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Emerging role and clinical implication of mRNA scavenger decapping enzyme in colorectal cancer

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ABSTRACT

Background: Turnover of RNA is a regulated process that in part controls gene expression. This process is partly controlled by the scavenger decapping enzyme (DcpS). This study aimed to investigate the expression of DcpS in colorectal cancer (CRC) tissue, to evaluate its prognostic significance in patients with CRC and to investigate potentially targeted genes by DcpS.
Methods: Immunohistochemical analysis was used to determine localization of DcpS in normal and CRC tissue, western blot analysis for quantification of protein expression and qPCR for mRNA expression in normal and CRC tissue and expression in cell lines after silencing using siRNA. Gene array analysis was used to study regulation of genes after silencing of DcpS. Proliferation was studied using BRDU.
Results: DcpS expression was localized to the epithelial cells of both control and cancer tissue. Tumor and paired control tissue samples from 100 patients who underwent surgical resection for primary colorectal adenocarcinomas were utilized. mRNA and protein of DcpS was significantly up-regulated in the patients with CRC and the mRNA level was higher in rectal cancer tissue compared to colon cancer tissue ($p < 0.05$). Lowest tertile levels of DcpS mRNA in cancer tissue was associated with a decreased cancer-specific survival rate with a hazard ratio (HR) of 4.7 (95% CI=1.02–12.3), independent of disease stage. The low level of DcpS mRNA was a predictor of poorer survival in patients with rectal and disseminated cancer and in patients receiving adjuvant treatment ($p < 0.05$). After silencing DcpS in Caco-2 cancer cells, altered expression of several genes associated with RNA, cell cycle regulation, alternative splicing and microRNA was observed and resulted in 23% increase in proliferation.
Conclusions: These results indicate that DcpS has potential as a prognostic factor for CRC but further studies in a broader cohort are warranted to evaluate the significance of the findings in the clinic.

1. Introduction

Colorectal cancer (CRC) is one of the most common and deadliest cancers worldwide [1]. The etiology of sporadic CRC is not completely known. In recent years, biomarkers have played an increasingly vital role in the detection of CRC and to guide the choice of treatment, predict prognosis and help in the selection of personalized therapy [2]. CRC is a heterogenous disease, and it is well known that various pathways contribute to the CRC pathogenesis including genetic and epigenetic changes which can affect the individual variability in CRC susceptibility [3,4]. RNA methylation inducing translational inhibition and mRNA degradation plays a crucial role in the gene expression and is, among other things, associated with gastrointestinal cancers [5]. One type of RNA methylation includes the formation of 7-Methylguanosine (m7G) which can be found in mRNA caps [6]. The mRNA turnover strategy involves removal of the poly(A) tail followed by decapping of the mRNA by the scavenger decapping enzyme DcpS [7]. DcpS is a 40 kDa nucleocytoplasmatic shuttling protein and is a member of the Histidin triad family (HIT) of pyrophosphatases [8]. DcpS release short mRNA fragments containing the 5′ mRNA cap structure, hydrolyzes the resulting cap structure following decay by the 3′→5′ mRNA decay pathway with following deadenylation and exosome-mediated turnover [7].

Both variation of mRNA splicing, and presence of microRNA (miRNA) are important for CRC progression including proliferation,

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apoptosis and metastasis [9,10]. Interestingly, it has been postulated that the presence of DcpS can modulate nuclear pre-mRNA splicing and control miRNA turnover in human cells [11,12]. There are other RNA decapping enzymes involved in the mRNA turnover. The silencing of the RNA decapping enzyme NUDT16 mediates c-Myc activation in T-cell acute lymphoblastic leukemia [13].

Knowledge of DcpS in relation to CRC is scarce. The aim of this study was to characterize expression of DcpS and its association with various clinical features in patients with CRC. Furthermore, we hypothesize that DcpS is a functional regulator of gene expression in CRC and investigated after silencing DcpS in colon cancer cells the potentially targeted pathways of potential importance in CRC carcinogenesis.

