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Overexpression of TSPAN8 in consensus molecular subtype 3 colorectal cancer

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ABSTRACT

Background: Recently, consensus molecular subtypes (CMSs) have been proposed as a robust transcriptome-based classification system for colorectal cancer (CRC). Tetraspanins (TSPANs) are transmembrane proteins. They have been associated with the development of numerous malignancies, including CRC, through their role as "master organizers" for multi-molecular membrane complexes. No previous study has investigated the correlation between TSPANs and CMS classification. Herein, we investigated the expression of *TSPANs* in patient-derived primary CRC tissues and their CMS classifications.

Methods: RNA samples were derived from primary CRC tissues (n = 100 patients diagnosed with colorectal adenocarcinoma) and subjected to RNA sequencing for transcriptome-based CMS classification and *TSPAN*-relevant analyses. Immunohistochemistry (IHC) and immunofluorescence (IF) stains were conducted to observe the protein expression level. To evaluate the relative biological pathways, gene-set enrichment analysis was performed.

Results: Of the highly expressed *TSPAN* genes in CRC tissues (*TSPAN8*, *TSPAN29*, and *TSPAN30*), *TSPAN8* was notably overexpressed in CMS3-classified primary tissues. The overexpression of TSPAN8 protein in CMS3 CRC was also observed by IHC and IF staining. As a result of gene-set enrichment analysis, TSPAN8 may potentially play a role in organizing signaling complexes for kinase-based metabolic deregulation in CMS3 CRC.

Conclusions: The present study reports the overexpression of TSPAN8 in CMS3 CRC. This study proposes TSPAN8 as a subtype-specific biomarker for CMS3 CRC. This finding provides a foundation for future CMS-based studies of CRC, a complex disease and the second leading cause of cancer mortality worldwide.

1. Introduction

Colorectal cancer (CRC) is a complex disease and the second leading cause of global cancer mortality (Rawla et al., 2019). Thus, there is an urgent need to develop novel prevention, detection, and therapeutic strategies for CRC (Hossain et al., 2022). Since clinical and molecular

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Table 1
Clinical data of CRC patients and CMS classifications.

Characteristics	Number
Sex	
Male	56
Female	44
Age	
Range (years)	23 to 91
Average ± standard deviation (years)	62.66 ± 13.32
Tumor location	
Left-sided colon	41
Right-sided colon	17
Rectum	42
Stage	
1	1
2	11
3	74
4	14
CMS	
1	5
2	46
3	18
4	29
Mixed/indeterminate	2

heterogeneity is a key feature and the main challenge for CRC, molecular classifications have been widely proposed to fully understand and overcome this complex disease (Singh et al., 2019; Chan and Buczacki, 2021). In particular, Guinney et al. recently proposed a consensus molecular subtype (CMS) system: a transcriptome-based classification of CRC into 4 subtypes (Guinney et al., 2015). "CMS1" is distinguished by microsatellite instability (MSI), high CpG island methylator phenotype (CIMP) levels, hypermutation, *BRAF* mutations, and immune infiltration and activation. "CMS2" is characterized by high somatic copy number alteration (SCNA) and activation of the WNT and MYC pathways. "CMS3" exhibits mixed MSI status, low CIMP, low SCNA, *KRAS* mutations, and metabolic deregulation. In particular, this subtype often exhibits alterations in key metabolic pathways, including increased glycolysis and changes in lipid metabolism. The enhanced glycolytic activity, even in the presence of oxygen, is expected to be more pronounced in CMS3, influencing energy metabolism. Dysregulation in lipid metabolism involves alterations in lipid synthesis and breakdown, impacting cellular processes such as membrane composition and signaling. "CMS4" shows high SCNA, stromal infiltration, TGF-β activation, and angiogenesis. The CMS system is considered one of the most robust classification systems for translational research, clinical stratification, and the therapeutic management of CRC (Guinney et al., 2015; Ten Hoorn et al., 2022).

Transmembrane proteins spanning the entire cell membrane contribute to numerous cellular functions in biological and pathological processes (Cournia et al., 2015). In the field of cancer, transmembrane proteins have been reported to be involved in many malignancies through various mechanisms and pathways, possibly leading to clinical implications and applications (Lin et al., 2019). Tetraspanins (TSPANs), also referred to as transmembrane 4 superfamily (TM4SF) proteins, are characterized by 4 transmembrane domains and 2 extracellular loops (one shorter and the other longer) (Hemler, 2008; Min et al., 2006). TSPANs are highly expressed in particular types of cells and tissues (Hemler, 2008; Min et al., 2006). They have been proposed as "master organizers" of the plasma membrane via *in cis* association with a limited number of partners (eg, other TSPANs, integrins, receptors, enzymes,

