Original paper

Lowered expression level of *INSC* predicts poor prognosis in patients with colon cancer

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The aim of this study was to investigate the prognostic value of inscuteable spindle orientation adaptor protein (*INSC*) in colon cancer (CC).
Firstly, transcriptional change of *INSC* was analysed using the data from public databases. Next, *INSC* protein expression was assessed by immunohistochemistry. Its correlation with clinicopathological features and the prognostic values of patients were also investigated. Then, an *INSC*-based nomogram was built to predict CC prognosis.
Compared to normal tissues, *INSC* was significantly downregulated at the transcriptional level in CC tissues. A low *INSC* mRNA level not only positively correlated with TNM stage (tumour-nodus-metastases), advanced T stage, and *N* stage, but also with the shorter 5- and 8-year overall survival (OS) and disease-specific survival. Concerning protein level, *INSC* downregulation was confirmed in CC samples. In terms of the correlation with N stage and 5- and 8-year OS, it was also consistent with mRNA levels. Cox regression analysis indicated that *INSC* protein expression was an independent prognostic factor for OS. The nomogram showed better prognostic accuracy and clinical net benefit for 5-year OS than TNM staging.
Altogether, downregulation of *INSC* is related to inferior clinicopathological features and patient outcomes, and it may be a novel independent prognostic biomarker in CC.

Key words: *INSC*, colon cancer, prognosis, biomarker, nomogram.

Introduction

Colon cancer (CC) is one of the most frequently diagnosed types of malignancy and the leading cause of cancer-related death in populous countries such as China and the United States [1, 2]. In China, the last few decades have seen a growing trend towards the incidence and mortality rates of CC, and changes in dietary habits, choices of unhealthy lifestyles, and lack of effective screening for early cancer detection and therapy are partly responsible for this trend [2]. In the United States, about 104,610 cases of newly diagnosed CC were estimated in 2020 [3]. Although CC shows histological similarity to rectal cancer and both are often discussed together, the 5-year overall survival (OS) for CC is lower than that for rectal cancer. Compared to patients with rectal cancer, CC patients presenting with later appearance of symptoms should partly contribute to more advanced cancer stages at diagnosis and worse prognosis [1, 4], so this malignant disease is increasingly recognized as a serious worldwide public health concern [5].

It is well known that TNM stage (tumour- nodus- metastases) combined with several established prognostic factors (e.g. histological grade) provide the principal guideline for the prognosis stratification

and choice of proper treatment for CC [1, 6–8]. As targeted therapies guided by genetic testing have been widely recommended, survival gains for some patients with distant metastases have become increasingly evident [9–11]. Nevertheless, due to the high heterogeneity and complex molecular mechanisms of CC, a variety of daunting challenges remain in clinical practice. For example, patients with the same TNM stage or similar clinicopathological features may have varying responses to treatment and suffer different survival outcomes, indicating that the TNM stage or the established prognostic factors cannot fully explain the prognosis [12, 13]. Hence, there is a critical need to develop novel effective biomarkers for risk stratification as prognostic predictors to assist in selecting optimal treatment regimes in CC patients.

The general consensus is that either precise positioning of the mitotic spindle or the establishment and maintenance of cell polarity are essential in numerous biological processes, including cell type differentiation, maintaining tissue integrity, and tissue homeostasis [14–16]. Human inscuteable spindle orientation adaptor protein *(INSC)* is a homologue of the Drosophila *INSC* gene, which was first discovered as a neural precursor gene of the Drosophila in 1996 [17]. Inscuteable spindle orientation adaptor protein protein controls cell polarity and spindle orientation by interacting with the components from 2 systems, respectively [18]. Similar molecular mechanisms that govern spindle orientation and cell fate determination are functionally conserved from Drosophila to mammals [19, 20]. Accumulating literature has demonstrated that changes in epithelial polarity programs and abnormal spindle positioning might be involved in human diseases, including the oncogenic process [14–16]. However, to the best of our knowledge, no literature regarding the clinicopathological significance of *INSC* in human cancer, especially in CC, has yet been reported. Thus, in this study, bioinformatics and immunohistochemistry were performed for a comprehensive analysis of *INSC* expression status and its significant correlations with clinicopathological features and prognosis.

Material and methods

Public data mining of inscuteable spindle orientation adaptor protein mRNA

Firstly, RNA-Seq data (Level 3 data) from a total of 479 CC tissues in stages I to IV and 41 paracancerous tissues with clinical information were retrieved from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/). The samples meeting any of the following criteria were excluded: (1) non-primary tumour samples; (2) those that had undergone any anti-cancer treatment before surgery; and (3) those with insufficient clinical parameters, including age, gender, T stage, N stage, M stage, tumour location, and OS. Meanwhile, duplicate measurements for one case were averaged, and the mean value was used for further analysis. Secondly, to make samples comparable, the raw gene read counts were converted into $\log_2$-transformed transcripts per million (TPM, $\log_2[\text{TPM} + 1]$) values. Thirdly, Ensemble gene ID numbers were annotated to gene symbols by using the human reference genome file (Homo sapiens.GRCh38.96.chr.gtf). Simultaneously, 3 mRNA microarray datasets were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/; Accession Number GSE83889, GSE44076 and GSE32323), respectively [21–23]. The probes were annotated to gene symbols based on the corresponding platform annotation files. When the same gene was mapped by multiple probes, the maximum value was selected. After data normalization, *INSC* mRNA expressions were extracted from each dataset.

Tissue samples in the tissue microarray cohort

The human CC tissue microarray (TMA) (Catalogue: HColA180Su17) used in this study was purchased from Outdo Biotech Co., Ltd. (Shanghai, China), and it contained 101 cancerous tissues and 79 paired adjacent non-tumour tissues. All cases enrolled in this tissue microarray had no preoperative therapy and signed an informed consent form. Shanghai Outdo Biotech Co. Ltd. produced the tissue chip, which was approved for the use in medical research by the institutional Ethics Committee (Grant No. YB M-05-02). Pathological staging was reclassified according to the American Joint Committee on Cancer TNM Staging Manual for colon cancer, 8th edition.

Immunohistochemical staining

Immunohistochemical (IHC) staining of *INSC* was performed using the kit (Catalogue: SP-9000; Zhongshan Jinqiao, Beijing, China), as described in our previous study [24]. In short, after drying at 60°C for 2 hours, the TMA slide was dewaxed in xylene and sequentially rehydrated using graded ethanol and distilled water. Then, sodium citrate buffer was used for high-pressure antigen repairing. Next, 3% H_2O_2 was used to block the activity of endogenous peroxidase, and normal goat serum was used to eliminate non-specific antibody binding. After incubation with the rabbit anti-*INSC* primary antibody (1 : 200 dilution; Catalogue: HPA039769; Sigma-Aldrich, USA) overnight at 4°C, the slide was incubated for 30 min at room temperature with the secondary antibody. Following reaction with horseradish peroxidase-labelled streptavidin, the slide was stained with diaminobenzidine tetrahydrochloride solution for

5 minutes and then counterstained with haematoxylin. Finally, the dehydration and sealing procedures were carried out.

