Original Article

ZNF326 as a potential prognostic and predictive biomarker in stage II colorectal cancer

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Background: Adjuvant chemotherapy is considered for stage II colorectal cancer (CRC) patients with poor prognostic risk factors. However, current stratification algorithms are still insufficient to identify high-risk patients.

Methods: We conducted a screening strategy to define ZNF326 based on quantitative proteomics in 11 paired CRC patients selected by a nested case-control design, and tested the association between *ZNF326* expression level with the prognosis of stage II CRC patients and the benefit from adjuvant chemotherapy in public datasets; further investigation was conducted through subgroup analyses.

Results: We found that low *ZNF326* expression was significantly associated with a lower 5-year overall survival (OS) rate among stage II patients in both the discovery [P=0.008; hazard ratio (HR): 3.13, 95% confidence interval (CI): 1.29–7.58] and validation (P=0.025; HR: 1.98, 95% CI: 1.08–3.65) cohorts. In the Cox multivariable analysis, low *ZNF326* expression was both associated with shorter OS after adjustment for age, sex, and adjuvant chemotherapy in the discovery and validation data sets. Subgroup analyses yielded largely similar results. In a pooled database, the rate of 5-year OS was higher among stage II *ZNF326*-high tumors who were treated with adjuvant chemotherapy than it was among those who were not treated with adjuvant chemotherapy (P=0.011; HR: 0.28, 95% CI: 0.10–0.80).

Conclusions: *ZNF326* has the potential to be used in clinical practice for risk classification. *ZNF326*-low expression level identified a subgroup of patients with high-risk stage II CRC who appeared to less benefit from adjuvant chemotherapy.

Keywords: *ZNF326*; colorectal cancer (CRC); proteomics; prognostic marker; adjuvant chemotherapy

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Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide with more than 1.9 million new cases diagnosed annually (1) and the second leading cause of cancer-related death (1) with a 5-year mortality rate of about 40% (2); it represents a great health burden in China (3,4). Based on current guidelines, most stage II CRC patients are treated surgically without adjuvant chemotherapy. Whether a stage II CRC patient should receive adjuvant chemotherapy after surgery largely depends on the recurrence risk assessed by a group of factors (5,6). Still, a considerable proportion of stage II patients who are evaluated as low-risk later experience recurrence and progression. The decision to treat stage II CRC patients with adjuvant chemotherapy has been one of the most challenging (7-10) and controversial issues in oncology over the past 20 years. Therefore, biomarkers are needed to more accurately identify patients with stage II CRC who are suitable for adjuvant chemotherapy.

Many specialist bodies have published separate guidelines for the adjuvant treatment of stage II CRC and there is considerable variation (11-13). The guidelines recommend considering adjuvant chemotherapy for stage II patients with high-risk clinicopathologic features, such as T4 tumors, poorly differentiated histology, lymphatic/vascular/perineural invasion, obstruction, perforation, and inadequate lymph node sampling, although the definition of "high-risk" is not standardized (14). However, with the exception of T4 tumors, these factors are not sufficient to identify those patients with stage II CRC who may be candidates for adjuvant chemotherapy (15) Significant efforts have been made to identify new biomarkers, including gene expression signatures, microRNA profiling and circulating tumor DNA, to assess patients' risk of relapse over the past few decades.. However, they often struggle to become routine clinical tests, perhaps because of low technical reproducibility, the requirement of fresh-frozen tissues, or the lack of validation in large prospective trials (16-20). As a result, there is a clinical need for more precise biomarkers to determine which patients will benefit from adjuvant chemotherapy.

As the effector molecules of genes, proteins carry out biological functions of the genes in the cell. Thus, proteomics could provide more direct evidence and better solutions to cancer problems (21). Compared with genomics, the verification and interpretation provided by proteomics are closer to phenotype, and virtually all existing drug targets are proteins (22,23). We have previously reported that at least 5 proteins, including the *ZNF326*, varied on the tumor tissues between the recurrence and non-recurrence CRC patients through a nested case-control cohort that combined the proteomics (24). However, the prognostic value of ZNF326 in stage II CRC patients is unclear.

In this study, ZNF326 was selected as a candidate biomarker from our last study. We evaluated its prognostic impact using 2 gene expression data sets for training and validation in different cohorts of CRC patients. We also assessed the association of *ZNF326* expression status and the benefit from adjuvant chemotherapy in stage II CRC patients. We present this article in accordance with the REMARK reporting checklist (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-908/rc).