2. Materials and methods

2.1. Study population and specimen collection

This study utilized tumor and paired control tissue samples from 100 patients who underwent surgical resection for primary colorectal adenocarcinomas between February 2014 and January 2017 at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Follow-up for the estimation of cancer specific survival ended on the date of death or in May 2023. Tumor tissue and adjacent control mucosa (about 5 cm from the tumor) from each patient, were excised and immediately frozen at − 78 °C until analysis. All tissue samples intended for RNA analysis were stored in RNA protect tissue reagent (Qiagen) to maintain good RNA quality. The tumors were classified according to American Joint Committee on Cancer (AJCC) [14]. Clinicopathological characteristics of the patients are shown in the Table 1. The investigation was approved by the Regional Ethical Review Board in Linköping, Sweden and informed consent was obtained from each of the participants. All research was performed in accordance with relevant guidelines/regulations and in accordance with the Declaration of Helsinki.

2.2. Western blot

Nineteen frozen tumor and paired control tissue were thawed and homogenized in ice cold RIPA lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) containing a protease inhibitor cocktail according to the manufacturer's instructions. The lysate was placed on ice for 30 min and then centrifuged at 18000 x g for 10 min. The protein content of the supernatant fluid was determined for each sample using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

The DcpS protein expression was analyzed in the lysates from the cancer tissue and paired normal tissue using rabbit anti-DcpS antibody (# PA5–30566, Thermo Fisher Scientific, Sweden), diluted 1:1000 following a standard protocol as described previously [15].

Table 1
Clinicopathological characteristics of 100 patients with CRC.

Characteristic	No. of patients (%)
Gender	
Male	50 (50)
Female	50 (50)
Age (years)	
≤70	41 (41)
>70	59 (59)
Tumor differentiation	
High/Medium	75 (75)
Poor	25 (25)
TNM stage	
I	16 (16)
II	43 (43)
III	37 (37)
IV	4 (4)
Localization	
Colon	53 (53)
Rectum	47 (47)
Histologic type	
Non-mucinous	86 (86)
Mucinous	14 (14)
Adjuvant treatment	
Yes	30 (30)
No	70 (70)

2.3. Immunohistochemistry

Ten formalin-fixed and paraffin-embedded tumor samples from the archives of the Department of Pathology County Hospital Ryhov, Jönköping, Sweden were available for immunohistochemical staining to study the cell type origin of the DcpS expression. The paraffin blocks specimens were cut into 3.5 μm sections and stained with rabbit anti-DcpS antibody (Invitrogen, Thermo Fisher Scientific) diluted 1:500 using standard protocol as described previously [16].

2.4. RT-qPCR

CRC tissue (n = 100) and adjacent control tissues samples (n = 85), of which 81 were paired. RNA was extracted using RNeasy Mini kit (Qiagen) in accordance with the manufacturer's instructions. Total RNA was reverse transcribed using Super Script III kit (#11752, Thermo Fisher Scientific) and the complementary DNA was amplified through RT-qPCR using probes for DcpS (Hs00204009 ml) that was normalized against GAPDH (Hs02758991_g1) (Thermo Fisher Scientific).

2.5. Silencing of DcpS in Caco-2 cells

Caco-2 cells (ATCC) were grown in DMEM supplemented with 10% FBS serum and seeded out in 96-well plates 0.01 × 10^6 cells. After attachment, cells were starved for 24 h in media containing 0.1% FBS to synchronize cells. Medium was thereafter replaced with medium with 10% FBS and transfected with 5 nmol Silencer Select siRNA (#122649, Thermo Fisher Scientific) or negative control (# 4390846, Thermo Fisher Scientific) mixed with Lipofetamine RNAiMAX reagent (#13778030, Thermo Fisher Scientific) for 24 and 48 h, according to the instructions. Cells were then analyzed for proliferation and viability as described below or frozen down in − 80 °C for RNA analysis. RNA quality and concentration was determined using TapeStation (Agilent).

Transfection efficiency was determined by analysis of DcpS mRNA expression by qPCR. Gene array analysis was carried out using Clariom D arrays (Thermo Fisher Scientific) after silencing DcpS in Caco-2 cells after 24 h to determine alterations in splicing and gene expression and was carried out by the core facility for Bioinformatics and Expression Analysis (Karolinska Insitute, Huddinge, Sweden). Functional annotation of miRNA was performed using David bioinformatics resource (www.David.ncifcrf.org). Analysis of potential pathways on altered genes was performed using Reactome pathway analysis tool (www.Reactome.org).