Two experienced pathologists, blinded to clinicopathological characteristics and survival outcome, independently assessed the TMA immunostaining. The membranous and cytoplasmic staining were evaluated for *INSC* protein expression. The immunohistochemical staining was scored based on the intensity and percentage of the positive cells, using the following criteria. Five fields under high magnification were randomly selected to count for each specimen. The intensity scores were assigned as 0 (none), 1 (weak), 2 (moderate), and 3 (strong). The percentage scores were categorized as follows: $< 5\%$, 0; 5–25%, 1; 26–50%, 2; 51–75%, 3; and $> 75\%$, 4. The sum of the percentage score and the intensity score produced a total score for each case. For further analysis, all samples were categorized into an *INSC* low-expression group (with total score ≤ 4) and a high-expression group (with total score > 4).

Statistical analysis

R software (version 4.0) was used for all statistical analyses. Wilcoxon matched test was performed for differential analysis of the paired samples. *P*-values were derived from Pearson's χ^2 test or Fisher's exact probability test to examine the association between *INSC* expression and clinicopathological factors in CC patients. Survival analyses for OS and disease-specific survival (DSS) were separately conducted via the Kaplan-Meier method with the log-rank test. Prognostic factors associated with OS were identified by uni- and multivariate Cox proportional hazards regression analysis. Hazard ratios (HR) and 95% confidence intervals (95% CI) were calculated, respectively. The nomogram based on the multivariate Cox proportional hazards regression analysis was constructed with R package "regplot". The performance of the nomogram was explored by combined evaluation of the area under the time-dependent receiver operating characteristic (ROC) area under curve (AUC) and the calibration plot. The receiver operating characteristic curve analysis using the approach in the "timeROC" R package was adopted to compare the discrimination ability between nomogram and conventional staging or each of the remaining prognostic factors [25]. Decision curve analysis (DCA) was performed with the R package "ggDCA". The level of significance in all analyses was defined as 0.05 (2-sided).

Ethical statement

This work was supported by the Talent Introduction Project of Chongqing Medical and Pharmaceutical College (Grant No. ygz2021118).

Ethical approvals for the study were obtained from the Ethics Committee of Chongqing Medical and Pharmaceutical College (Grant No. KYLLSC20230418034). All procedures conformed to the Declaration of Helsinki (as revised in 2013).

Results

Inscuteable spindle orientation adaptor protein was downregulated at the transcriptional level and associated with clinicopathological features in colon cancer

Firstly, compared to normal colon tissues, the gene expression values of *INSC* were notably decreased in the non-paired tumour samples ($p < 0.001$) (Fig. 1A). Secondly, in 40 paired CC tissues from the TCGA colon adenocarcinoma cohort, we found that the *INSC* mRNA level (TPM) was significantly lower in CC tissues than that in the paired normal samples ($p < 0.001$) (Fig. 1B). Likewise, mRNA downregulation of *INSC* in colon and rectal cancer was found in paired tumour and normal samples from 3 other independent cohorts: GSE44076 ($p = 0.002$) (Fig.1C), GSE83889 ($p < 0.001$) (Fig. 1D), and GSE32323 ($p = 0.033$) (Fig. 1E). Taken together, the above results imply that an abnormal expression patterns of *INSC* might be involved in CC tumourigenesis.

Downregulated inscuteable spindle orientation adaptor protein mRNA expression was associated with unfavourable survival outcome and clinicopathological features

For survival analysis, we included a total of 424 eligible patients from the TCGA cohort. Inscuteable spindle orientation adaptor protein mRNA expression values were initially treated as continuous variables and were used to evaluate the predictive value for OS with the utilization of 5-year time-dependent ROC curve analysis. Then, the optimum cut-off value for 5-year OS was determined to be 0.970, with the maximum Youden index of 0.317, the AUC of 0.655 (95%CI: 0.568–0.743), a sensitivity of 65.6%, and a specificity of 66.1%. Next, the samples were grouped into low ($n = 232$) and high ($n = 192$) *INSC* mRNA level groups based on this cut-off value. As shown in the Kaplan-Meier survival curves (Fig. 2A, B), the 5- and 8-year OS rates were significantly lower among patients in the low *INSC* mRNA level group than those in high-level group (log-rank test $p = 0.002$ and $= 0.009$, respectively). Meanwhile, the survival curves also demonstrated that low *INSC* mRNA level was obviously related to worse 5- and 8-year DSS of CC patients (both log-rank test $p < 0.05$) (Fig. 2C, D). These trends suggest that the transcriptional level of *INSC* was negatively related to the survival outcomes of CC patients.