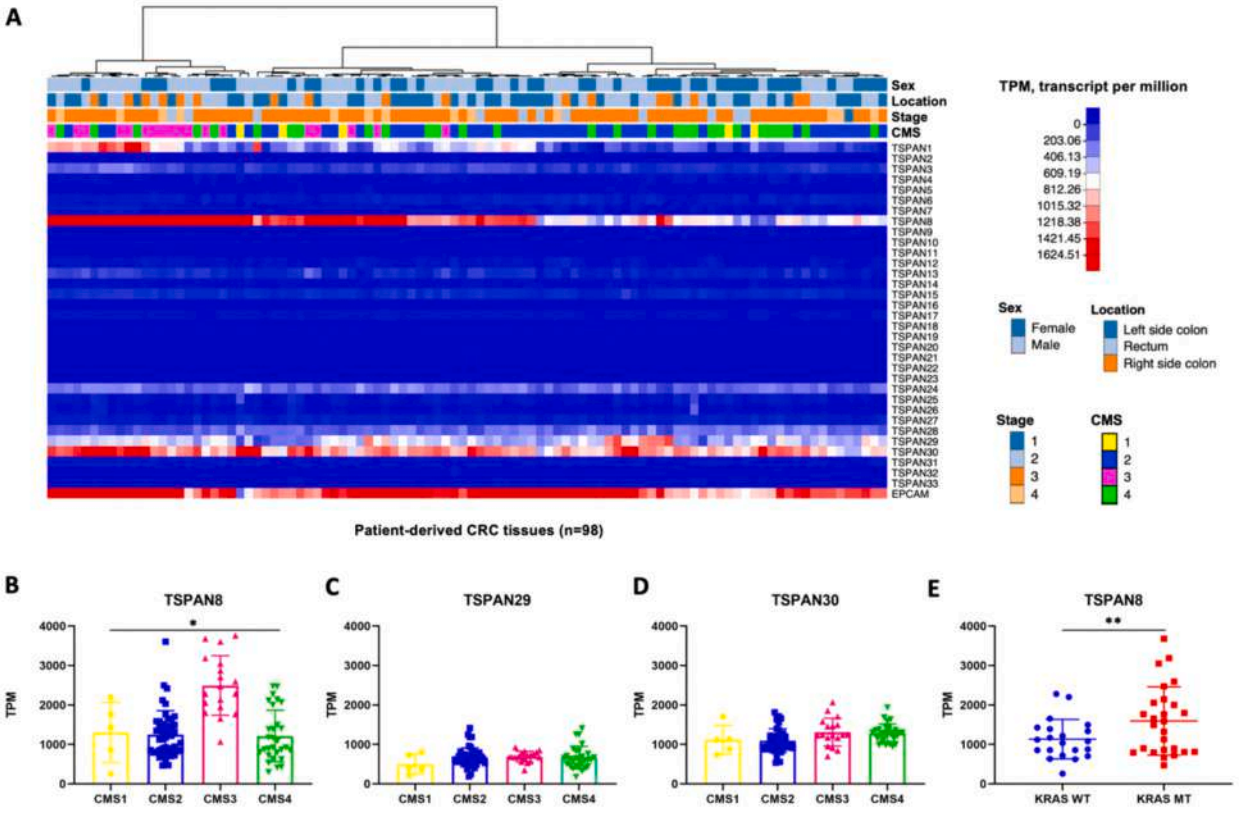

Fig. 1. RNA-Seq-based expression of *TSPAN* genes in CMS-classified primary CRC tissues. (A) Heat map of *TSPAN* gene expression in CMS-classified primary CRC tissues. The heat map was clustered and generated by a web-based next-generation clustered heat map (NG-CHM) tool (https://build.ngchm.net/NGCHM-web-builder/). (B) *TSPAN8* gene expression in CMS-classified primary CRC tissues. * indicates *P* value < 0.0001, ANOVA test. (C) *TSPAN29* gene expression in CMS-classified primary CRC tissues. (D) *TSPAN30* gene expression in CMS-classified primary CRC tissues. (E) *TSPAN8* gene expression in *KRAS* status-classified CRC tissues. WT, wide type. MT, mutant. ** indicates *P* value < 0.05, *t*-test. Each dot represents individual data. Error bars show the mean and standard deviation.

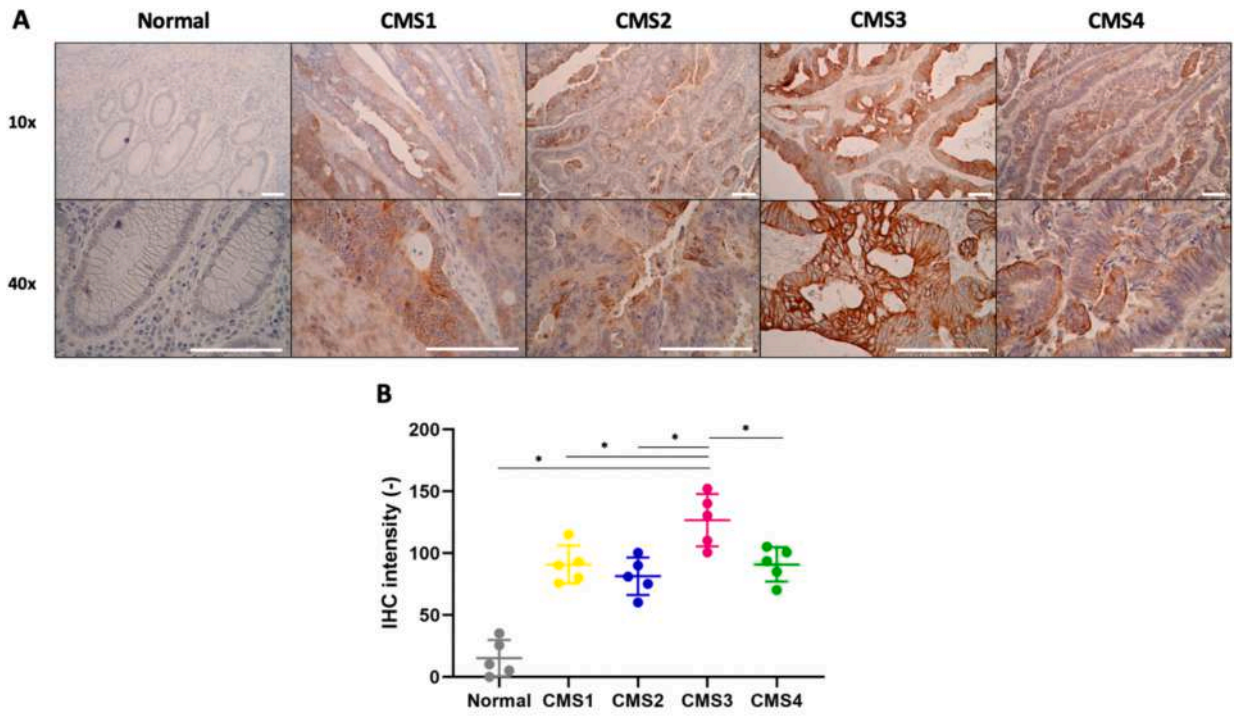

Fig. 2. Immunohistochemistry (IHC) staining of TSPAN8 protein in CMS-classified primary CRC tissues. (A) Representative TSPAN8-stained IHC images of normal-, CMS1-, CMS2-, CMS3-, and CMS4-classified tissues. Scale bar, 100 μm. Microscope magnifications were 10× and 40× for the upper and lower images, respectively. (B) Quantified IHC intensity in TSPAN8-stained IHC images of normal-, CMS1-, CMS2-, CMS3-, and CMS4-classified tissues. * indicates *P* value < 0.05, t-test. Each dot represents individual data. Error bars show the mean and standard deviation.

and signaling proteins) (Hemler, 2005). The outcome is the formation of TSPAN-enriched microdomains (TEMs) or webs for partner-dependent functions (Van Deventer et al., 2017). TSPANs are essential for fundamental cellular phenomena such as adhesion, migration, fusion, proliferation, differentiation, immune activation, signaling, malignancy, and infection (Charrin et al., 2014; Zöller, 2009). Interestingly, some TSPAN members—TSPAN1, TSPAN6, TSPAN8, and TSPAN30—are highly expressed in CRC (Titu et al., 2021; Andrijes et al., 2021); however, their expression related to CMS subtypes has not been investigated. TSPAN1 and TSPAN8 were reported to promote CRC development, while TSPAN6 and TSPAN30 were described as suppressing CRC progression (Titu et al., 2021; Andrijes et al., 2021; Zhang et al., 2020). Thus, further studies of TSPANs may be beneficial to identify biomarkers and therapeutic targets for CRC (Titu et al., 2021; Malla et al., 2018).

No previous study has examined the correlation between *TSPANs* and CMS. Therefore, the present work aimed to analyze *TSPANs* in patient-derived primary colorectal adenocarcinoma tissues and their CMS classifications. We found that TSPAN8 was predominantly overexpressed in CMS3 CRC and potentially involved in kinase-based metabolic deregulation. Our research suggests a possible role of TSPAN8 and its potential as a molecular subtype-specific biomarker in CMS3 CRC. Its use as a biomarker would facilitate further investigations for CMS3 CRC.