2.6. Proliferation and viability of Caco-2 cells after DcpS silencing

For analysis of proliferation, 10 μl of BRDU reagent (#11444611001), diluted 1:1000, was added to each 96-well with cells treated as mentioned above, and incubated for 4 h in accordance to the instructions (Roche, Sigma-Aldrich, Sweden). Cells were fixed and denatured and anti-BrdU-POD antibody was added followed by washing and adding of substrate. Proliferation was analyzed against a standard curve at 450 nm with 690 nm as reference, after silencing of DcpS in relation to control.

For analysis of viability, 10 μl AlamarBlue (#A50100, Thermo Fisher Scientific) was added to each 96-well plate and incubated for 24 h and analyzed at 570 nm with 600 nm as reference, according to the instructions.

2.7. Statistical analysis

The comparisons between groups regarding protein and mRNA expression DcpS were performed with non-parametric tests. The Wilcoxon's signed-rank test and the Mann-Whitney *U*-test were used for the analysis of the related and independent parameters. Survival analysis was performed by Kaplan-Meier analysis with the log-rank test and Cox's regression. Statistical analysis was performed using Stata Statistical Software Release 15, Stata Corp. College Station, TX, USA and SPSS software for Windows, version 14.0 for (SPSS Inc., Chicago, IL, USA). Results were considered significant at $p < 0.05$.

3. Results

3.1. Protein and gene expression of DcpS in cancer and adjacent tissue in relation to clinical feature

To assess whether DcpS is expressed by colorectal tissue and cancer, immunohistochemical analysis and Western blot analysis were carried out. Cancer and adjacent control tissue showed positive and negative to weak immunohistochemical staining, respectively (Fig. 1A-E). Predominantly cytoplasmic staining was evident in the epithelial cells of both control and cancer tissue.

In a subset of 19 patients, protein levels of DcpS was up-regulated in tumor tissue in 14 the of cases (74%), with a 2-fold ($p < 0.01$) higher level in cancer tissue compared to normal tissue (Fig. 1F and G).

Using RT-qPCR, the expression of DcpS mRNA was higher in tumor tissue compared with control paired tissue ($p < 0.001$) (Fig. 2A). Evaluation of the relative expression (tumor vs. adjacent paired tissue) mRNA showed an up-regulation in 52 of 81 (64%) matched samples of the patients. There was also a higher expression of DcpS mRNA in rectum compared to colon cancer tissue ($p < 0.001$) (Fig. 2B). Interestingly, there was a lower expression of DcpS mRNA among those patients who eventually died from their cancer ($p < 0.05$) (Fig. 2C).

There were no other significant relationships between reported clinical characteristics (as shown in Table 1) and mRNA DcpS levels.

3.2. Survival analysis according to the level of DcpS mRNA in cancer tissue

When patients were grouped into tertiles regarding the levels (low, medium and high) of DcpS mRNA there was a gradual decrease of the cancer-specific survival where the patients with low levels had lower cancer specific survival rate (72%) compared to those with highest tertile levels (93%), according to Kaplan-Meier analyses with log-rank test (Fig. 3A). Cancer specific survival was associated with a hazard ratio (HR) of 4.7 (95% CI=1.02–12.3), $p < 0.05$. After adjusting for disease stage, the results remained, HR= 5.0 (95% CI=1.07–23.0), $p < 0.05$. Stratification analysis of association between low level of DcpS mRNA and survival showed worse survival for rectal and disseminated (stage III+IV) cancer and in patients receiving adjuvant treatment (Fig. 3B-D).

3.3. Effect of silencing of DcpS in Caco-2 cells

Caco-2 and HT-29 colon cancer cells were evaluated by qPCR to determine if they expressed DcpS and which cell type had the highest basal expression for further silencing strategy. Both cell lines expressed DcpS at equal levels (Fig. 4A). The pro-inflammatory cytokines IFN-γ was used as stimuli to see if the expression could be altered. The effects of IFN-γ was different in the used cell lines. IFN-γ tended to decrease DcpS expression in the Caco-2 cells and increase it in the more proliferative HT-29 cells. Caco-2 cells were chosen for further experiments.