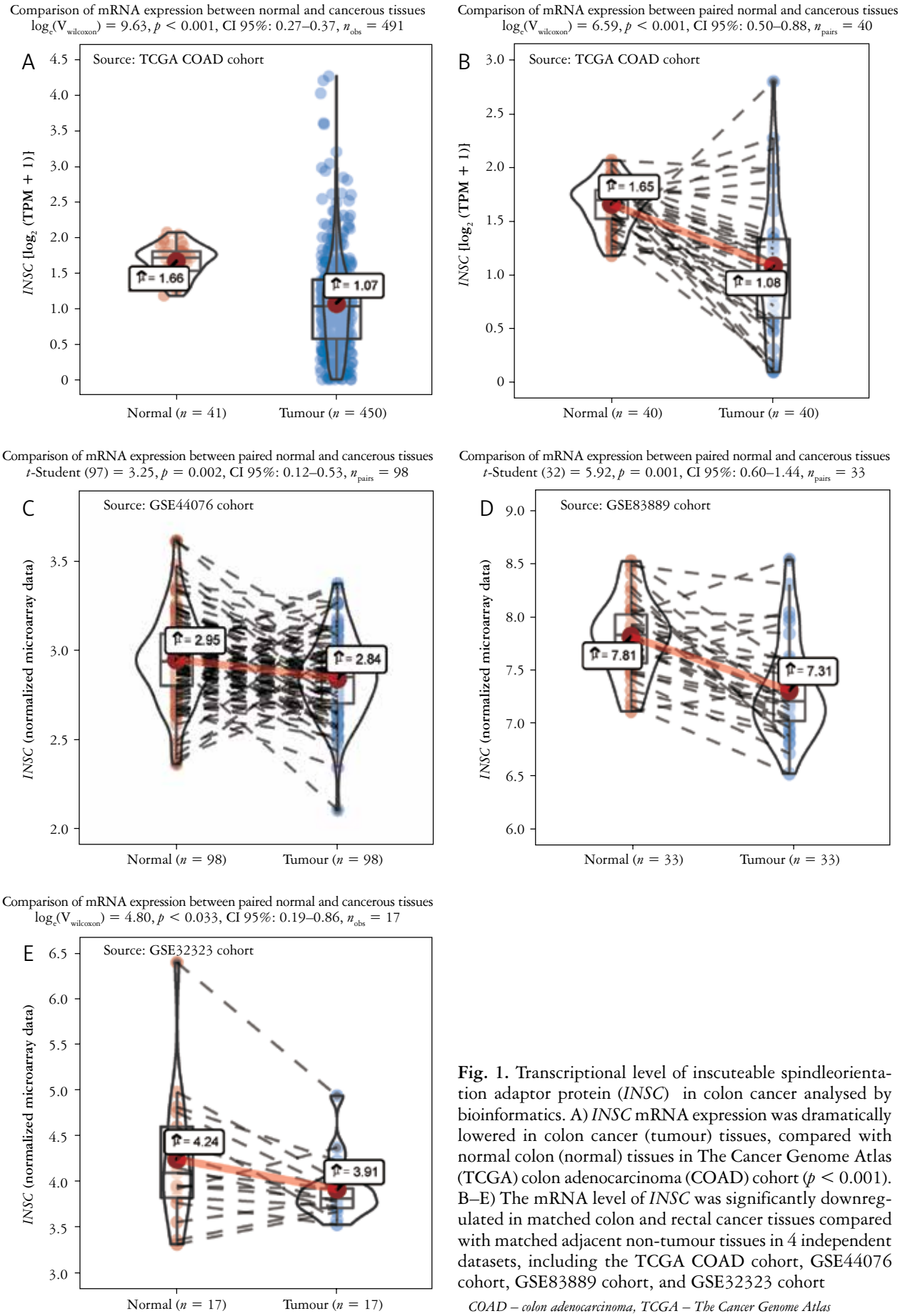


Fig. 1. Transcriptional level of inscuteable spindleorientation adaptor protein (*INSC*) in colon cancer analysed by bioinformatics. A) *INSC* mRNA expression was dramatically lowered in colon cancer (tumour) tissues, compared with normal colon (normal) tissues in The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) cohort ($p < 0.001$). B–E) The mRNA level of *INSC* was significantly downregulated in matched colon and rectal cancer tissues compared with matched adjacent non-tumour tissues in 4 independent datasets, including the TCGA COAD cohort, GSE44076 cohort, GSE83889 cohort, and GSE32323 cohort

COAD – colon adenocarcinoma, TCGA – The Cancer Genome Atlas

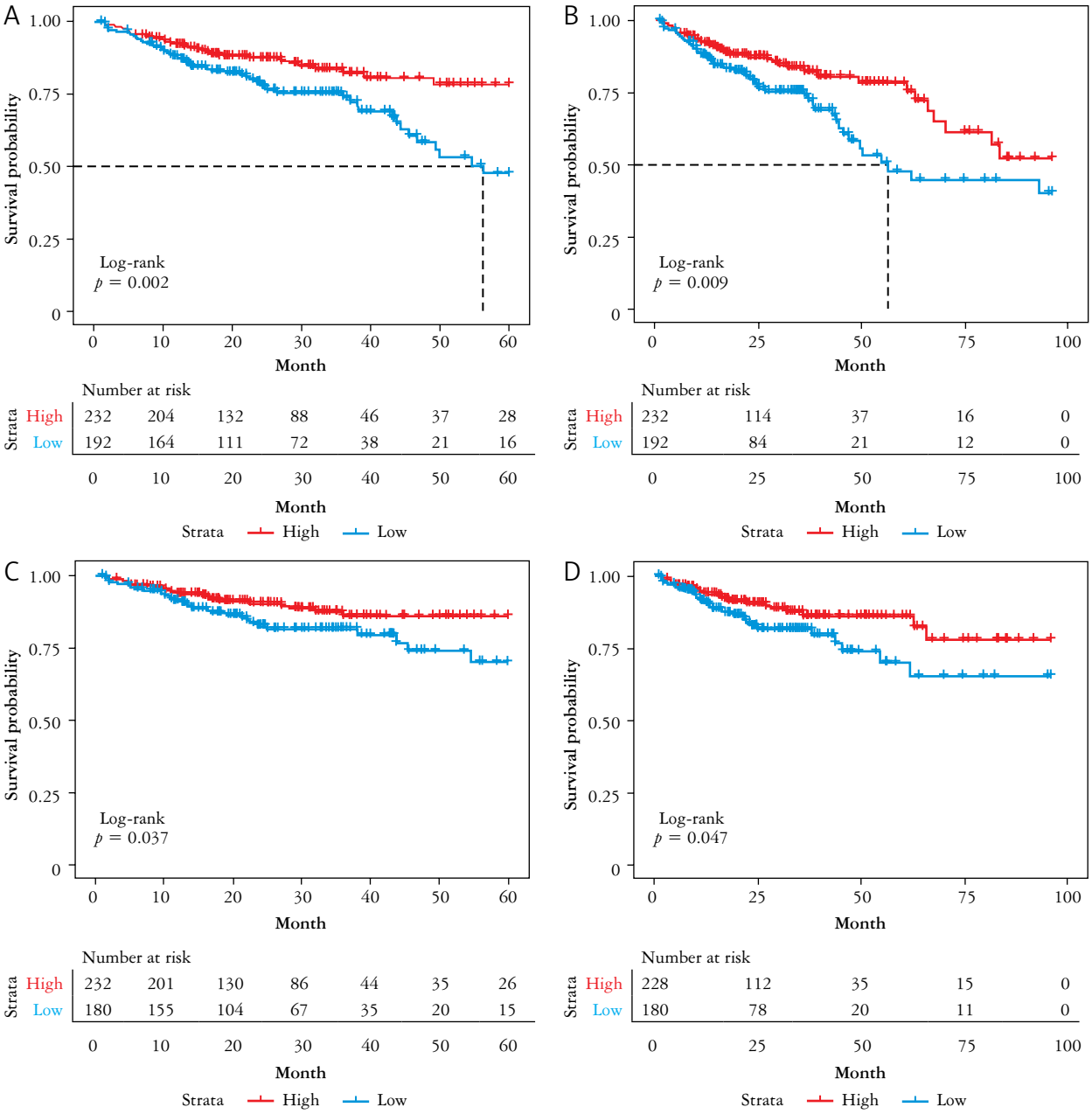


Fig. 2. Downregulation of inscuteable spindle orientation adaptor protein *(INSC)* mRNA was related to a worse prognosis in colon cancer patients from The Cancer Genome Atlas cohort. A, B) Kaplan-Meier analysis indicated that the 5- and 8-year overall survival rates were significantly lower in patients with low mRNA level than those with high mRNA level ($p = 0.002$ and $= 0.009$, respectively). C, D) Kaplan-Meier analysis showed that low mRNA expression group of *INSC* was closely associated with 5- and 8-year reduced DSS, compared with high mRNA expression group ($p = 0.037$ and $= 0.047$, respectively). All p-values derived from the log-rank tests

Additionally, 402 of these samples with complete clinical information were used to analyse the relationship between *INSC* mRNA levels and various clinicopathological factors in the TCGA dataset. As depicted in Table I, low *INSC* mRNA level was associated with advanced TNM stage ($p = 0.005$), T stage ($p < 0.001$), and N stage ($p = 0.048$). However, no other clinicopathological parameters correlated statistically significantly with low *INSC* expression. These results suggested that *INSC* might participate in the progression of CC.