2. Materials and methods

2.1. Patient-derived tissues and storage

Tissue specimens were sourced from 100 CRC patients, who were diagnosed with colorectal adenocarcinoma and treated according to the National Comprehensive Cancer Network guidelines at Siriraj Hospital, Thailand. All CRC patients in this study provided written informed consent. In the case of fresh frozen tissues, primary CRC tissues were resected from colonoscopy with biopsy or surgical operation (≤ 0.5 cm in any single dimension), immediately washed with phosphate-buffered saline, immersed in chilled RNAlater (Invitrogen, MA, USA), and stored at −80 °C at the Siriraj Biobank, Faculty of Medicine Siriraj Hospital. For formalin-fixed, paraffin-embedded (FFPE) tissues, the resected tissues were fixed in 10% formalin, dehydrated by ethyl alcohol, and embedded in hot paraffin wax. They were stored at the Department of Pathology, Faculty of Medicine Siriraj Hospital. Tumor-distant normal mucosa tissues from the same patients were also collected for comparison.

2.2. RNA sequencing (RNA-Seq)

RNA extraction from fresh frozen tissues was conducted according to a previously-described protocol (Acharayothin et al., 2023). Lysing matrix Z and a FastPrep-24 5G system (MP Biomedicals, CA, USA) with a speed of 6.0 m/s (40-s ON and 3-min OFF cycles) were applied to lyse each primary CRC tissue (approximately 30 mg) until a clear lysate was obtained. Next, the RNeasy mini kit (Qiagen, Hilden, Germany) was used to extract total RNA following the manufacturer's instructions. The concentration and purity of the extracted RNA solutions were assessed by a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, MA, USA). An Agilent 2100 bioanalyzer and an Agilent RNA 6000 Nano LabChip kit (Agilent Technologies, CA, USA) were employed to determine the RNA integrity number (RIN). Suitable RNA solutions (over 1 μg of RNA and an RIN higher than 7) were lyophilized. The lyophilized RNA samples were transported to Novogene Co Ltd. (Singapore) to prepare the RNA library and perform sequencing. In brief, total RNA was subjected to messenger RNA isolation by poly-T oligo-attached magnetic beads. After fragmentation, random hexamer primers were used to prepare first-strand cDNA. Next, deoxyuridine triphosphate was used to synthesize second-strand cDNA. Following end repair, A-tailing, adapter ligation, size selection, enzyme digestion, amplification, and purification, the library was checked for quantification by Qubit and real-time PCR, as well as for size distribution by bioanalyzer. After RNA library construction, RNA-Seq was conducted with an Illumina HiSeq 2500

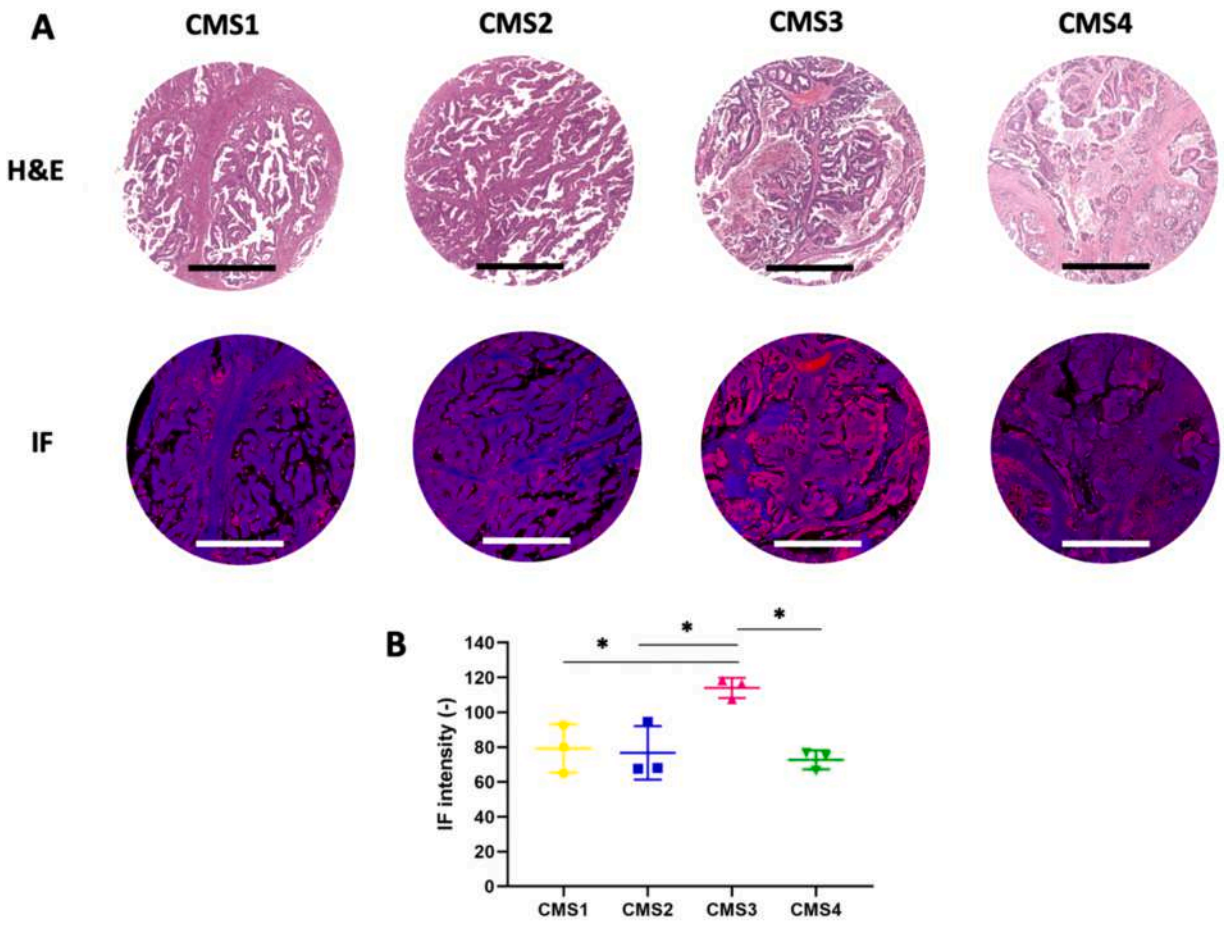

Fig. 3. Immunofluorescence (IF) staining of TSPAN8 protein in CMS-classified primary CRC tissues. (A) Representative H&E and TSPAN8-stained IF images of CMS1-, CMS2-, CMS3-, and CMS4-classified tissues. Red, TSPAN8. Blue, nucleic acid-staining SYTO13. Scale bar, 1000 μm. Microscope magnification was 10×. (B) Quantified IF intensity in TSPAN8-stained IF images of CMS1-, CMS2-, CMS3-, and CMS4-classified tissues. * indicates *P* value < 0.05, t-test. Each dot represents individual data. Error bars show the mean and standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

platform (Illumina, San Diego, CA, USA) using a 2 × 150 bp paired-end configuration to provide 8.0 Gb raw data per sample. The FASTQ files we obtained were used to generate a normalized transcript per million (TPM) value of 29,972 genes. Our FASTQ files are publicly available from the Gene Expression Omnibus (accession number GSE220148; samples: GSM6792596–GSM6792695).