Highlight box

Key findings

- Our study identified *ZNF326* as a potential prognostic and predictive biomarker in stage II colorectal cancer (CRC) patients. Stage II CRC patients with low *ZNF326* expression levels exhibited a poor 5-year overall survival (OS) rate compared to those with high *ZNF326* expression. Furthermore, patients in stage II with high *ZNF326* expression levels are more likely to derive benefits from adjuvant chemotherapy compared to low *ZNF326* expression.

What is known and what is new?

- Current stratification algorithms are still insufficient to identify stage II CRC patients with poor prognostic risk factors.
- We implemented a screening strategy utilizing quantitative proteomics to identify ZNF326 in 11 paired CRC patients selected by a nested case-control design. Subsequently, we investigated the association between *ZNF326* expression level with the prognosis of stage II CRC patients and the benefit from adjuvant chemotherapy.

What is the implication, and what should change now?

- *ZNF326* holds promise for clinical application in risk classification among stage II CRC patients.

Methods

Study design

ZNF326 was selected as a candidate biomarker based on previous proteomics analyses and literature reviews (24). Briefly, we established a Yunnan CRC cohort comprising stage I–III CRC patients at Yunnan Cancer Hospital between December 2010 and February 2019. Then,

propensity score matching (PSM) was used to adjust for critical variables between post-operative and non-post-operative metastatic patients. A nested case-control cohort was employed, and 11 pairs of patients were selected through PSM. Subsequently, we focused on the intersection of differentially expressed proteins identified using Student's *t*-test and metastasis-related protein verified by univariate Cox regression analysis. ZNF326 was selected for further analysis. According to the literature search and our basic research, these genes are rarely reported in most cancers. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Yunnan Cancer Hospital Ethics Committee (No. KY2019141). The requirement for informed consent was waived by the Ethics Committee owing to the retrospective nature of the study.

The following processes were employed to analyze *ZNF326*. Firstly, we used X-Tile (Yale School of Medicine, New Haven, CT, USA) to identify an optimal cutoff for *ZNF326* expression level in the discovery cohort of the stage II CRC patients. Secondly, we tested its association with the prognosis of stage II CRC patients in a discovery data set, and verified the association in a different cohort of stage II CRC patients as the validation data set. Then, we analyzed the correlation between the *ZNF326* expression level and existing prognostic factors. Subgroup analyses were used to assess the robustness of the risk estimations. Thirdly, a pooled database was used for analyses of the association of *ZNF326* and the benefit from adjuvant chemotherapy in stage II CRC patients. *Figure 1* shows the study flowchart.

Datasets collection

CRC tissue gene expression profiles, annotated with clinical and pathological information, were obtained from two independent sources: The Cancer Genome Atlas (TCGA) for the discovery dataset and GSE40967 in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI-GEO) for the validation dataset. Combined data pooled by three data sets, namely, GSE29623, GSE40967, and GSE103479, were found to satisfy our criteria [including pathological stage, available information on *ZNF326* gene expression, adjuvant chemotherapy, date of overall survival (OS), and follow-up duration] was used to explore the relationship with adjuvant chemotherapy. The detailed workflows are described in Figures S1-S4. A comprehensive depiction of the patient cohorts from the 2 independent sources is available in Table S1.

We downloaded from the TCGA portal (https://portal.gdc.cancer.gov/) and the NCBI-GEO dataset (https://www.ncbi.nlm.nih.gov/geo/) transcriptome profiles in fragments per kilobase million (FPKM) format and corresponding clinical information. The NCBI-GEO datasets used for the multiple dataset analysis were based on different platforms. Therefore, we combined the three datasets by normalizing them using the Robust Multichip Average (RMA) algorithm (25) and removing batch effects using the affy and sva R packages (26,27) to increase the sample size and avoid less reliable results. Meanwhile, we averaged the probes corresponding to the same gene.

Identification of the optimal cutoff for ZNF326 expression level

Due to the variation of sequencing between the TCGA and NCBI-GEO datasets, we used the Z-score to balance the difference before the data analysis. *ZNF326* expression levels were stratified into *ZNF326*-high and *ZNF326*-low subgroups according to the *ZNF326* expression. X-tile (28) was used to determine the threshold of *ZNF326* expression in TCGA dataset and we applied this threshold to the NCBI-GEO dataset.

Association analysis of ZNF326 with prognosis in the discovery and validation data sets

We explored the association between *ZNF326* expression levels and OS in both of these data sets. Subgroup analyses were performed based on adjuvant chemotherapy, age, gender, tumor location, genetic mutations in the *BRAF*, *KARS*, and *TP53* genes, and mismatch repair (MMR), with tests for interaction by the Cox regression model in GSE40967.