After transfection of siRNA DcpS for 24 h and 48 h DcpS was silenced by 95% and 97%, respectively (Fig. 4B). This was associated with a decrease in total RNA content of the cells (Fig. 4C) and the proliferation

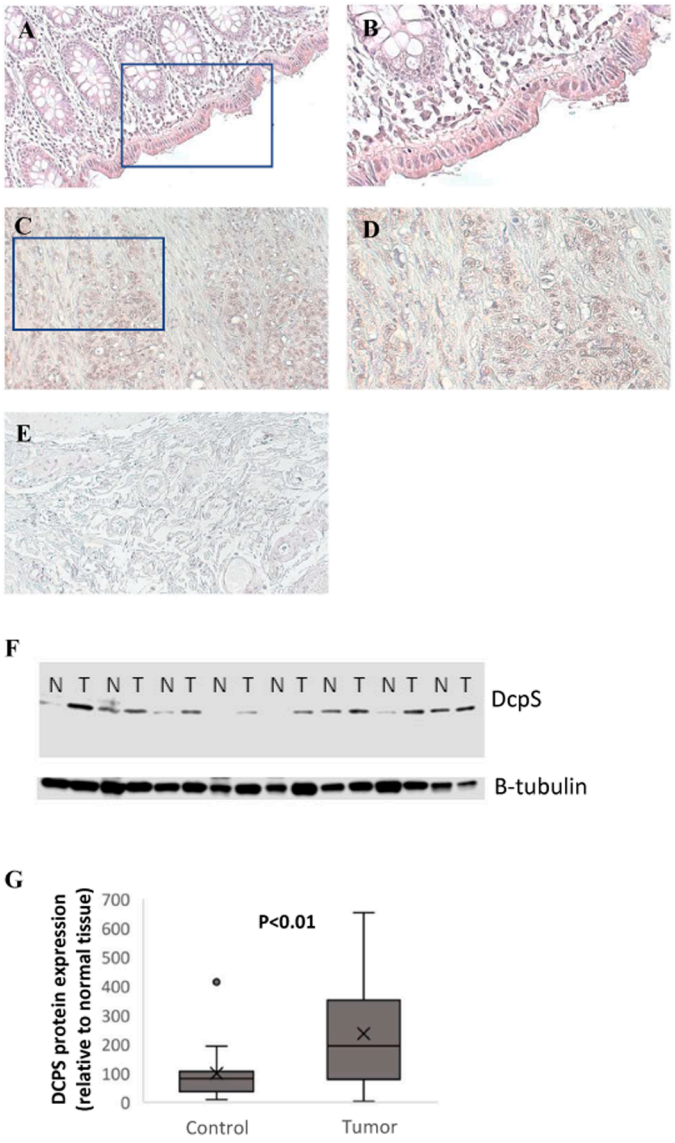

Fig. 1. DcpS protein expression in colorectal cancer and control tissue. (A and B) DcpS staining in adjacent normal tissue. (C and D) DcpS staining in colorectal cancer. (E) Negative control omitting DcpS antibody. (F) Western blot analysis of DcpS and Beta-tubulin for loading. (G) Relative expression of DcpS from quantification of Western blot analysis.

was significantly increased by 23% after transfection of siRNA DcpS for 48 h (Fig. 4D) without affecting viability (data not shown).

To investigate potentially targeted pathways after silencing DcpS in Caco-2 cells gene array.

analysis covering genes with EntrezGene ID with an expression over 8, in any of the groups, that 1018 genes were altered after silencing DcpS. One hundred of the most significant altered genes are described in Supplementary Table 1. Among these 1018 genes, only 144 were up-regulated whereas the majority were down-regulated. Expression of 108 miRNAs, 251 small nuclear or nucleolar RNAs, 53 circRNA and 50 lincRNA were altered. The role of small nuclear and nucleolar RNA, circRNA and lincRNA is largely unknown except for miRNAs where functional annotation using David bioinformatics resource indicate that these miRNAs are involved in gene silencing, mRNA binding involved in posttranscriptional gene silencing. RNA-induced silencing complex, miRNAs in cancer, mRNA 3′-UTR binding, miRNA mediated inhibition of translation, mRNA cleavage involved in gene silencing by miRNA and extracellular space. In addition to these 1018 genes, 4852 non-coding