2.3. CMS classification

The deep cancer subtype classification (DeepCC) R package with the functional spectra-based cancer stratification model was employed to identify the CMS of each primary CRC sample, based on its RNA-Seq gene expression profile (Gao et al., 2019). The DeepCC R package provides a 95% confidence interval and other assessment items (eg, sensitivity, specificity, and balanced accuracy). To train the DeepCC R package, previously reported CMS and RNA-Seq data from The Cancer Genome Atlas (TCGA) were utilized (Guinney et al., 2015). The trained package was finally inputted with the obtained RNA-Seq data to indicate the CMS classification (CMS1, CMS2, CMS3, or CMS4) for each primary CRC sample in our cohort.

2.4. KRAS mutation screening

Due to limited availability of tissue samples, the remaining CRC tissues ($n = 47$) underwent testing to determine their *KRAS* mutational status. The therascreen *KRAS* kit from Qiagen, Hilden, Germany, was utilized to analyze mutations in exon 2 (specifically codons 12 and 13) of the *KRAS* gene. This kit employs allele-specific amplification through an amplification refractory mutation system, following the manufacturer's instructions. Any samples testing negative underwent subsequent Sanger sequencing to detect *KRAS* mutations in exon 3 (codon 61).

2.5. Immunohistochemistry, hematoxylin and eosin, and immunofluorescence staining

To prepare an FFPE slide, an FFPE tissue block was sectioned at 5 μm and mounted on a glass slide. The slide was deparaffinized and rehydrated by a wash series of limonene, 100% ethanol, 95% ethanol, and ultrapure water, in that order. An antigen retrieval process was then conducted by placing the slide in a jar containing citrate buffer (0.1 M; pH 6.0) in a heated pressure cooker.

For immunohistochemistry (IHC) staining, a prepared FFPE slide was dried, drawn by a hydrophobic barrier around the tissue, and covered by a blocking solution (Dako, Agilent Technologies, CA, USA). The tissue was placed in a humidity chamber and covered with a diluted primary antibody solution (anti-TSPAN8, ab230448, 1/100) at 37 °C for 1 h. The tissue was subsequently covered with Dako HRP solution (30 min), followed by Dako DAB solution (5 min), in a light-protected humidity chamber. Finally, the tissue was covered with a solution of Dako hematoxylin (5 min), rinsed with water, mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, MA, USA), and observed under an AxioStar plus microscope fitted with a Canon EOS 1100D

Table 2
Top 10 ARCHS4 kinases coexpression pathways and enriched overlap genes in CMS3 of CRC by Enrichr (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021).

Pathway	*P* value	Overlap genes*
ERN2 human kinase ARCHS4 coexpression	4.66×10^{-16}	*PIGR, FCGBP, ST6GALNAC1, MS4A8, WFDC2, LRRC31, SELENBP1, CAPN9, SCIN, MUC2,* ***TSPAN8****, VSIG2, AGR2, AGR3, CLCA1, FAM3D, ZBTB7C, NXPE4, CHST6, REG4, NXPE1, DLEC1, VWA3B, B3GALT5, SNTN, CCDC60*
STYK1 human kinase ARCHS4 coexpression	4.40×10^{-15}	*PIGR, FCGBP, ST6GALNAC1, REP15, KLK3, CWH43, LRRC31, SELENBP1, CAPN9, MUC2,* ***TSPAN8****, VSIG2, AGR2, AGR3, CLCA1, FAM3D, SPDEF, SPINK4, NXPE4, REG4, NXPE1, B3GALT5, SLC9A2, FABP2, MRAP2*
PTK6 human kinase ARCHS4 coexpression	1.09×10^{-9}	*PIGR, NXPE4, FCGBP, ST6GALNAC1, REG4, NXPE1, B3GALT5, LRRC31, SLC9A2, SLC9A4, XKR9, SCIN, MUC2,* ***TSPAN8****, FFAR4, AGR2, CLCA1, FAM3D, ZBTB7C*
MST1R human kinase ARCHS4 coexpression	7.11×10^{-9}	*SPINK4, PIGR, NXPE4, FCGBP, ST6GALNAC1, REG4, NXPE1, B3GALT5, SLC9A2, SELENBP1, CAPN9, MUC2,* ***TSPAN8****, VSIG2, AGR2, CLCA1, FAM3D, CTSE*
ERBB3 human kinase ARCHS4 coexpression	4.36×10^{-8}	*PIGR, FCGBP, ST6GALNAC1, REG4, B3GALT5, FMO5, LRRC31, SLC9A2, SELENBP1, CAPN9, MUC2, MLPH,* ***TSPAN8****, AGR2, CLCA1, FAM3D, SPDEF*
TRPM6 human kinase ARCHS4 coexpression	1.35×10^{-6}	*PIGR, NXPE4, FCGBP, ST6GALNAC1, NXPE1, C9ORF66, B3GALT5, FMO5, SELENBP1, FABP2, SCIN, MUC2,* ***TSPAN8****, CLCA1, FAM3D*
NEK10 human kinase ARCHS4 coexpression	5.38×10^{-4}	*CHST6, SNTN, CAPN9, MS4A8, AGR3, DLEC1, VWA3B, C1ORF168, CCDC60, WFDC2, ZBTB7C*
ACVR1C human kinase ARCHS4 coexpression	5.38×10^{-4}	*G6PC2, UCN3, ATP8A1, KIAA1324, HEPACAM2, MTUS2, KSR2, KCTD4, TDRD5, CRYM, NOL4*
MYO3A human kinase ARCHS4 coexpression	0.00194	*AGBL4, G6PC2, UCN3, SCGB2A1, ARX, KIAA1324, HEPACAM2, SCG5, SLC16A9, NOL4*
SGK2 human kinase ARCHS4 coexpression	0.00638	*SELENBP1, NXPE4, FCGBP, ALDH1L1,* ***TSPAN8****, NXPE1, ANG, HMGCS2, TRHDE*

* *TSPAN8* is displayed in the bold font.

digital camera (Carl Zeiss Co Ltd., Oberkochen, Germany). ImageJ Fiji software (National Institutes of Health, MD, USA) was utilized to quantify the IHC intensity in the IHC images using a protocol proposed in a previous report (Crowe and Yue, 2019).