Correlation analyses of ZNF326 expression with existing prognostic factors

The relationship between *ZNF326* expression with prognostic factors, including age, gender, genetic mutations in the *BRAF*, *KARS*, and *TP53* genes, and MMR, was investigated in GSE40967. The distribution of *ZNF326* expression in binary prognostic factors was compared using violin plots and *t*-test, and the correlations between *ZNF326* expression and age were analyzed based on scatterplot and Pearson's correlation analysis. Meanwhile, we explored the expression of *ZNF326* in the tumor samples and normal samples based on the Human Protein Atlas (HPA)

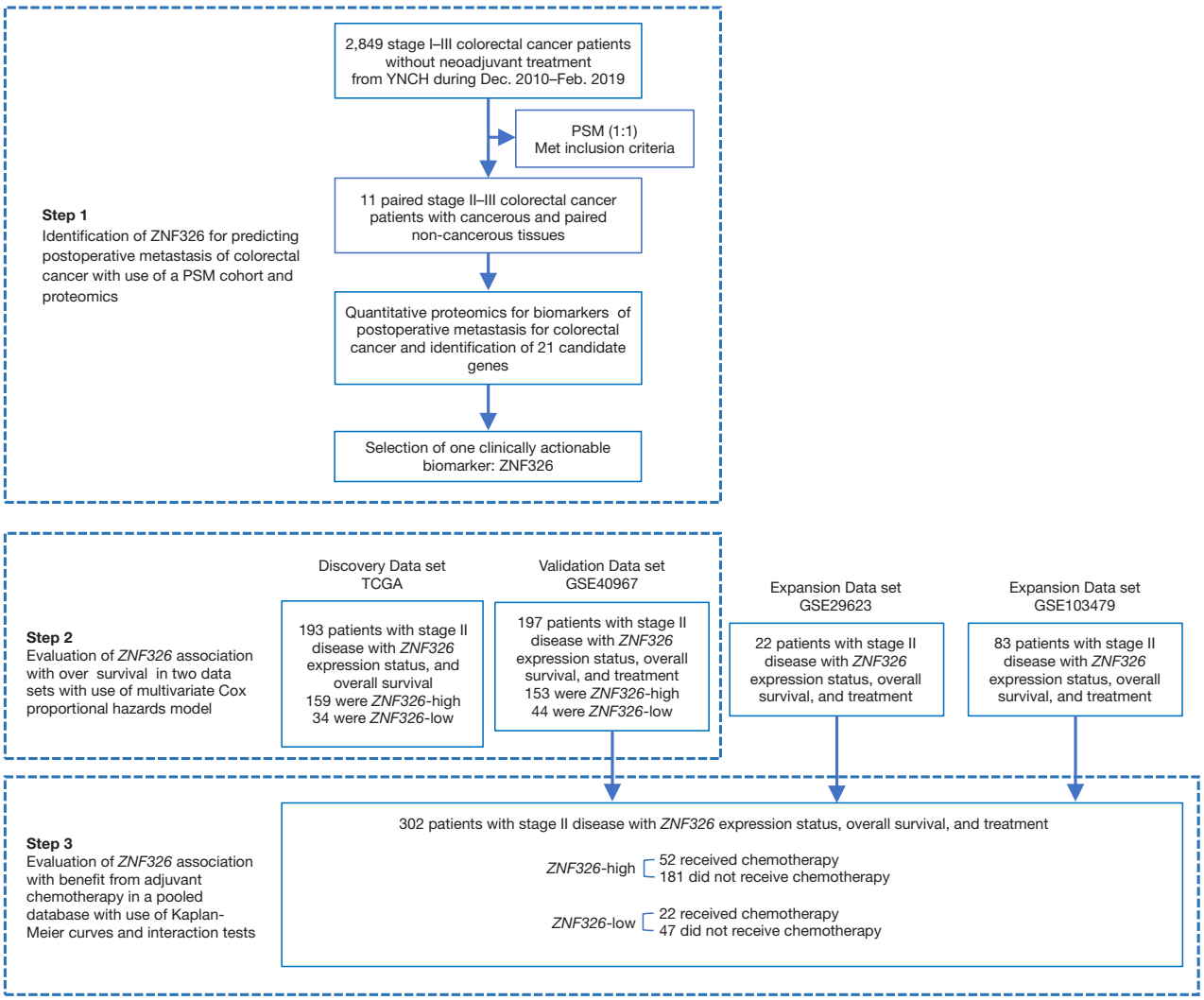

Figure 1 Workflow for the discovery and validation of *ZNF326* in patients with stage II colorectal cancer. YNCH, Yunnan Cancer Hospital; PSM, propensity score matching; TCGA, The Cancer Genome Atlas.

database (https://www.proteinatlas.org/). Many articles have demonstrated that chemokines and their receptors regulate tumor progression and metastasis. Correlations also were calculated between *ZNF326* expression and chemokines across human cancers based on Tumor and Immune System Interaction Database (29) (TISIDB, http://cis.hku.hk/TISIDB).