Low protein expression of inscuteable spindle orientation adaptor protein correlated with inferior clinicopathological parameters in patients with colon cancer

Given that protein expression is not always consistent with mRNA expression, IHC assays were used to detect the *INSC* protein expression level in the TMA cohort. Eight tissue samples (2 tumour and 6 normal samples) were missed during antigen retrieval. Finally, a total of 99 CC samples and 73 paired nor-

Table I. Correlation between *insc*uteable spindle orientation adaptor protein mRNA level and clinicopathologic features in The Cancer Genome Atlas cohort

Characteristics	Number	High mRNA, level	Low mRNA, level	*p*-value
		n = 227 (%)	n = 175 (%)	
Age				0.705#
< 60	113	66 (29.1)	47 (26.9)	
≥ 60	289	161 (70.9)	128 (73.1)	
Gender				0.558#
Female	187	109 (48.0)	78 (44.6)	
Male	215	118 (52.0)	97 (55.4)	
Histological type				0.495##
Adenocarcinoma	356	200 (86.6)	156 (83.9)	
Mucinous adenocarcinoma	57	28 (12.1)	29 (15.6)	
Unknown	4	3 (1.3)	1 (0.5)	
TNM stage				0.005*#
Stage I	69	52 (22.9)	17 (9.7)	
Stage II	159	87(38.3)	72 (41.1)	
Stage III	118	61 (26.9)	57 (32.6)	
Stage IV	56	27 (11.9)	29 (16.6)	
T stage				<0.001*##
T1	8	6 (2.6)	2 (1.1)	
T2	70	53 (23.3)	17 (9.7)	
T3	277	150 (66.1)	127 (72.6)	
T4	47	18 (7.9)	29 (16.6)	
N stage				0.048*#
N0	235	144 (63.4)	91 (52.0)	
N1	95	50 (22.0)	45 (25.7)	
N2	72	33 (14.5)	39 (22.3)	
M stage				0.231#
M0	346	200 (88.1)	146 (83.4)	
M1	56	27 (11.9)	29 (16.6)	
Tumour location				0.891#
Left	165	92 (40.5)	73 (41.7)	
Right	237	135 (59.5)	102 (58.3)	

TNM – tumour-node-metastasis
** Statistically significant*
 # Exact χ^2 test
Fisher's exact test

mal tissues were included in the study, including 49 (49.5%) men and 50 (50.5%) women. Their median age at diagnosis was 69 years with an age range of 43 to 91 years.

All the normal colon epithelia were positively stained for *INSC* with total scores of more than 4, predominantly localized in the cytoplasm as well as the cytomembrane region of the epithelial cell (73/73, 100%), whereas the stroma showed no significant staining (Fig. 3A). Although *INSC* subcellular localization in human CC tissues was similar to that found in normal colon epithelia, a significant portion of the samples had considerably weakened *INSC* immunostaining (Fig. 3B–F). Of the 99 samples, low *INSC* expression was found in 53 samples (53.5%; scores 0 to 1, 19 cases; scores 2 to 4, 34 cases), while high *INSC* expression

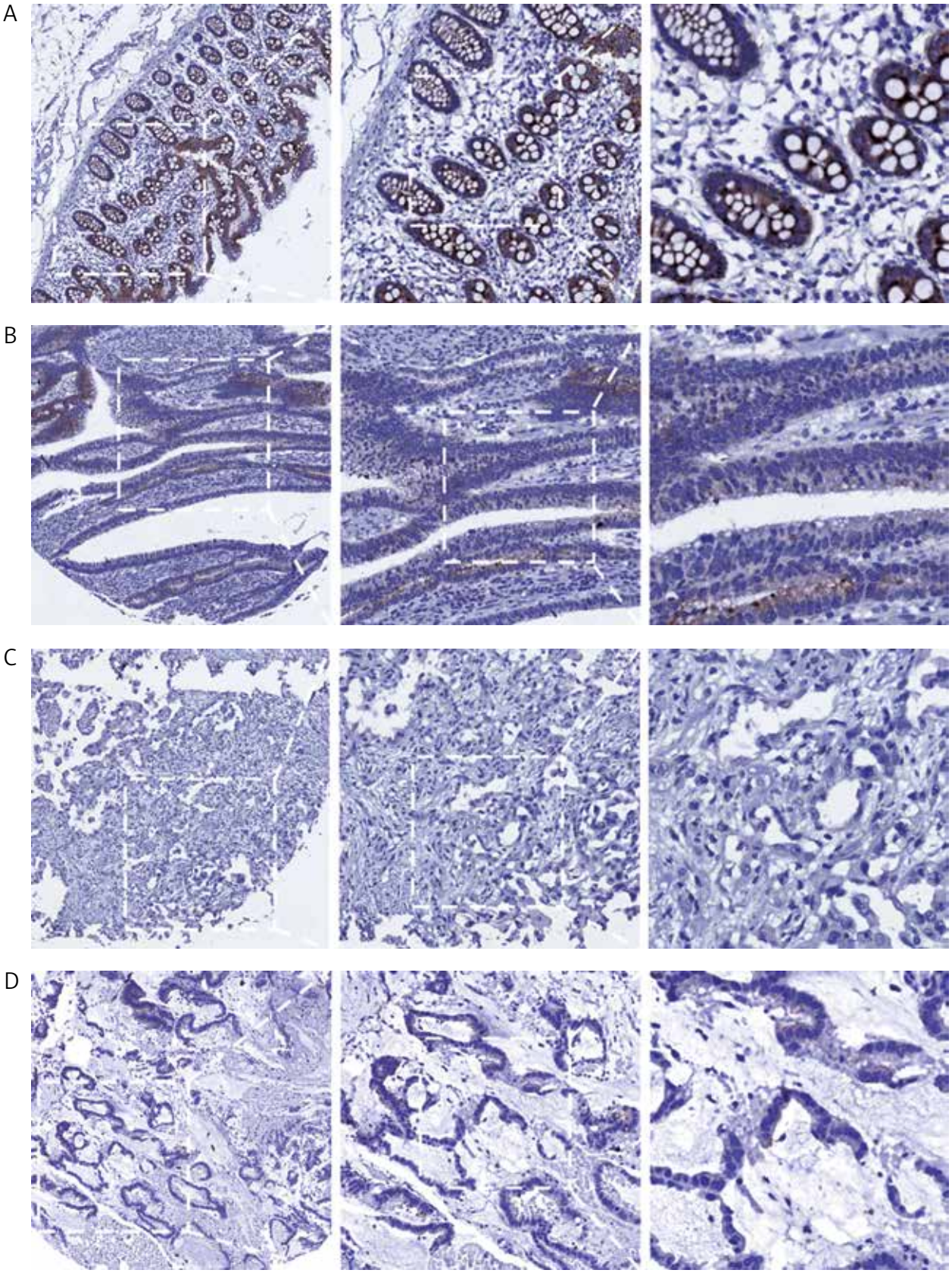
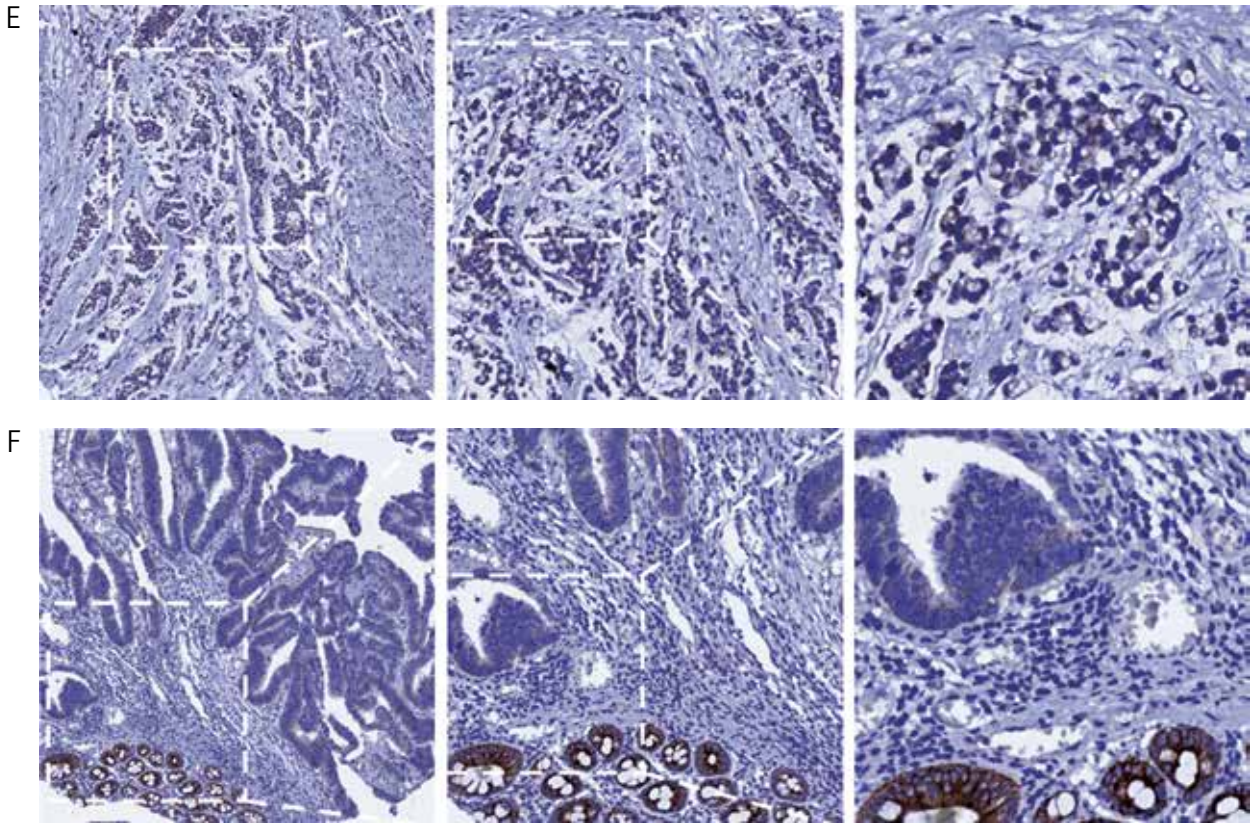