For hematoxylin and eosin (H&E) staining, a prepared FFPE slide was stained with hematoxylin to visualize nuclei (blue-purple) and eosin to stain cytoplasm and extracellular matrix (pink) (Dako, Agilent Technologies, CA, USA). After staining, the slide was briefly immersed in acid alcohol to differentiate staining. The slide was then passed through graded alcohols (70, 95 and 100% ethanol) and m-Xylene (Sigma-Aldrich Corp, MO, USA) for dehydration and clearing, respectively. A coverslip was added using ProLong Gold Antifade Mountant (Thermo Fisher Scientific, MA, USA) for microscopic examination. The tissue histology was examined with the previously mentioned digital camera-integrated microscope.

For immunofluorescence (IF) staining, the tissue was surrounded by a hydrophobic barrier and covered with a diluted solution of a primary antibody (1/100, anti-Tspan-8 antibody, ab230448; Abcam, Cambridge, UK) at 37 °C for 1 h in a humidity chamber. Then, the tissue was covered with a dilution of a secondary antibody (1/100, goat anti-rabbit IgG H&L with Alexa Fluor 647, ab150079; Abcam, Cambridge, UK). It was kept at room temperature for 1 h in a light-protected humidity chamber. This was followed by washes in phosphate-buffered saline. Nucleic staining was performed by applying a dilution of SYTO13 (1/1000, Cat#14010; Lumiprobe, MD, USA) at room temperature for 20 min in a light-protected humidity chamber. The IF-stained slide was scanned and observed under the GeoMx instrument (NanoString Technologies Inc., WA, USA). To quantify the IF intensity in the IF images, ImageJ Fiji software (National Institutes of Health, MD, USA) was used.

2.6. Gene-set enrichment analysis

Enrichr, a comprehensive gene-set enrichment analysis web server (https://maayanlab.cloud/Enrichr/, assessment date: 23 January 2023), was utilized to examine the biological pathways associated with *TSPAN8* in CMS3 CRC (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021). According to the obtained RNA-Seq data, the enriched genes ($P < 0.05$; over 1-fold increase) in CMS3 compared with CMS1, CMS2, and CMS4 were first indicated and applied to the Enrichr web server.

2.7. Statistical analysis

Statistical analysis was performed according to data type and distribution. *P* value of <0.05 was considered statistically significant. The analysis was performed using IBM SPSS Statistics (SPSS Inc., IL, USA) and/or Prism (GraphPad Software, Inc., CA, USA). ANOVA test was applied for analyzing differential gene expression levels of *TSPANs* in CMS subtypes. *t*-test was utilized to assess differences in *TSPAN8* expression according to *KRAS* mutational status, as well as in the quantified IHC and IF intensities of TSPAN8-stained images. Gene expression correlation was analyzed using the built-in correlation function in Prism (GraphPad Software, Inc., CA, USA).

3. Results

3.1. Participant backgrounds and CMS classifications

Table 1 summarizes the clinical profiles of the CRC patients. Their mean age was 62.66 years; all underwent treatments based on the National Comprehensive Cancer Network guidelines. Primary CRC tumor tissues from left-sided colon, right-sided colon, and rectum were derived from both male and female participants (Table 1). All stages of cancer were represented among the participants, with most cancers classified as stage 3 (Table 1). As a result of the CMS classification via RNA-Seq-based comprehensive gene expression profiles, all primary CRC tumor tissues were classified into CMS1, CMS2, CMS3, CMS4, or mixed/indeterminate (Table 1). CMS2 (46%) predominated, followed by CMS4 (29%), CMS3 (18%), and CMS1 (5%) (Table 1).

3.2. RNA-Seq-based expression of TSPAN genes in CMS-classified primary CRC tissues

Fig. 1 depicts the RNA-Seq-based expression of all human *TSPAN* genes (33 genes; *TSPAN1* to *TSPAN33*) in the primary CRC tissues, clustered by columns. The *TSPAN8*, *TSPAN29*, and *TSPAN30* genes were highly expressed in the primary CRC tissues, with a TPM average exceeding 1 standard deviation from the overall average (Fig. 1A). The *TSPAN8*, *TSPAN29*, and *TSPAN30* genes had expression levels comparable to the *EPCAM* gene for the "epithelial cellular adhesion molecule" (EpCAM) protein which is a well-known cell surface tumor marker of epithelial origins (Fig. 1A) (Baeuerle and Gires, 2007). Interestingly, the *TSPAN8* gene and CMS3 were clustered on the heatmap's left side (Fig. 1A). To further demonstrate the relationship between the highly-expressed *TSPANs* and CMS, the expression levels of the *TSPAN8*, *TSPAN29*, and *TSPAN30* genes were separately and relatively plotted with CMS (Fig. 1B–D). Figs. 1B–D illustrate the intercorrelations among the highly expressed *TSPAN* genes and the CMS groups. In particular, *TSPAN8* was overexpressed in CMS3 of primary CRC tissues (Fig. 1B). As a result of additional analysis to assess *TSPAN8* expression according to