ZNF326 expression and benefit from adjuvant chemotherapy

By pooling 3 datasets (GSE40967, GSE29623, and GSE103479), we investigated the association between *ZNF326* expression, assessed either at the messenger RNA (mRNA) level, and OS in patients who received or did not receive adjuvant chemotherapy in the NCBI-GEO dataset to assess whether patients with *ZNF326*-low tumors might benefit from adjuvant chemotherapy.

Statistical analysis

Qualitative and quantitative variables were compared between the 2 patient groups using χ^2 test and *t*-test, respectively. Univariate and multivariate survival analyses were conducted using the Cox proportional hazards regression model. Survival plots were generated with the

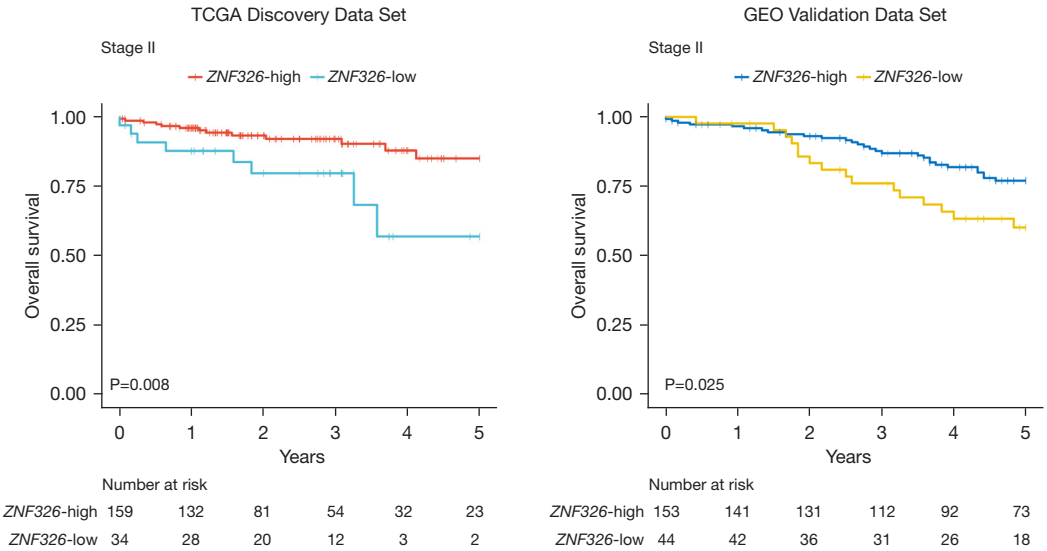

Figure 2 Kaplan-Meier curves showing the relevance between 5-year OS and *ZNF326* gene expression status in colorectal cancer, using TCGA and GEO data. Left: TCGA dataset. Right: GSE40967 dataset. OS, overall survival; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus.

use of Kaplan-Meier method and compared by log-rank test. Statistical analyses were performed using R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria) and corresponding R packages.

Results

Optimal cutoff for ZNF326 expression level

According to our last study involving a nested case-control cohort combining the proteomics, 21 proteins varied on the tumor tissues between the recurrence and non-recurrence CRC patients. Based on this, we selected ZNF326 as a candidate biomarker. Previous studies had indicated that *ZNF326* expression was associated with specific disease processes, such as in non-small cell lung cancer (NSCLC), triple-negative breast cancer (TNBC), glioma, and schizophrenia (30-34). Figure S5 shows the distribution in metastatic and non-metastatic patients, tumor and tumor-adjacent tissues.

The optimal cutoff value for *ZNF326*-scaled expression count at dissection was identified using X-tile software in the discovery cohort of the stage II CRC patients (n=193). As shown in Figure S6, the optimal cutoff value of *ZNF326* was –0.72. Then, the level of *ZNF326* was divided into *ZNF326*-high and *ZNF326*-low groups using cutoff value: there were 159 (82.4%) patients with *ZNF326* above –0.72 and 34 (17.6%) patients with *ZNF326* less than or equal to –0.72, which indicated significant differences among the cutoff value.