Fig. 3. Representative images of inscuteable spindle orientation adaptor protein (*INSC*) expression in normal colon tissues and colon cancer tissues. A) The strong immunoreactivity of *INSC* in normal colon mucosa. B–E) The images representing a portion of the colon cancer (CC) samples with negative expression or low expression, including well-differentiated colonic adenocarcinoma (B), poorly-differentiated colonic adenocarcinoma (C), mucinous adenocarcinoma (D)



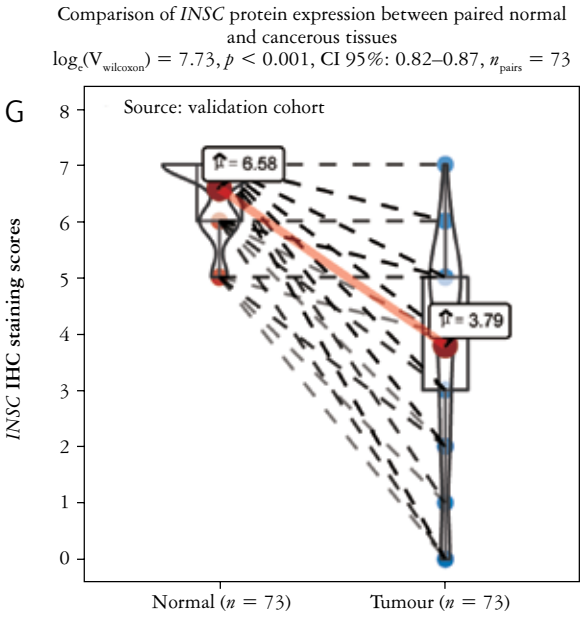


Fig. 3. Cont. Signet ring cell carcinoma (E). F) A representative image of *INSC* staining in paracancerous tissues and carcinoma tissues of CC patients. G) The analysis for the differences in immunohistochemical scoring between paired samples using Wilcoxon Signed-rank test ($p < 0.001$). Original magnifications 100×, 200×, and 400×, respectively

IHC – immunohistochemical, INSC – inscuteable spindle orientation adaptor

was observed in the remaining 46 cases (46.5%, scores 5 to 7). A paired differential analysis identified that, compared to matched normal tissues, *INSC* displayed significantly lower levels of protein expression in cancer tissues ($p < 0.001$) (Fig. 3G), which concurs with mRNA expression status.

As for the clinical features, *INSC* protein expression was associated with N stage ($p = 0.041$) but not with other factors of CC patients, such as age, gender, histological type, T stage, and TNM stage (all p-values > 0.05). Although a trend toward an increase in tumour volume in *INSC* low expression group was seen, the difference did not reach statistical significance ($p = 0.051$). The results are summarized in Table II.

Decreased inscuteable spindle orientation adaptor protein expression was an independent prognostic factor for overall survival

In the TMA cohort, the 5- and 8-year OS rates of the entire cohort were 53.5% and 48.5%, respectively. In accordance with the prognostic significance

Table II. Association of *insc*uteable spindle orientation adaptor protein level with clinicopathologic features in the tissue microarray cohort

Characteristics	Number	High protein, level	Low protein, level	*p*-value
		n = 46 (%)	n = 52 (%)	
Age				0.588#
< 60	16	9 (19.6)	7 (13.5)	
≥ 60	82	37 (80.4)	45 (86.5)	
Gender				0.544#
Female	49	25 (54.3)	24 (46.2)	
Male	49	21 (45.7)	28 (53.8)	
Tumour volume [cm^3]				0.051##
< 32	57	32 (69.6)	25 (48.1)	
≥ 32	40	14 (30.4)	26 (50.0)	
Unknown	1	0 (0.00)	1 (1.92)	
Histological type				0.681##
Adenocarcinoma	92	44 (95.7)	48 (92.3)	
Non- adenocarcinoma	6	2 (4.35)	4 (7.69)	
TNM stage				0.594##
Stage I	6	4 (8.7)	2 (3.8)	
Stage II	51	25 (54.3)	26 (50.0)	
Stage III	40	17 (37.0)	23 (44.2)	
Stage IV	1	0 (0.0)	1 (1.9)	
T stage				0.695##
T1	1	1 (2.17)	0 (0.00)	
T2	5	3 (6.52)	2 (3.85)	
T3	73	34 (73.9)	39 (75.0)	
T4	19	8 (17.4)	11 (21.2)	
N stage				0.041*##
N0	57	29 (63.0)	28 (53.8)	
N1	31	16 (34.8)	15 (28.8)	
N2	10	1 (2.17%)	9 (17.3)	

TNM – tumour-node-metastasis
** Statistically significant*
 # Exact χ^2 test
Fisher's exact test

of *INSC* mRNA expression, the Kaplan-Meier survival curve revealed a significant positive relationship between *INSC* expression and OS in CC patients. Cases with low *INSC* protein expression achieved significantly worse 5- and 8-year OS rates than those with high expression (log-rank test $p = 0.015$ and $p = 0.005$, respectively) (Fig. 4A, B).

Then, the Cox proportional hazards regression analysis was conducted to investigate the influence of each predictive factor for OS in CC patients. The univariate Cox regression analysis indicated that TNM stage (HR, 2.244, 95% CI: 1.435–3.509; $p < 0.001$), N stage (HR, 2.040, 95% CI: 1.419–2.932; $p < 0.001$), tumour differentiation (HR, 2.965, 95% CI: 1.568–5.606; $p < 0.001$), and low *INSC* expression (HR, 2.264, 95% CI: 1.260–4.070; $p = 0.006$) significantly shortened the OS of CC patients. Further, the multivariate analysis identified that *INSC* downregulation was a potential independent prognostic risk factor for OS in CC patients (HR, 1.961, 95% CI: 1.077–3.571; $p = 0.028$). Detailed results can be found in Figure 4C.