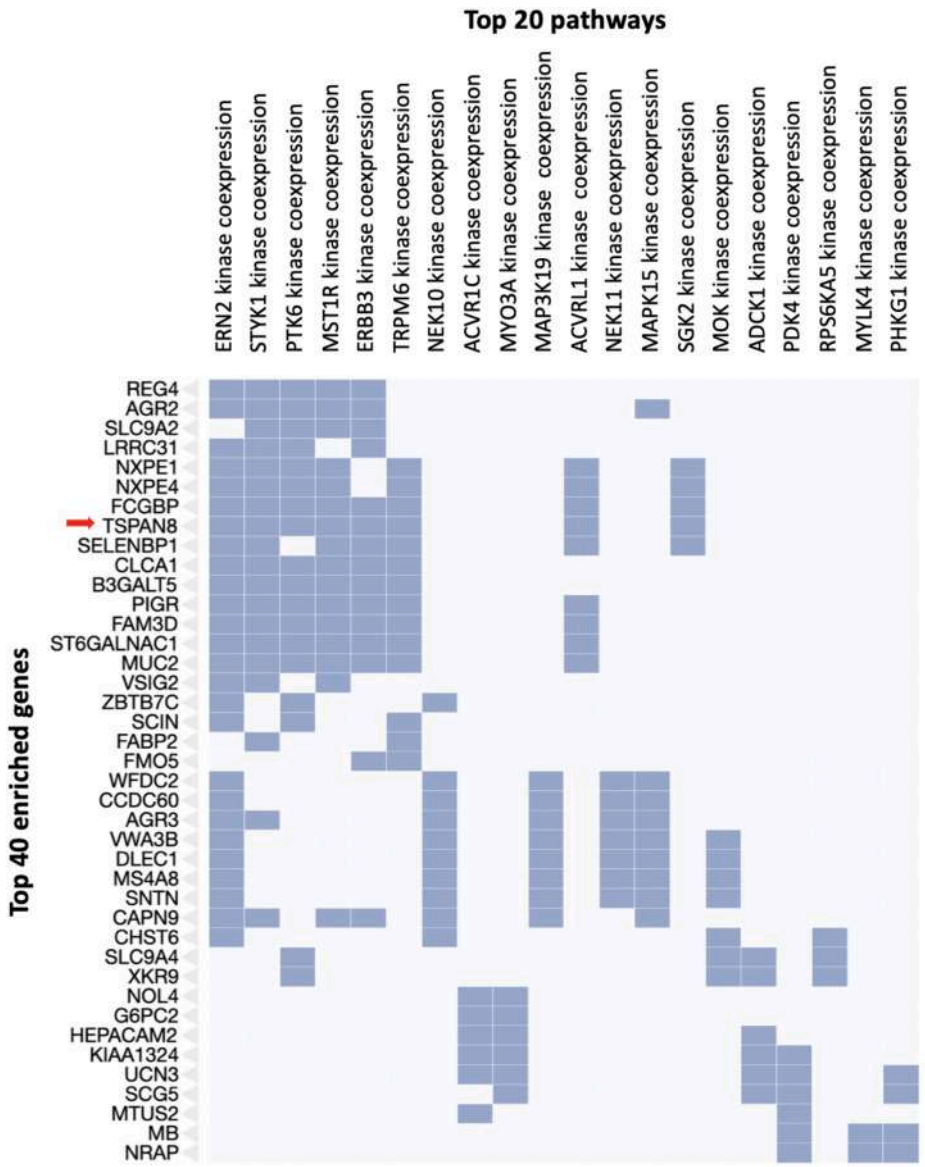

Fig. 4. Clustergram of top 20 ARCHS4 kinase coexpression pathways and top enriched 40 genes in CMS3 of CRC by Enrichr (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021). The red arrow indicates *TSPAN8* gene. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

known *KRAS* mutational status in our study cohort, we found that *TSPAN8* was also overexpressed in *KRAS* mutant-classified CRC tissues (Fig. 1E). This observation further strengthens the potential association between *TSPAN8* expression and *KRAS* mutations in CRC. These results suggest that TSPAN8 could be a candidate subtype-specific biomarker for metabolic CMS3 in CRC.

3.3. Overexpression of TSPAN8 protein in CMS3 of primary CRC tissues

To validate the overexpression of TSPAN8 protein in CMS3 of primary CRC tissues, IHC and IF staining was performed. Fig. 2A presents representative IHC images of TSPAN8 staining in normal and CMS1-, CMS2-, CMS3-, and CMS4-classified tissues. According to a protocol described in an earlier report (Crowe and Yue, 2019), the IHC intensity was quantified by ImageJ Fiji software (Fig. 2B). The TSPAN8 protein was particularly overexpressed in CMS3 CRC, consistent with the above RNA-Seq data (Fig. 1B). Furthermore, the overexpression of TSPAN8 protein in CMS3 CRC tissue was significantly observed by IF staining (Fig. 3). These results reveal that not only the *TSPAN8* gene but also the TSPAN8 protein is overexpressed in metabolic CMS3 CRC.

3.4. Potential involvement of TSPAN8 in kinase-based metabolic deregulation in CMS3 CRC

Gene-set enrichment analysis was performed to suggest the possible role of TSPAN8 in metabolic CMS3 CRC. Enriched genes (222 genes upregulated over 1-fold increase with $P < 0.05$; Supplementary File 1) in CMS3 compared with CMS1, CMS2, and CMS4 were listed and applied to Enrichr (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021). From all RNA-Seq and combining the chromatin immunoprecipitation sequencing (ChIP-Seq) sample and the signature search (ARCHS4) database (Lachmann et al., 2018), 23 ARCHS4 kinase coexpression pathways showed statistical significance ($P < 0.05$) in CMS3 CRC (Supplementary File 1). Table 2 shows the top 10 ARCHS4 kinase coexpression pathways and overlapping genes. Fig. 4 depicts the top 20 ARCHS4 kinase coexpression pathways and the top 40 enriched genes in CMS3 CRC. Interestingly, in 8 of the 23 significant pathways, *TSPAN8* was found in the gene sets co-expressed with *ERN2*, *STYK1*, *PTK6*,

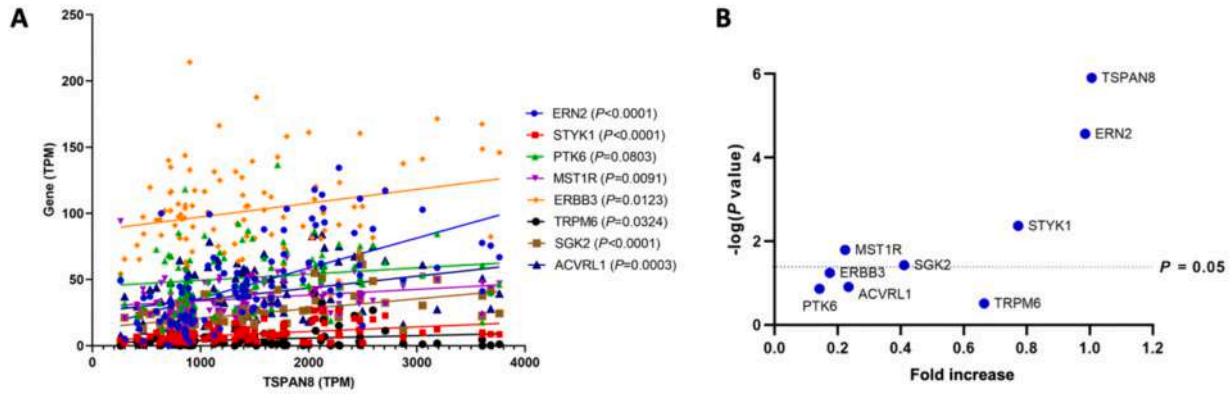

Fig. 5. (A) Scatterplot showing expression correlation between *TSPAN8* and *ERN2, STYK1, PTK6, MST1R, ERBB3, TRPM6, SGK2,* or *ACVRL1* across all CMS-classified tissues. *P* value (*P*) is shown in the bracket. (B) Fold increases of *TSPAN8* and *TSPAN8*-coexpressed human kinase genes in CMS3 CRC. The x-axis is fold change of gene expression in CMS3 compared with the other CMS groups. The y-axis is the -log10 (*P* value) results. Genes of interest are annotated within the plot. The dots above the dashed line denote genes that are statistically upregulated in CMS3.