ZNF326 expression and OS in the TCGA discovery data set

We aimed to explore the association between *ZNF326* expression and OS among stage II patients in the TCGA discovery data set. The 5-year OS of the 2 groups was compared using Kaplan-Meier curves. As shown in *Figure 2*, the rate of 5-year OS was lower among 34 patients (17.6%) with *ZNF326*-low level than among those 159 (82.4%) patients with *ZNF326*-high level (P=0.008; hazard ratio (HR): 3.13, 95% confidence interval (CI): 1.29–7.58]. In a multivariate analysis that includes age and sex as confounding variables, shown in *Table 1*, the HR for OS among stage II patients with *ZNF326*-low versus *ZNF326*-high was 2.77 (95% CI: 1.11–6.94; P=0.029).

ZNF326 expression and survival in the NCBI-GEO validation data set

To evaluate the robustness of our findings, we performed analysis within the GSE40967 in NCBI-GEO data set. As shown in *Figure 2*, low *ZNF326* expression was associated with poorer 5-year OS in stage II patients (P=0.025; HR: 1.98, 95%

Table 1 Univariate and multivariable Cox analyses for overall survival among patients in stage II disease in TCGA and GSE40967 datasets

Dataset	Variable	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
TCGA	Age[a]	1.12 (1.05–1.18)	<0.001	1.11 (1.05–1.18)	<0.001
	Male *vs.* female	1.09 (0.46–2.58)	0.849	1.20 (0.49–2.92)	0.690
	*ZNF326*_low *vs. ZNF326*_high	3.13 (1.29–7.58)	0.012	2.77 (1.11–6.94)	0.029
GSE40967	Age[a]	1.03 (1.00–1.05)	0.041	1.03 (1.00–1.06)	0.032
	Male *vs.* female	1.07 (0.59–1.94)	0.817	1.22 (0.66–2.24)	0.521
	*ZNF326*_low *vs. ZNF326*_high	1.98 (1.08–3.65)	0.028	2.21 (1.19–4.10)	0.012
	Adjuvant chemotherapy[b]	0.76 (0.35–1.63)	0.484	0.83 (0.37–1.85)	0.641

[a], continuous variable. [b], yes *vs.* no. TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval.

CI: 1.08–3.65). After adjustment for sex, age, and adjuvant chemotherapy, as shown in *Table 1*, multivariate analysis confirmed that low *ZNF326* expression status was associated with poor prognosis and an independent prognostic factor for 5-year OS (HR: 2.21, 95% CI: 1.19–4.10).

Correlation analyses of ZNF326 expression level with existing prognostic factors and chemokines

To evaluate the prognostic ability of *ZNF326*, we assessed the correlation between the expression of *ZNF326* and clinical and pathological features from the GSE40967 cohort (age, sex, genetic mutations in the *BRAF*, *KARS*, and *TP53* genes, and MMR), and *ZNF326* was found to be independent of those prognostic factors (all P>0.05, Figure S7). Next, the expression of *ZNF326* was validated in the tumor samples and normal samples in HPA database. As shown in *Figure 3*, *ZNF326* was lowly expressed in normal colon and rectum tissues. We also explored correlations between *ZNF326* expression and chemokines across human cancers based on the TISIDB database. As shown in Figure S8, most chemokines were negatively correlated with the expression of *ZNF326* and CCL23 was significantly associated with *ZNF326* expression in CRC (r=−0.59, P<0.001).

Subgroup analyses

We performed subgroup analysis to further investigate the association between clinical and pathological characteristics with *ZNF326* expression levels in GSE40967. As displayed in *Figure 4*, the *ZNF326*-low expression level was significantly associated with the lower 5-year OS in patients treated with adjuvant chemotherapy (HR: 6.13, 95% CI: 1.23–30.46), age less than 60 years old (HR: 3.75, 95% CI: 1.06–13.30), and female patients (HR: 3.46, 95% CI: 1.40–8.52). It was also correlated to the 5-year OS in patients with wild-type *BRAF* gene (HR: 2.31, 95% CI: 1.20–4.45) and patients with mutated *KRAS* gene (HR: 3.53, 95% CI: 1.36–9.16).

ZNF326 expression and benefit from adjuvant chemotherapy

To further clarify whether stage II patients with different *ZNF326* expression levels might benefit from adjuvant chemotherapy, we explored the association between *ZNF326* status and OS among stage II patients who either did or did not accept adjuvant chemotherapy in the NCBI-GEO database. As displayed in *Figure 5*, treatment with adjuvant chemotherapy was associated with a higher rate of OS in all stage II patients (P=0.021; HR: 0.46, 95% CI: 0.24–0.90) and the *ZNF326*-high patients (P=0.011; HR: 0.28, 95% CI: 0.10–0.80). Meanwhile, adjuvant chemotherapy was independent of a higher rate of OS in *ZNF326*-low patients (P=0.472; HR: 0.71, 95% CI: 0.28–1.81), which highlights that stage II patients with *ZNF326*-high status are more likely to benefit from adjuvant chemotherapy than stage II patients with *ZNF326*-low level.