The *INSC*-based nomogram displayed superiority in predicting 5-year overall survival for colon cancer patients

To further analyse the prognostic value of *INSC*, we built a nomogram incorporating its protein expression levels and several clinicopathological features (T stage,

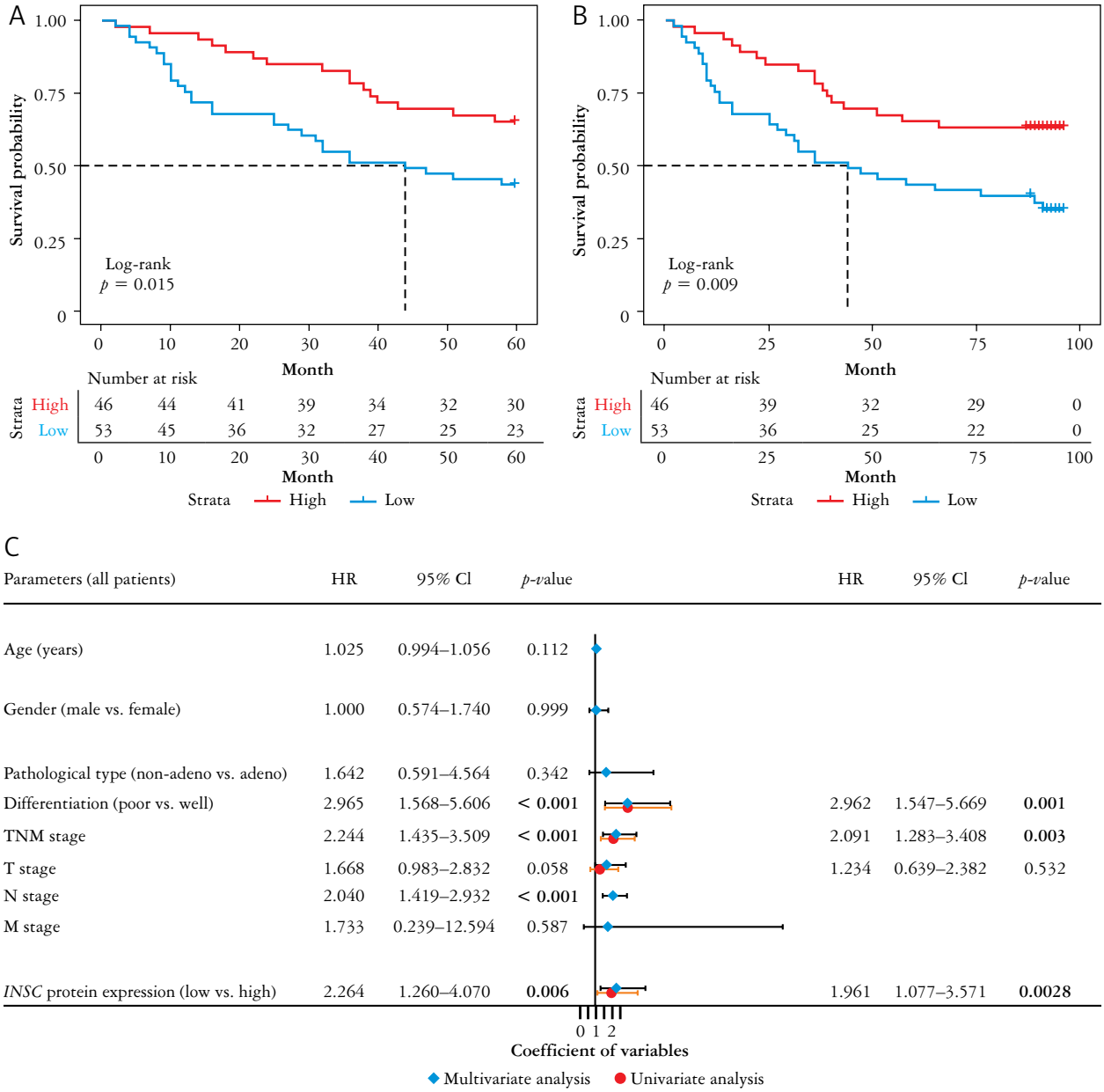


Fig. 4. Lowered protein expression of inscuteable spindle orientation adaptor protein (*INSC*) predicted poor survival outcomes. A, B) Kaplan-Meier curves plotted according to *INSC* protein status showed that the patients with low *INSC* expression exhibited the 5-and 8-year shorter overall survival (OS) than those with high *INSC* expression (log-rank test log-rank test $p = 0.015$ and $p = 0.005$, respectively). C) Forest plots of univariate and multivariate Cox regression analysis for OS. The univariate analysis showed that along with *INSC* expression, tumour differentiation, TNM stage, and N stage also correlated with poor OS. The multivariate analysis indicated that *INSC* protein expression is an independently prognostic factor for OS in colon cancer patients

CI – confidence interval HR – hazard ratio, INSC – inscuteable spindle orientation adaptor

N stage, and tumour differentiation) for predicting 5-year OS of CC patients in the TMA cohort (Fig. 5A). Regarding predictive performance, the time-dependent ROC curves demonstrated that our nomogram exhibited better discriminatory capabilities, with an AUC of 0.741 (95% CI: 64.38–83.86), than TNM stage (AUC: 0.648; 95% CI: 55.22–74.42), T stage (AUC: 0.589; 95% CI: 50.49–67.40), N stage (AUC: 0.653; 95% CI: 55.65–74.87), and tumour differentiation (AUC: 0.613; 95% CI: 53.97–68.52; all p-values < 0.05) (Fig. 5B). For internal validation, the calibration curve for OS prediction at 5 years was plotted by 1000-resampled bootstrap, and it fitted well with the actual outcomes (Fig. 5C). In addition, DCA, a common method for assessment of the clinical usefulness of the nomogram, indicated that the *INSC*-based nomogram increased the positive net benefits with a wider range of threshold probability for predicting 5-year OS in CC patients (Fig. 5D).