MST1R, *ERBB3*, *TRPM6*, *SGK2*, and *ACVRL1* human kinases, and it was possibly associated with their pathways in CMS3 CRC (Table 2, Fig. 4, and Supplementary File 1). As shown in the clustergram (Fig. 4), these 8 human kinases and the overlapping genes and molecules are possible candidates for co-association with TSPAN8 in the metabolic deregulation of CMS3 CRC. Fig. 5A depicts a scatterplot illustrating the expression correlation between *TSPAN8* and *ERN2, STYK1, PTK6, MST1R, ERBB3, TRPM6, SGK2,* or *ACVRL1* across all CMS-classified tissues. Notably, *ERN2, STYK1, MST1R, ERBB3, TRPM6, SGK2,* and *ACVRL1* exhibited significant expression correlation with *TSPAN8* (Fig. 5A). In addition to the *TSPAN8* coexpression and correlation, *ERN2*, *STYK1*, *MST1R*, and *SGK2* genes were significantly up-regulated in CMS3 CRC according to our RNA-Seq data (Fig. 5B). Therefore, ERN2, STYK1, MST1R, and SGK2 molecules are likely to be significant partners of TSPAN8 in metabolic CMS3. Collectively, TSPAN8 may organize signaling membrane complexes for kinase-based metabolic deregulation in CMS3 CRC.

4. Discussion

Despite advances in diagnostic and therapeutic interventions, CRC—particularly colorectal adenocarcinoma—remains the most common gastrointestinal malignancy and the second leading cause of cancer mortality globally (Rawla et al., 2019; Hossain et al., 2022). The major issues associated with CRC are its diverse molecular makeup and its wide range of clinical outcomes and drug responses (Singh et al., 2019; Chan and Buczacki, 2021). To overcome those heterogeneities, a CMS-based classification system with clear biological interpretability has recently been proposed for clinical stratification and subtype-based targeted interventions (Guinney et al., 2015). Through their role in organizing multi-molecular membrane complexes, some TSPANs have been recognized as being involved in many malignancies, including CRC, and are proposed as potential therapeutic targets (Charrin et al., 2014; Zöller, 2009; Titu et al., 2021; Andrijes et al., 2021; Zhang et al., 2020; Malla et al., 2018; Heo and Lee, 2020; Suwatthanarak et al., 2023a; Suwatthanarak et al., 2023b). The current work is the first attempt to investigate the correlation between CMS, the most robust classification system for CRC, and *TSPANs*.

First, RNA-Seq was performed to evaluate gene expression in patient-derived primary CRC tissues ($n = 100$ colorectal adenocarcinoma patients) and to classify CMS. We found that 98 samples (98%) could be classified with single CMS, while 2 samples were identified as mixed or indeterminate (2%). In our cohort, the distribution of CMS groups was CMS1: 5%, CMS2: 46%, CMS3: 18%, and CMS4: 29%. Guinney et al. first reported the CMS distribution: CMS1: 14%, CMS2: 37%, CMS3: 13%, CMS4: 23%, and mixed or indeterminate: 7% (Guinney et al., 2015). Although the CMS percentage distribution of the present investigation differed from that of the previous report (Guinney et al., 2015), the CMS proportions were generally consistent, and CMS2 was still the most common CMS in both studies. The variations may arise from diversity in ethnicity, sampling protocol/location, sample size (ours: starting from n = 100; Guinney et al.: starting from $n = 858$), sample type (ours: fresh-frozen; Guinney et al.: fresh-frozen and FFPE), and gene expression platform (ours: RNA-Seq; Guinney et al.: RNA-Seq and microarray) (Guinney et al., 2015). Our classification results represent the CMS distribution of Thai patients, who were diagnosed and treated at Siriraj Hospital, the largest hospital in Thailand.

The expression of *TSPAN* genes was next examined to determine the correlations between CMS and *TSPANs*. We found that the *TSPAN8*, *TSPAN29*, and *TSPAN30* genes were highly expressed in primary CRC tissues, consistent with earlier reports (Titu et al., 2021; Zhang et al., 2020; Zhan et al., 2019). Of the highly expressed *TSPAN* genes, the *TSPAN8* gene showed statistically significant overexpression in CMS3 compared with the other CMS groups. As results of IHC and IF staining, the TSPAN8 protein was highly detected in the CMS3 group rather than the other CMS groups, supporting the RNA-Seq result. The IHC results also showed that the TSPAN8 protein was upregulated in CRC tissues compared with normal tissues. This finding agrees with the research by Zhan et al., which examined the expression of TSPAN8 upregulated in CRC tissues and cells, compared with normal adjacent tissues and normal colorectal epithelial cells, respectively (Zhan et al., 2019). The association of elevated TSPAN8 levels with tumor progression and invasion spans across a spectrum of cancers, including CRC, pancreatic cancer, gastric cancer, hepatocellular carcinoma, breast cancer, and ovarian cancer (Titu et al., 2021). The precise mechanisms through which TSPAN8 contributes to tumor progression may vary among these cancers, reflecting the complexity of its involvement in cellular processes. To the best of our knowledge, the overexpression of TSPAN8 in CMS3 CRC has not been reported. Therefore, this study reports the overexpression of TSPAN8 in CMS3 CRC for the first time. Consequently, our findings open the door to further investigations of TSPAN8 as a subtype-based targeted biomarker for CMS3 CRC.

We later conducted gene-set enrichment analysis to explore the potential role of TSPAN8 in metabolic CMS3 CRC. A list of the enriched genes in CMS3, compared with the other CMS groups, was applied to the Enrichr web server (Chen et al., 2013; Kuleshov et al., 2016; Xie et al., 2021). This enabled us to gain mechanistic insights into the gene lists and identify relative biological pathways enriched in CMS3 CRC from our RNA-Seq data. CMS3 in CRC is associated with a distinct metabolic phenotype characterized by heightened glycolytic activity, increased