Discussion

Although current published clinical guidelines suggest that adjuvant chemotherapy may be appropriate for stage II

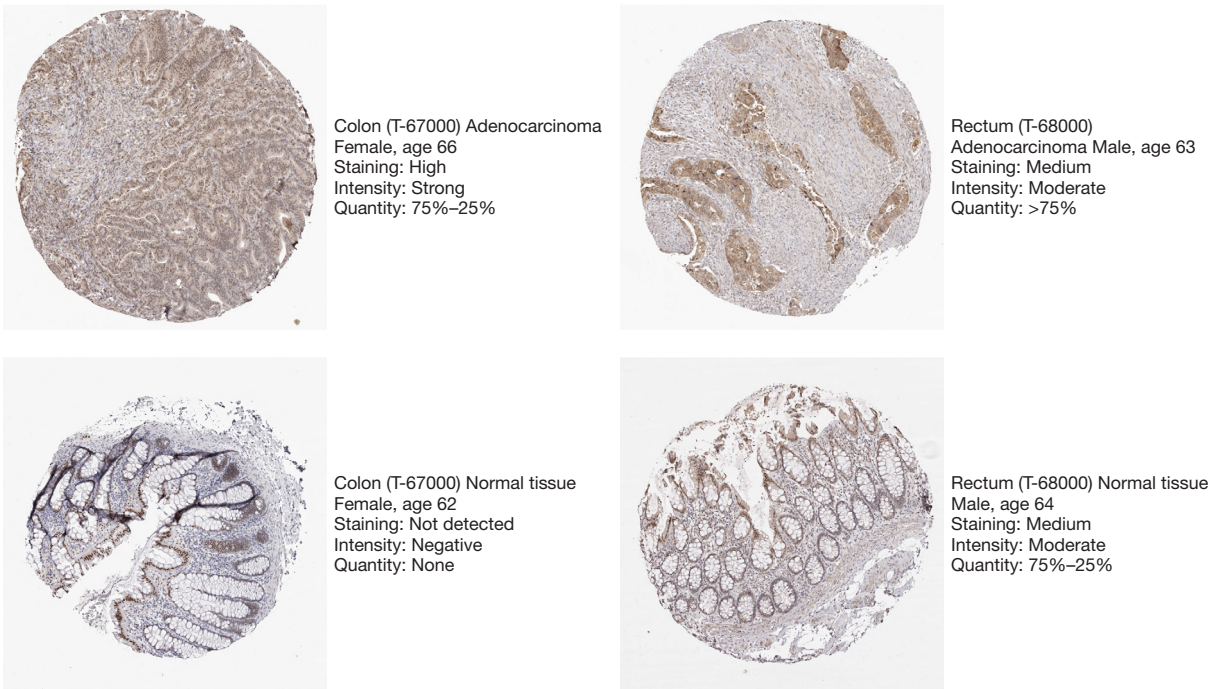

Figure 3 Immunohistochemistry staining with magnification ×400 of ZNF326 proteins in normal tissues and adenocarcinoma samples of colon and rectal cancer, downloaded in the Human Protein Atlas database.

Characteristics	N (%)	Event (%)	HR (95%CI)	P value for interaction
Adjuvant Chemotherapy				
No−Chemo	157 (79.70)	37 (82.22)	1.57 (0.76−3.25)	0.116
Chemo	40 (20.30)	8 (17.78)	6.13 (1.23−30.46)	
Age				
≤60	48 (24.37)	10 (22.22)	3.75 (1.06−13.30)	0.300
>60	149 (75.63)	35 (77.78)	1.66 (0.80−3.45)	
Gender				
Male	111 (56.35)	26 (57.78)	1.21 (0.51−2.88)	0.095
Female	86 (43.65)	19 (42.22)	3.46 (1.40−8.52)	
Tumor Location				
Distal	120 (60.91)	27 (60.00)	1.69 (0.76−3.76)	0.558
Proximal	77 (39.09)	18 (40.00)	2.43 (0.94−6.28)	
BRAF Status				
BRAF.WT	161 (89.44)	37 (88.10)	2.31 (1.20−4.45)	0.332
BRAF.M	19 (10.56)	5 (11.90)	0.72 (0.08−6.52)	
KRAS Status				
KRAS.WT	123 (64.74)	28 (62.22)	1.30 (0.57−2.96)	0.119
KRAS.M	67 (35.26)	17 (37.78)	3.53 (1.36−9.16)	
TP53 Status				
TP53.WT	52 (53.06)	17 (58.62)	1.76 (0.62−4.99)	0.685
TP53.M	46 (46.94)	12 (41.38)	2.29 (0.74−7.10)	
MMR Status				
pMMR	137 (82.04)	31 (77.50)	2.03 (0.97−4.25)	0.849
dMMR	30 (17.96)	9 (22.50)	1.64 (0.34−7.94)	