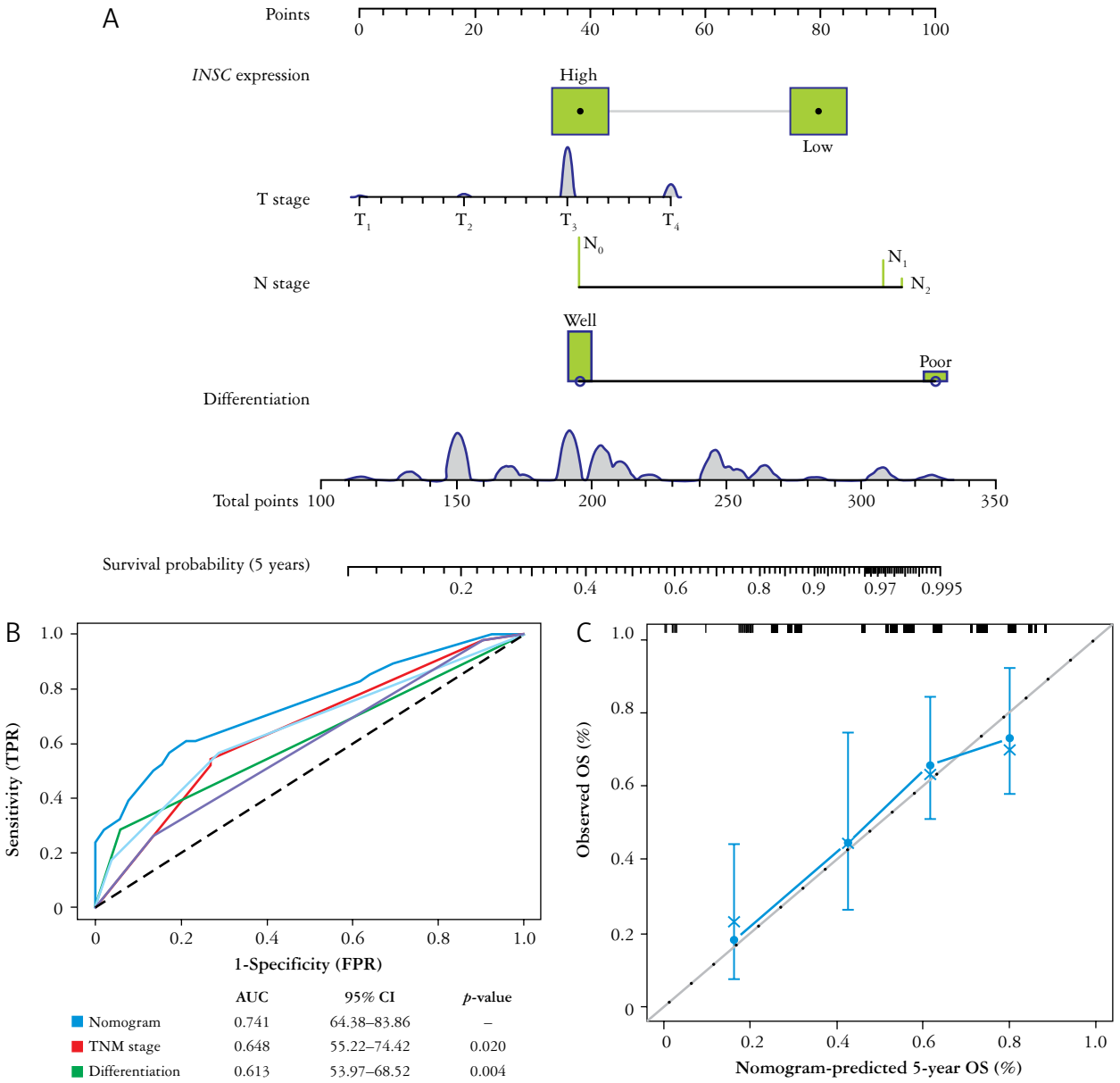


Fig. 5. Development of *INSC*-based prognostic nomogram for predicting 5-year survival in tissue microarray cohort. A) The nomogram predicted 5-year overall survival (OS) for colon cancer patients. The point value of each variable could be assigned by drawing a vertical line to the top row (points scale line). The sum of these values was found on the total points axis, and the likelihood of 5-year OS could be determined by drawing a vertical line from the total points axis downward to the probability axis. B) Time-dependent receiver operating characteristic curve for comparison of discriminatory capabilities between nomogram and each of the remaining prognostic factors. C) The calibration curve for the probability of 5-year OS. The x- and y-axis represent predicted survival generated from the nomogram and actual survival, respectively. The diagonal line represents a perfect consistency between the prediction and observation. D) Decision curve analyses for evaluation of the clinical usefulness of the nomogram. The horizontal dashed yellow line at the bottom and the dashed grey line represented the assumptions that no case or all cases would experience the event, respectively. The solid lines indicated that the positive net benefit of nomogram or other prognostic factors across a range of threshold probabilities in terms of 5-year OS, respectively

CI – confidence interval, FPR – false positive rate, INSC – inscuteable spindle orientation adaptor, OS – overall survival, TNM – tumour-nodus-metastases, TPR – true positive rate

Discussion

In the present study, we described the following steps to validate whether *INSC* is a potential prognostic biomarker for CC patients. Firstly, decreased *INSC* mRNA level in cancerous tissues relative to the normal controls was uncovered by mining the publicly available databases. Secondly, statistically significant correlations between low mRNA level and inferior clinical characteristics as well as poor survival outcomes were found. Thirdly, given that the real functions of encoding genes are to encode specific proteins executing practical functions, we performed IHC staining and analyses of immunostaining scores. The results further identified that *INSC* downregulation at the protein level is a common event in CC samples. Similarly to its transcription level, patients with low *INSC* protein expression showed a higher frequency of regional lymph node involvement and worse OS than those with a high protein level, suggesting that overexpression of *INSC* might halt disease progression and improve survival outcomes. Next, multivariate analysis revealed that the lower level of *INSC* represented an independent poor prognostic factor for OS in CC patients. Furthermore, one *INSC*-based nomogram was developed in this study, which performed better than conventional staging systems or other prognostic factors in terms of the prediction of 5-year survival and clinical net benefit. Meanwhile, a reasonable fit degree was observed in the calibration plot indicating good calibration of this model.

As an adaptor protein, the homologues of *INSC* are exclusively present in organisms from insects to mammals [25]. The contribution of dysregulated *INSC* expression to defective cell division and system development has been identified in previous in vitro and in vivo studies. In Drosophila neuroblasts, *INSC* mutant is thought to contribute to incorrect spindle orientation and defective cell polarity [26, 27]. Prior experimental evidence has shown that *INSC* putatively participates in the regulation of the activity of downstream Pins (partner of *INSC*) pathways involved in mitotic spindle positioning in Drosophila S2 cells [28]. Mammalian *INSC* may also play an important role in mitotic spindle orientation and asymmetric cell division [25]. To exemplify, reduced neurogenesis and defective cortical organization in association with loss of *INSC* expression are observed in mice. In contrast, *INSC* overexpression results in the expansion of the neuronal cell pool [29].

So far, only a few existing structure-function analyses have recognized that the N-terminal portion of human *INSC* is involved in binding to LGN protein [30], while the conserved C-terminal motif is implicated in interaction with the PDZ domains of Par3 [31, 32]. Much less is known about its expression status and clinical significance in human disease, including malignancy. Here, we provide the first insight into the potential role of *INSC* in CC. Although there is little concordance in the association with other clinicopathologic variables, both the mRNA and protein level of *INSC* significantly correlate with N stage, suggesting its potential participation in tumour progression. It has been well documented that alterations of cell polarity proteins induce cancer progression and greater invasiveness [33], which might, to some extent, account for this relationship. Accordingly, it is not surprising that low *INSC* expression is associated with a poor prognosis of patients with CC. Moreover, one previous report has suggested that a lack of *INSC* expression caused the inaccurate localization and segregation of Numb during the asymmetric division of neuroblasts, and deficient Numb at the end of cytokinesis. Conversely, reduced Notch activity was found in *INSC* clones [34]. Considering the inhibitory effect of Numb on the Notch signalling [35, 36], it appears plausible, although speculative, that downregulation of *INSC* may promote tumourigenesis and cancer progression via Numb/Notch signalling pathway in CC. Further studies are warranted to clarify the molecular mechanisms underlying *INSC* functions on CC.

