correlated with poor survival and was more common in patients with high alanine aminotransferase levels, and high alpha-fetoprotein levels.	(54)
ACSM4	Triple-negative breast cancer	Not determined	The higher expression levels of the ACSM4 enzyme were shown to correlate significantly with poor prognosis in TNBC patients.	(56)
ACSM5	Breast cancer	Low	ACSM5 presented significant diagnostic potential in the detection of breast cancer and its expression level of luminal A subtype breast cancer samples.	(50)
ACSM5	Breast cancer	Low	Gene set enrichment analysis and differentially expressed genes analysis revealed that the ACSM5 gene is significantly downregulated and negatively enriched in breast cancer samples and is suggested as a diagnostic and prognostic biomarker.	(51)
ACSM6	Bladder cancer	Not determined	One of the target genes of PPARγ is the ACSM6 gene, which its expression correlated positively with an increased mRNA level of PPARγ.	(57)
ACSM6	Bladder cancer	Not determined	ACSM6 was predicted as a prognostic biomarker with help of LASSO algorithms.	(58)

ACSM, Acyl-CoA synthetase medium-chain-fatty acid-CoA ligase 1; AKT, protein kinase B; PPARγ, peroxisome proliferator- activated receptor gamma.

By differential expression analysis, we found that ACSS2 and ACSS3 had low expression levels in COAD samples, while ACSS1 had a high expression level in tumor samples compared to normal tissue samples. As presented in Table 3, the metabolism and utilization of acetate in different cancers have been studied. A study reported upregulated level of ACSS1 enzyme in hepatocellular cancer tissues compared to noncancerous liver samples (58). Treatment of colon cancer cells was reported to be associated with the increased expression level of ACSS2 and no change in ACSS1 expression level (59). Another study suggested that the up-regulation of ACSS1 could be involved in the progression of bladder cancer (60). The expression level of the ACSS2 enzyme was reported as high in cervical squamous cell carcinoma and cisplatin-resistant patients with bladder cancer, while its expression was low in gastric cancer tissue samples (61-63). The expression level of the ACSS3 enzyme was also determined as high and low in bladder and prostate cancers respectively (26,64). ACSS enzymes are known to activate acetate molecules in different compartments of the cells, and utilization of acetate molecules is an important step for the survival and progression of most cancer cells. According to the outcomes of our study and previous studies done by other groups on ACSS enzymes, it can be understood that the three members of ACSS enzymes have alternative expression patterns across different types of cancers, and each cancer holds its own metabolic profile.

ACSBG1 and ACSBG2 enzymes are two less familiar members of ACS enzymes in humans, which have shown ACS activity and can activate LCFAs. ACSBG1 and ACSBG2 enzymes share a high sequence similarity, but their differential expression in cancers is still unknown (65). A study suggested that ACSBG1 might act as a metabolic checkpoint in regulatory T cells and its genetical deletion was associated with mitochondrial dysfunction (66). In this study, we found that ACSBG1 expression level in COAD samples was significantly low and ACSBG2 had a higher expression level in tumor samples compared to normal tissue samples.

Table 3. Review of ACSS enzymes expression and mechanism in a variety of cancers.

Enzyme	Type of cancer	Expression level	Mechanism	Reference
ACSS1	Colon cancer	Not determined	In HT29 cells, the expression of ACSS2 was enhanced by acetate but it didn't change ACSS1 expression.	(60)
ACSS1	Hepatocellular carcinoma	High	Hepatocellular carcinoma patients with higher levels of ACSS1 enzyme had a lower rate of glutamate utilization compared to high ACSS1-expressing patients.	(59)
ACSS2	Cervical squamous cell carcinoma	High	ACSS2 has been suggested as a prognostic biomarker. Also, it was shown that patients with higher levels of ACSS2 have shorter survival periods.	(62)
ACSS2	Gastric cancer	Low (in 62% of the cancer tissues)	ACSS2 can be used as a prognostic marker in gastric cancer. It was shown also that patients with low levels of ACSS2 had poor survival.	(63)
ACSS2	Bladder cancer	High (in cisplatin-resistant patient tissues)	Suppression of ACSS2 in T24R cells correlated with reduced synthesis of fatty acids but had no effect in T24S cells.	(64)
ACSS3	Bladder cancer	High	Down-regulation of ACSS3 resulted in the suppression of cellular growth. Metabolic stress (hypoxia) upregulated ACSS3 and ACSS2 expression levels.	(26)
ACSS3	Prostate cancer	Low	Prostate cancer cells demonstrated a higher level of methylation within the promoter region of ACSS3 compared to normal prostate cells. Which might provide a possible explanation for the downregulation of ACSS3 in prostate cancer.	(65)

ACSS, Acyl-CoA synthetase short-chain-fatty acid-CoA ligase 1.