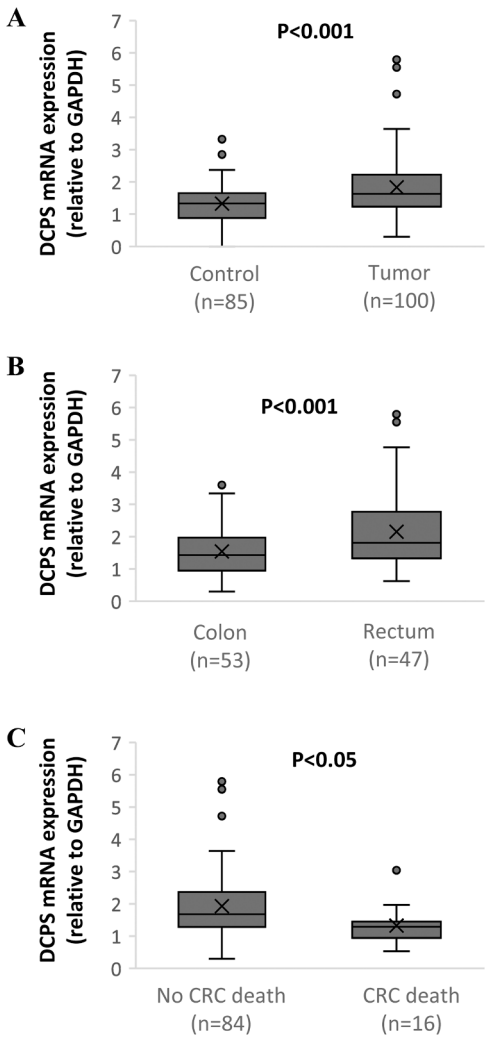

Fig. 2. Expression of DcpS mRNA. (A) Expression of DcpS mRNA in colorectal cancer and control tissue, (B) in cancer tissue localized to rectum and colon, (C) in tumor tissue from patients diagnosed with cancer specific death. DcpS mRNA was normalized to GAPDH expression. Abbreviations: CRC; colorectal cancer.

RNAs were altered. Reactome pathway analysis revealed that the most potential pathways are in connection with RNA and cell cycle regulation (Table 2). 4977 genes were altered with regards to expression on exons within their respective genes. Reactome pathway analysis of these genes show an association with collagen biosynthesis and extracellular matrix as well as MET receptor tyrosine kinase signaling (Table 3). Several of the pathways indicate that DcpS regulates pathways of importance in CRC carcinogenesis.

4. Discussion

Up to now, there has been little information available about the connection between DcpS and its clinical implication on CRC. The present study assesses the potential association of DcpS in CRC and reflect the prognosis related to patients with CRC and tries to functionally describe a potential role in colorectal carcinogenesis.

Evaluation of the relative (tumor vs. normal paired tissue) expression of mRNA and protein regarding DcpS showed an up-regulation in the tumor tissue. We also noted an expression of DcpS mRNA in Caco-2 cells, confirming that cancer epithelial cells can express the factor. Recently, a study has been published on potential genes related to the regulation of RNA methylation focused on the modification of m7G [17]. It was shown here that DcpS is one of several genes and that the expression of