Additionally, this study had some shortcomings. Firstly, the sample size in TMA was still relatively small and only one patient with stage 4 disease was included. Secondly, no functional experiments were executed in vitro and in vivo to elucidated whether *INSC* has tumour-suppressive function and related molecular mechanisms in CC. Lastly, further assessment in external validations are needed to verify our findings.

Conclusions

In conclusion, this study revealed that human *INSC* gene is frequently downregulated at both mRNA and protein levels in CC. Low *INSC* expression was closely related to worse clinicopathological features and survival outcomes. Our findings suggested that *INSC* could act as a novel independent prognostic factor for OS. Therefore, detection of *INSC* expression might be helpful for clinicians to more accurately estimate the individual prognosis of CC patients. Its possibility of being a potential therapeutic target deserves further investigation.

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References

1. Siegel RL, Miller KD, Goding Sauer A, et al. Colorectal cancer statistics, 2020. CA Cancer J Clin 2020; 70: 145-164.
2. Chen H, Li N, Ren J, et al. Participation and yield of a population-based colorectal cancer screening programme in China. Gut 2019; 68: 1450-1457.
3. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. CA Cancer J Clin 2020; 70: 7-30.
4. Costantini L, Molinari R, Farinon B, Merendino N. Retinoic acids in the treatment of most lethal solid cancers. J Clin Med 2020; 9: 360.
5. Fitzmaurice C, Abate D, Abbasi N, et al. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the global burden of disease study. JAMA Oncol 2019; 5: 1749-1768.
6. Perna C, Navarro A, Ruz-Caracuel I, et al. Molecular heterogeneity of high grade colorectal adenocarcinoma. Cancers 2021; 13: 233.
7. Mayo E, Llanos AA, Yi X, et al. Prognostic value of tumour deposit and perineural invasion status in colorectal cancer patients: a SEER-based population study. Histopathology 2016; 69: 230-238.
8. Al-Sukhni E, Attwood K, Gabriel EM, et al. Lymphovascular and perineural invasion are associated with poor prognostic features and outcomes in colorectal cancer: a retrospective cohort study. Int J Surg 2017; 37: 42-49.
9. Allegra CJ, Rumble RB, Hamilton SR, et al. Extended RAS gene mutation testing in metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy: American Society of Clinical Oncology Provisional Clinical Opinion Update 2015. J Clin Oncol 2016; 34: 179-185.
10. Santini D, Spoto C, Loupakis F, et al. High concordance of BRAF status between primary colorectal tumours and related metastatic sites: implications for clinical practice. Ann Oncol 2010; 21: 1565.
11. Valtorta E, Martino C, Sartore-Bianchi A, et al. Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. Mod Pathol 2015; 28: 1481-1491.
12. Punt CJ, Koopman M, Vermeulen L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. Nat Rev Clin Oncol 2017; 14: 235-246.
13. Weiser MR, Gonen M, Chou JF, et al. Predicting survival after curative colectomy for cancer: individualizing colon cancer staging. J Clin Oncol 2011; 29: 4796-4802.
14. Rejon C, Al-Masri M, McCaffrey L. Cell polarity proteins in breast cancer progression. J Cell Biochem 2016; 117: 2215-2223.
15. Knoblich JA. Asymmetric cell division: recent developments and their implications for tumour biology. Nat Rev Mol Cell Biol 2010; 11: 849-860.
16. Ellenbroek SI, Iden S, Collard JG. Cell polarity proteins and cancer. Semin Cancer Biol 2012; 22: 208-215.
17. Kraut R, Campos-Ortega JA. *INSC*uteable, a neural precursor gene of Drosophila, encodes a candidate for a cytoskeleton adaptor protein. Dev Biol 1996; 174: 65-81.
18. Siller KH, Doe CQ. Spindle orientation during asymmetric cell division. Nat Cell Biol 2009; 11: 365-374.
19. Postiglione MP, Juschke C, Xie Y, et al. Mouse *INSC*uteable induces apical-basal spindle orientation to facilitate intermediate progenitor generation in the developing neocortex. Neuron 2011; 72: 269-284.
20. Lechler T, Fuchs E. Asymmetric cell divisions promote stratification and differentiation of mammalian skin. Nature 2005; 437: 275-280.
21. Kwon Y, Park M, Jang M, et al. Prognosis of stage III colorectal carcinomas with FOLFOX adjuvant chemotherapy can be predicted by molecular subtype. Oncotarget 2017; 8: 39367-39381.
22. Sole X, Crous-Bou M, Cordero D, et al. Discovery and validation of new potential biomarkers for early detection of colon cancer. PLoS One 2014; 9: e106748.
23. Khamas A, Ishikawa T, Shimokawa K, et al. Screening for epigenetically masked genes in colorectal cancer Using 5-Aza-2'-deoxycytidine, microarray and gene expression profile. Cancer Genom Proteom 2012; 9: 67-75.
24. Han X, Tang J, Chen T, et al. Restoration of GATA4 expression impedes breast cancer progression by transcriptional repression of ReLA and inhibition of NF-kappaB signaling. J Cell Biochem 2019; 120: 917-927.
25. Yuzawa S, Kamakura S, Iwakiri Y, et al. Structural basis for interaction between the conserved cell polarity proteins *INSC*uteable and Leu-Gly-Asn repeat-enriched protein (LGN). Proc Natl Acad Sci U S A 2011; 108: 19210-19215.
26. Kraut R, Chia W, Jan LY, et al. Role of *INSC*uteable in orienting asymmetric cell divisions in Drosophila. Nature 1996; 383: 50-55.
27. Siegrist SE, Doe CQ. Microtubule-induced Pins/Galphai cortical polarity in Drosophila neuroblasts. Cell 2005; 123: 1323-1335.
28. Mauser JF, Prehoda KE. *INSC*uteable regulates the Pins-Mud spindle orientation pathway. PLoS One 2012; 7: e29611.
29. Ishibashi R, Kozuki S, Kamakura S, et al. c-Rel regulates *INSC*uteable gene expression during mouse embryonic stem cell differentiation. J Biol Chem 2016; 297: 3333-3345.
30. Zigman M, Cayouette M, Charalambous C, et al. Mammalian *INSC*uteable regulates spindle orientation and cell fate in the developing retina. Neuron 2005; 48: 539-545.
31. Zheng Z, Wan Q, Liu J, et al. Evidence for dynein and astral microtubule-mediated cortical release and transport of Galphai/LGN/NuMA complex in mitotic cells. Mol Biol Cell 2013; 24: 901-913.
32. Kamakura S, Nomura M, Hayase J, et al. The cell polarity protein m*INSC* regulates neutrophil chemotaxis via a noncanonical G protein signaling pathway. Dev Cell 2013; 26: 292-302.
33. Martin-Belmonte F, Perez-Moreno M. Epithelial cell polarity, stem cells and cancer. Nat Rev Cancer 2011; 12: 23-38.
34. An H, Ge W, Xi Y, et al. *INSC*uteable maintains type I neuroblast lineage identity via Numb/Notch signaling in the Drosophila larval brain. J Genet Genomics 2017; 44: 151-162.
35. Flores AN, McDermott N, Meunier A, et al. NUMB inhibition of NOTCH signalling as a therapeutic target in prostate cancer. Nat Rev Urol 2014; 11: 499-507.
36. Man J, Yu X, Huang H, et al. Hypoxic induction of vasorin regulates notch1 turnover to maintain glioma stem-like cells. Cell Stem Cell 2018; 22: 104-118 e106.

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