Currently, a limited number of research has been performed on the molecular functions and the expression levels of ACSBG enzymes in cancer cells. Our study revealed altered expression levels of ACSBG1 and ACSBG2 enzymes in COAD samples for the first time and further investigations and efforts are demanded in order to better understand the association between the expression levels of ACSBG enzymes with biological processes and progression of different types of cancer cells.

ACSF2 and ACSF3 enzymes belong to ACSM enzymes. While data severely lacks about the function and expression patterns of these two enzymes in different cancers, some groups have predicted that ACSF2 may participate in neural differentiation (67) and the methylation levels of ACSF2 was higher in patients with hepatocellular carcinoma (68). By differential expression analysis, we reported for the first time that ACSF2 and ACSF3 enzymes had significantly lower expression levels in COAD samples compared to normal tissue samples.

AACS is known as a ketone body-utilizing enzyme and some studies have suggested that it is involved in cholesterol homeostasis in mice, but its expression pattern and physiological role in normal and cancerous human cells are still significantly unknown (69,70). A recent study has reported that patients with hepatocellular cancer with higher expression levels of AACS enzyme had shorter survival (68). In the current study, we found no significant difference in expression levels of AACS enzyme between COAD and normal tissue samples.

By employing the Kaplan-Meier method in the survival analysis we found that among the different members of ACS enzymes, only ACSM3 (*P*-value = 0.03) and ACSM5 (*P*-value = 0.05) had significant prognostic potential in COAD samples, and patients with higher expression levels of ACSM3 or ACSM5 had a significantly longer survival period. In a previous study, we also reported that the ACSM5 enzyme had a significant diagnostic and prognostic potential in breast cancer patients and higher expression levels of the ACSM5 enzyme were associated with a longer survival period in breast cancer patients (50). To assess the diagnostic potential of ACS enzymes in COAD, an ROC test was performed and we found that ACSM5 (AUC = 0.96, *P*-value < 0.0001), ACSS2 (AUC = 0.90, *P*-value < 0.0001), and ACSF2 (AUC = 0.90, *P*-value < 0.0001) enzymes had stronger diagnostic potential compared to rest of the ACS enzymes. Considering the predicted associations between the expression levels of ACSM3 and ACSM5 enzymes with the survival period of patients with COAD cancer, future investigations are suggested to be taken in order to clarify the connection between the expression levels of ACSM enzymes with the progression and survival of cancer cells.

By analysis of GO definitions, KEGG pathway, and PPI network analysis of ACS enzymes with top DEGs, we found that the biological process of most of the genes was associated with the biosynthesis of LCFA-CoA and acyl-CoA molecules, while their molecular functions were mostly associated with LCFA-CoA and butyrate-CoA ligase activities. The cellular component for most of the genes was predicted in mitochondrial matric and peroxisomal membrane. KEGG pathway analysis suggested that a great ratio of the genes was involved in the metabolism and biosynthesis of butyrate and FA molecules, respectively. PPI network analysis revealed a great interaction between different members of ACSL, ACSS, and ACSBG enzymes. While several studies have highlighted the prognostic potential of ACS enzymes, further investigations are still needed to clarify the molecular functions and differential expression level of ACS enzymes in the progression and development of many types of cancers. Especially future investigations are suggested to study the expression levels, and biological and molecular functions of ACSBG, ACSF, and AACS enzymes in different human cancer cell lines.

CONCLUSION

In conclusion, our study, for the first time, demonstrated the differential expression pattern of 17 different ACS genes in COAD, while no study has yet reported the expression levels of less familiar members of ACS enzymes, such as ACSFs, ACSBGs, and AACS genes in COAD.

Differential expression analysis clarified the differential expression levels of ACS enzymes in cancer samples. ACSL6 and ACSM5 genes had the most significant LogFC values among other ACS enzymes and were also shown by GSEA analysis to be notably enriched in tumor phenotype. GO analysis and PPI networks predicted that ACS enzymes and top DEGs interact with each other and are involved in the metabolic pathways of FAc. Our analysis, suggests ACSM3, ACSM5, ACSS2, and ACSF2 as possible prognostic and diagnostic biomarkers in COAD. More investigations are demanded to clarify the association between the molecular function and expression levels of ACS enzymes in the survival, growth, progression, and development of COAD cells.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

The study design was performed by H. Sirous and B. Yazdani; data analysis was carried out by B. Yazdani, E. Parsazad; interpretations of the data and bioinformatics analysis were performed by B. Yazdani, E. Parsazad, F. Esrafili, S. Ghafarzadeh, N. Razmavar, and H. Sirous; E. Parsazad, B. Yazdani, F. Esrafili, S. Ghafarzadeh, and N. Razmavar wrote the manuscript. The final version of the manuscript was approved by all authors.

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