dependence on glucose metabolism, and alterations in lipid metabolism. This metabolic profile often exhibits the Warburg effect, wherein cancer cells favor glycolysis for energy production even in the presence of oxygen. Additionally, dysregulation in lipid metabolism involves changes in lipid synthesis and breakdown, impacting cellular processes such as membrane composition and signaling. Thus, the CMS3-enriched gene set was found to be relevant to metabolic pathways by the BioPlanet 2019, WikiPathway 2021 Human, and KEGG 2021 Human databases (Supplementary File 1) (Huang et al., 2019; Martens et al., 2021; Kanehisa et al., 2021). This finding supported the accuracy of our CMS classifications. Unfortunately, the *TSPAN8* gene was not listed in the metabolic pathways from these three databases since experimental data on TSPAN8 are still limited. However, the *TSPAN8* gene was listed in some pathways in the ARCHS4 kinase coexpression resource generated from the top 300 genes in the ARCHS4 database, which are co-expressed with kinases (Lachmann et al., 2018). By extensively mining publicly available data from all human and mouse RNA-Seq experiments, ARCHS4 resource provides access to gene and transcript counts from the Gene Expression Omnibus and the Sequence Read Archive (Lachmann et al., 2018). As described in the Results section, the *TSPAN8* gene was significantly and statistically listed in the gene sets co-expressed with particular human kinase genes: *ERN2*, *STYK1*, *PTK6*, *MST1R*, *ERBB3*, *TRPM6*, *SGK2*, and *ACVRL1*, in CMS3. Notably, all 8 of these human kinases have been reported to be involved in CRC or other human malignancies (Xia et al., 2020; Essegian et al., 2020; Hu et al., 2015; Mathur et al., 2016; Cazes et al., 2022; Greally et al., 2018; Qin et al., 2020; Liu et al., 2020; Hanna et al., 2018). STYK1, MST1R, ERBB3, TRPM6, and ACVRL1 are receptor kinases, while ERN2, SGK2, and PTK6 are non-receptor kinases. Based on this finding, we propose that TSPAN8 may directly or indirectly associate with these 5 receptor kinases (STYK1, MST1R, ERBB3, TRPM6, and ACVRL1), overlapping molecules, and other molecules to form signaling membrane complexes that control proliferation, differentiation, and metabolism in CMS3 CRC. Furthermore, TSPAN8 may indirectly contribute to the pathways associated with ERN2 and SGK2 kinases in metabolic CMS3 CRC. In particular, ERN2, STYK1, MST1R, and SGK2 kinases, which also up-regulated in CMS3 CRC, may be the TSPAN8's major partners for metabolic deregulation in CMS3. Kinase signaling, especially via receptor tyrosine kinases, is a well-known driver of oncogenesis, tumor growth, and metabolism in numerous cancers, including CRC (Wiese and Hitosugi, 2018; García-Aranda and Redondo, 2019). In a recent report, TSPAN8 was associated with human metabolic regulation and diseases, including cancer (De et al., 2021). Furthermore, TSPAN8 has been associated with kinase signaling in some human malignancies (Heo and Lee, 2020; Wei et al., 2015). Therefore, in addition to the previously reported roles of TSPAN8 in CRC stemness, motility, progression, and metastasis (Zhang et al., 2020; Heo and Lee, 2020; Zhan et al., 2019), the current study suggests another candidate role of TSPAN8 in metabolic deregulation, particularly for CMS3.

As Bramsen et al. demonstrated that combining subtyping and subtype-specific biomarkers could improve patient prognostication and provide a strong basis for future studies in CRC (Bramsen et al., 2017), TSPAN8 appears to be a promising candidate biomarker. Moreover, TSPAN8 is an emerging therapeutic target in cancers for monoclonal antibody therapy and radioimmunotherapy (Heo and Lee, 2020; Maisonial-Besset et al., 2017). In our RNA-Seq data, while the previously reported ErbB tyrosine kinase genes in CRC, including *EGFR*, *ERBB2*, *ERBB3*, and *ERBB4*, were expressed with averages ranging between approximately 10 and 100 TPM, *TSPAN8* was expressed with an average exceeding 1000 TPM. Thus, the TSPAN8 protein may serve as a potential biomarker and therapeutic target for CMS3 CRC. (Roskoski Jr., 2014; Mishra et al., 2018; Xie and Bourne, 2015; Tanjak et al., 2023).

Due to the limited sample size and diversity, and the experimental evidence we have presented, the role of TSPAN8 in kinase-based metabolic deregulation in CMS3 CRC should be further clarified in both in vitro and in vivo assays. Moreover, the mRNA and protein expressions of TSPAN8 and related molecules in CMS3 CRC should be confirmed by other techniques, such as RT-PCR and Western blot, in the future.

In summary, we report the overexpression and potential role of TSPAN8 in CMS3 CRC, thereby providing a foundation for CMS3-based further research.

5. Conclusions

This study evaluated the transcriptomic correlation among TSPAN membrane organizers and CMS classification in primary tissues of colorectal adenocarcinoma. The *TSPAN8*, *TSPAN29*, and *TSPAN30* genes were highly expressed in CRC tissues. In particular, *TSPAN8* was overexpressed in CMS3 CRC. TSPAN8 overexpression was assessed by IHC and IF staining in CMS3 CRC tissues. Moreover, gene-set enrichment analysis findings suggest that TSPAN8 has the potential to organize signaling complexes for kinase-based metabolic deregulation in CMS3 CRC. Our findings provide advantages for molecular cancer biology related to CMS3 CRC.

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Ethics statement

This study was approved by the Siriraj Institutional Review Board (Si 105/2021) and performed in accordance with the International Guidelines for Human Research Protection, including the Declaration of Helsinki. Written informed consent was willingly provided by each participating patient.

Consent for publication

This manuscript does not contain any individual person's data in any form.

Author contributions

The authors confirm contribution to this study as follows: study conception and design: Thanawat Suwatthanarak, Pariyada Tanjak, Vitoon Chinswangwatanakul; investigation: Thanawat Suwatthanarak, Pariyada Tanjak, Onchira Acharayothin, Kullanist Thanormjit; resources; Amphun Chaiboonchoe, Jantappapa Chanthercrob, Ananya Pongpaibul, Wipapat Vicki Chalermwai, Atthaphorn Trakarnsanga, Asada Methasate; data collection: Thanawat Suwatthanarak, Pariyada Tanjak, Amphun Chaiboonchoe, Onchira Acharayothin, Kullanist Thanormjit; analysis and interpretation of results: Thanawat Suwatthanarak, Pariyada Tanjak, Amphun Chaiboonchoe, Tharathorn Suwatthanarak, Apichaya Niyomchan, Masayoshi Tanaka, Mina Okochi, Atthaphorn Trakarnsanga, Asada Methasate, Manop Pithukpakorn, Vitoon Chinswangwatanakul; funding: Thanawat Suwatthanarak, Pariyada Tanjak, Manop Pithukpakorn, Vitoon Chinswangwatanakul; draft manuscript preparation: Thanawat Suwatthanarak, Pariyada Tanjak, Kullanist Thanormjit; administration: Vitoon Chinswangwatanakul. All authors reviewed and approved the final version of the manuscript.

CRediT authorship contribution statement

Thanawat Suwatthanarak: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Pariyada Tanjak:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Amphun Chaiboonchoe:** Writing – review & editing, Resources, Formal analysis, Data curation. **Onchira Acharayothin:** Writing – review & editing, Methodology, Investigation, Data curation. **Kullanist Thanormjit:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Jantappapa Chanthercrob:** Writing – review & editing, Resources. **Tharathorn Suwatthanarak:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Apichaya Niyomchan:** Writing – review & editing, Formal analysis. **Masayoshi Tanaka:** Writing – review & editing, Formal analysis. **Mina Okochi:** Writing – review & editing, Formal analysis. **Ananya Pongpaibul:** Writing – review & editing, Resources. **Wipapat Vicki Chalermwai:** Writing – review & editing, Resources. **Atthaphorn Trakarnsanga:** Writing – review & editing, Resources, Formal analysis. **Asada Methasate:** Writing – review & editing, Resources, Formal analysis. **Manop Pithukpakorn:** Writing – review & editing, Funding acquisition, Formal analysis. **Vitoon Chinswangwatanakul:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2024.104911.

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