Figure 4 Forest plot for subgroup analysis evaluating the impact of *ZNF326* expression level on OS, using GSE40967 data. HR and corresponding 95% CI were estimated using the univariate Cox regression model. OS, overall survival; HR, hazard ratio; CI, confidence interval; WT, wild type; M, mutation; MMR, mismatch repair; pMMR, proficient MMR; dMMR, deficient MMR.

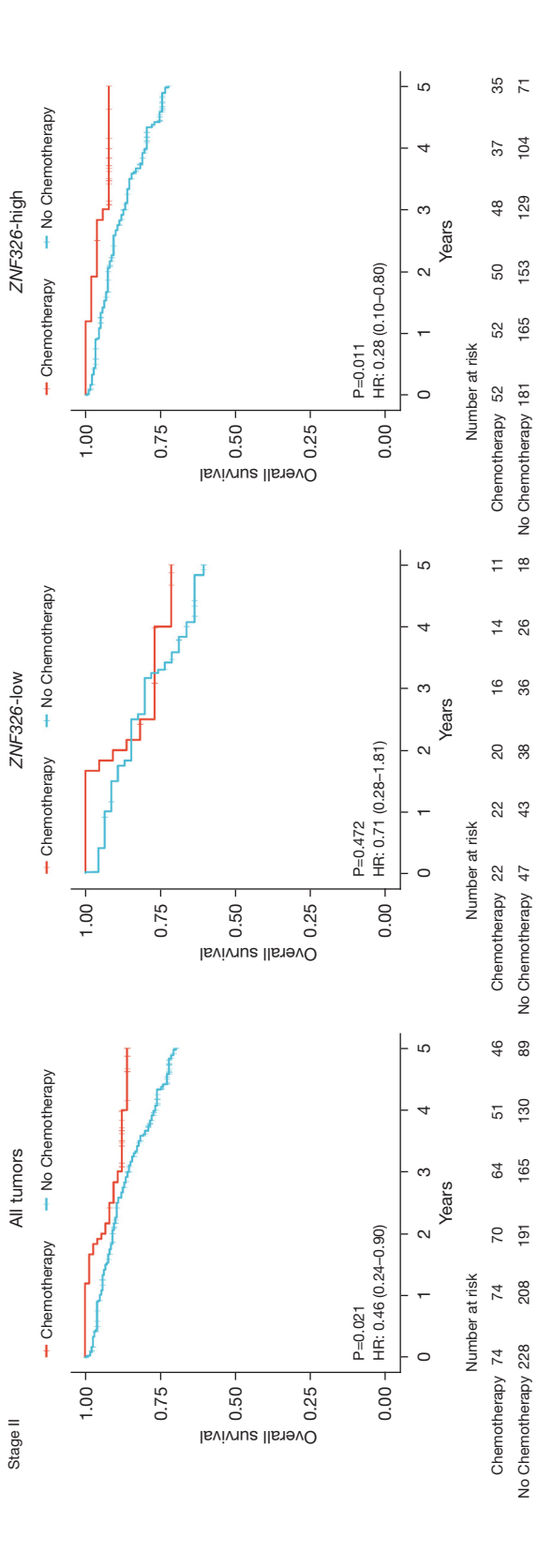

Figure 5 Relationship between *ZNF326* expression and benefit from adjuvant chemotherapy in patients with stage II disease using GEO datasets. GEO, Gene Expression Omnibus; HR, hazard ratio.

CRC patients with poor prognostic features, it is estimated that the adjuvant chemotherapy only provides a relatively small absolute benefit (3–4% patients with survival improvement) for stage II CRC patients (14,35). Accurately predicting the prognosis at the time of diagnosis is critical for clinicians to tailor the treatment plan for maximal efficacy and to determine surveillance strategies (36). However, prognostic markers in stage II CRC have been sparse and the available ones are not easily translated into the clinical setting (37).