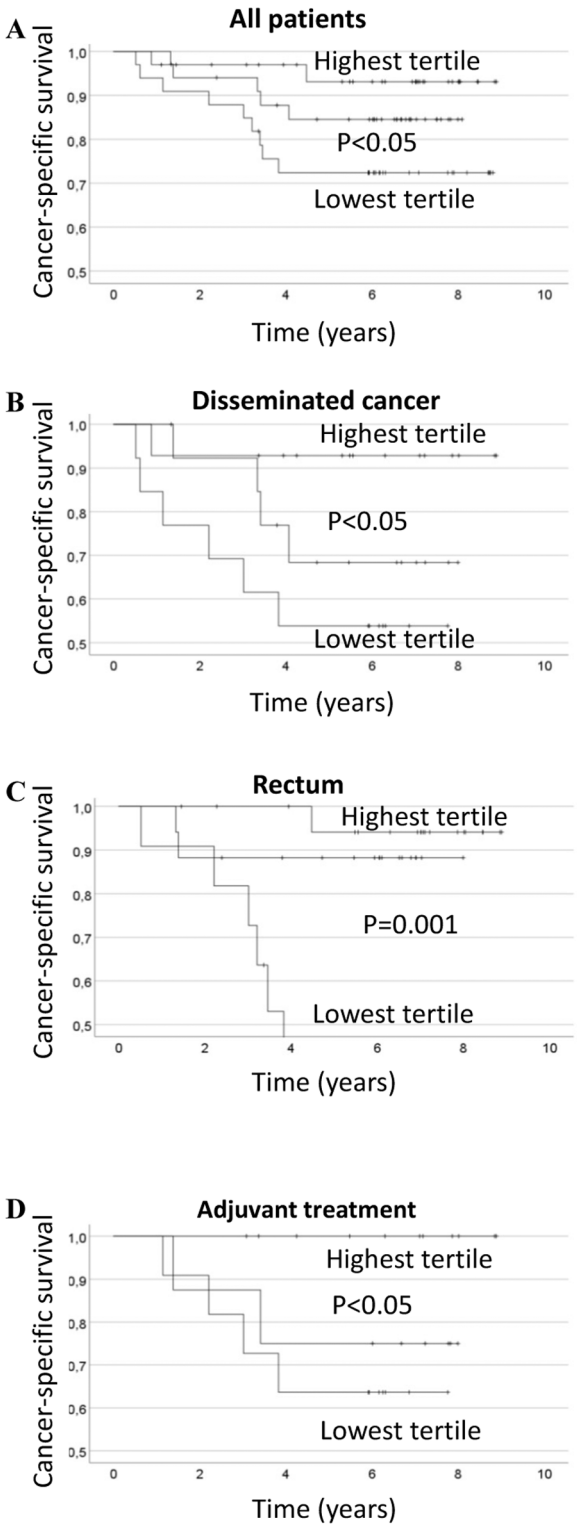

Fig. 3. DcpS influence cancer specific survival. (A) Cancer specific survival in all patients, (B) in patients with disseminated cancer (stadium III and IV), (C) in patients with cancer localized to rectum and (D) in patients with adjuvant treatment.

DcpS was higher in colon adenocarcinoma compared to regular colon tissue which is consistent with our results in patient tissues. However, our examination included rectal cancer and we observed a significantly higher level in rectal cancer tissue compared to colon cancer tissue. It has been suggested that adenocarcinoma of the colon and rectum should

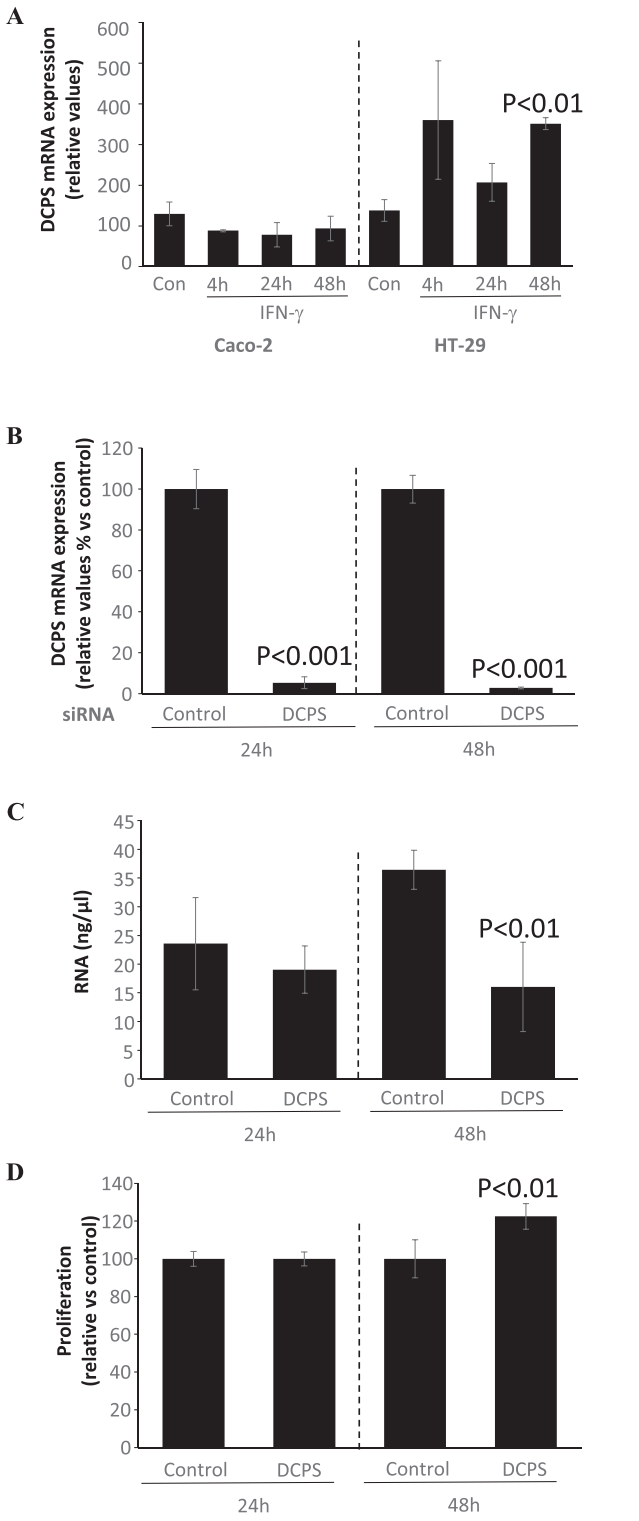

Fig. 4. DcpS mRNA expression in colon cancer cells. (A) DcpS mRNA expression in Caco-2 and HT-29 cells in unstimulated cells and IFN-γ stimulated cells during 4, 24 and 48 h. (B) DcpS mRNA expression after silencing of DcpS in relation to control siRNA in Caco-2 cells. (C) RNA content after silencing of DcpS in relation to control siRNA at 24 and 48 h in Caco-2 cells. (D). Effect of DcpS silencing on proliferation at 24 and 48 h in Caco-2 cells.

be considered as two distinct entities [18,19]. Possible differences in the carcinogenesis of colon and rectal carcinoma could explain the level difference of DcpS.

In this study, lower levels of DcpS in cancer tissue was associated with worse cancer-specific survival. This effect could partly be explained by the fact that low levels of DcpS lead to increased proliferation, which is supported by the fact that we showed an increased proliferation when silencing DcpS in Caco-2 cells. Specifically, we found that the low level showed a prognostic value in rectal cancer, disseminated cancer and in patients who received adjuvant treatment.

DcpS is a nucleo-cytoplasmatic shuttling protein with a broad functionality. Variation of mRNA splicing, and presence of miRNA regulate proliferation, apoptosis, invasion, metastasis and play an important role for CRC progression activity [9,10]. It has been reported that the

Table 2
Twenty-five most potential pathways in Caco2 cells after silencing of DcpS (sorted by p-value), found in Reactome pathway analysis.

Pathway name	Entities	p-value
Condensation of prophase chromosomes	16/54	0.00000558
RNA polymerase I promoter escape	15/60	0.0000729
B-WICH complex positively regulates rRNA expression	15/61	0.0000872
SIRT1 negatively regulates rRNA expression	13/44	0.000170
Transcriptional regulation by small RNAs	17/80	0.000172
Assembly of the ORC complex at the origin of replication	11/39	0.000237
RNA polymerase I promoter opening	10/33	0.000257
Inhibition of DNA recombination at telomere	12/47	0.000307
Recognition and association of DNA glycosylase with site containing an affected pyrimidine	11/41	0.000359
DNA methylation	10/35	0.000405
Abortive elongation of HIV-1 transcript in the absence of Tat	8/24	0.000565
Mitotic prophase	22/134	0.000730
Transcription of E2F targets under negative control by p107 (RBL1) and p130 (RBL2) in complex with HDAC1	7/20	0.000932
Gene silencing by RNA	20/119	0.000943
Packaging of telomere ends	9/33	0.001
Positive epigenetic regulation of rRNA expression	15/79	0.001
NoRC negatively regulates rRNA expression	15/79	0.001
HDACs deacetylate histones	13/63	0.001
Amyloid fiber formation	16/89	0.002
Cleavage of the damaged pyrimidine	11/50	0.002
Depyrimidination	11/50	0.002
RNA polymerase II pre-transcription events	15/82	0.002
PRC2 methylates histones and DNA	10/43	0.002
Defective pyroptosis	11/51	0.002
RNA polymerase I promoter clearance	15/84	0.002

Entities found: the number of curated molecules of the type selected with Results Type that are common between the submitted data set and the pathway named in column 1. Entities total: The total number of curated molecules of the type selected with Results Type within the pathway named in column 1. 557 out of 1030 identifiers in the sample were found in Reactome, where 1391 pathways were hit by at least one of them. All non-human identifiers have been converted to their human equivalent.

Table 3
Most potential pathways associated with alternative splicing in Caco-2 cells after silencing of DcpS (sorted by p-value), found in Reactome pathway analysis.

Pathway name	Entities	p-value
Collagen chain trimerization	32/44	0.00018
Degradation of the extracellular matrix	73/148	0.004
MET activates PTK2 signaling	21/32	0.007
MET promotes cell motility	23/195	0.013
NrCAM interaction	15/109	0.014
Collagen degradation	4/10	0.017
Laminin interaction	20/173	0.018
Non-integrin membrane ECM interaction	16/127	0.034
Collagen biosynthesis and modifying enzymes	4/11	0.038

Entities found: the number of curated molecules of the type selected with Results Type that are common between the submitted data set and the pathway named in column 1. Entities total: The total number of curated molecules of the type selected with Results Type within the pathway named in column 1. 2863 out of 4968 identifiers in the sample were found in Reactome, where 2159 pathways were hit by at least one of them. All non-human identifiers have been converted to their human equivalent.

presence of DcpS can modulate nuclear pre-mRNA splicing and control cytoplasmic miRNA turnover independently of its scavenger decapping activity [11,12]. After silencing of DcpS in Caco-2 cells we performed gene array analysis that showed an altered expression of several genes. Following reactome pathway analysis we found potential pathways associated with RNA and cell cycle regulation, alternative splicing and miRNA which is in accordance with its known role. For example, we found that DcpS affected expression of miRNA-373 and miRNA-1299, which both have been shown to have diverse functions in cancer and have been suggested to function as tumor suppressors in CRC [20,21].

The present study has some limitations. The patients were selected from one hospital and may not represent the general population. As some analysis is performed on smaller sub-groups the results should be interpreted with caution and should be regarded as hypothesis generating.

In conclusion, this study demonstrated that the gene expression of DcpS was up-regulated in CRC tissue compared to paired normal tissue and that the level of DcpS was independent of the disease stage but higher in rectal than colon cancer. Moreover, we can speculate whether a low level of DcpS has a modulating role regarding the clinical outcome with less favorable prognosis. This is especially seen in rectal cancer, disseminated cancer and in patients who received adjuvant treatment. As part of understanding the role and driving regulatory mechanisms of DcpS in CRC, we performed silencing of DcpS in a colon cancer cell line with subsequent gene array to study target genes. Based on our clinical findings and insight into possible pathways DcpS is associated with, this study can be the basis for a larger prospective study in which the role of DcpS as a biomarker is clarified.

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CRediT authorship contribution statement

Dick Wågsäter and **Jan Dimberg** designed and prepared the main manuscript. **Levar Shamoun** prepared clinical samples and **Gustaf Johansson, Dick Wågsäter** and **Levar Shamoun** performed laboratory work. **Gustaf Johansson, Dick Wågsäter** and **Jan Dimberg** analyzed data. Kalle Landerholm provided clinical insights and took responsibility for patient data and follow up. All authors were involved in interpretation of data, read and approved the final manuscript.

Declaration of Competing Interest

This work was supported by grants from Division of Medical Diagnostics of Region Jönköping County, Sweden. No. Futurum-970572 and Swedish Research Council (2019–01673). The founders did not influence the work and the authors declare no conflict of interest.

Data Availability

Datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.155009.

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