We employed a nested case-control design combining the proteomics to discover a prognostic biomarker in CRC patients. Based on literature search and previous basic experiments, *ZNF326* was selected as a biomarker. It was first discovered in *Drosophila*, where it is thought to play an important role in the differentiation of nerve cells (38). *ZNF326* has been shown to promote the epithelial-mesenchymal transition (EMT) and invasiveness of CRC cells, and CRC patients with high *ZNF326* expression level were positively correlated with tumor differentiation, tumor-node-metastasis (TNM) staging, and lymph node metastasis (39). Several previous studies have demonstrated that *ZNF326* expression levels are associated with prognosis of other cancers, including that it promotes the proliferation of NSCLC cells by regulating the expression of ERCC1 (30), plays a vital role in promoting the malignant phenotype of breast cancer cells by interacting with DBC1 and is associated with poor prognosis (31,40), and expresses highly in glioma cell lines and tissues and is closely related with advanced tumor grade in the patients (33).

Our results revealed that stage II CRC and *ZNF326*-low expression level were both associated with poor OS with adjustment of the existing prognostic factors in the discovery dataset and validation dataset. Hence, *ZNF326* was an independent prognostic biomarker. A study conducted by Uhlen *et al.* reported that *ZNF326* is a favorable prognostic gene symbol (P=0.007) in CRC patients based on a pathology atlas of the human cancer transcriptome (41), which is consistent with our finding. Besides, we detected an association of *ZNF326* expression level and the benefit from adjuvant chemotherapy in stage II CRC patients. Our finding confirmed that *ZNF326* was a predictive biomarker for the adjuvant chemotherapy.

To exclude existing prognostic factors that may impact *ZNF326* expression, correlation analyses were performed between *ZNF326* expression and prognostic factors based on *t*-test or Pearson's correlation analysis. Of those factors, age (r=0.1, P=0.16), sex (P=0.76), MMR

(P=0.38), mutational status of *BRAF* gene (P=0.70), *KRAS* gene (P=0.67), and *TP53* gene (P=0.38) were not significantly correlated with *ZNF326* expression; *ZNF326* was found to be independent of those prognostic factors. Different immune cell subsets are recruited into the tumor microenvironment via interactions between chemokines and chemokine receptors, and these populations have distinct effects on tumor progression and therapeutic outcomes (42). In this regard, we employed correlation analyses between *ZNF326* gene expression and chemokines across human cancers using TISIDB and found that expression of *ZNF326* in most cancers is negatively correlated with chemokines. Importantly, CCL23 was significantly associated with *ZNF326* expression in CRC patients (r=−0.59, P<0.001), which indicated that *ZNF326* may affect the prognosis of CRC patients by participating in immune processes.

Our findings suggest that stage II CRC patients with *ZNF326*-high expression status might benefit from adjuvant chemotherapy, therefore, *ZNF326* might be potential predictive biomarker for stage II CRC patients in the current clinical setting. Indeed, subgroup analysis was used to explore the relationship between other clinical and pathological features with adjuvant chemotherapy. It is worth noting that *ZNF326* expression demonstrated significant prognostic value among less than 60 years old and female patients, it was also correlated to the 5-year OS in patients with wild-type *BRAF* gene and mutated *KRAS* gene. Therefore, *ZNF326* expression status alone or in combination with conventional features, such as tumor age, sex, and genetic mutation status in *BRAF* and *KRAS* has the potential to improve prognosis and influence postoperative decisions.

Our study had several limitations. Firstly, as certain CRC patients were lacking recurrence-free survival (RFS) information in the discovery dataset, our current study focused on OS; thus, to a certain extent, the effect of *ZNF326* expression on RFS could not be explained. Second, information on some important clinicopathological features of patients in public cohorts, including TCGA and GEO, is incomplete. Third, because of the exploratory and retrospective nature of the study, a prospectively designed study is necessary to further validate the prognostic value of *ZNF326*.

Conclusions

With the use of independent cohort and public datasets comprising stage II CRC patients, extensive protein screening was performed and validated in conjunction with public databases. Our results indicated that the rate of 5-year OS was lower among stage II CRC patients with low *ZNF326* expression level than those with *ZNF326*-high status. Stage II patients with *ZNF326*-high status are more likely to benefit from adjuvant chemotherapy than those with low *ZNF326* expression level. In conclusion, *ZNF326* has the potential to be used in clinical practice for risk classification. However, given the prospectively designed of our study, our results need to be further validated.

Acknowledgments

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-908/rc

Data Sharing Statement: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-908/dss

Peer Review File: Available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-908/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jgo.amegroups.com/article/view/10.21037/jgo-23-908/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was

conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Yunnan Cancer Hospital Ethics Committee (No. KY2019141). The requirement for informed consent was waived by the Ethics Committee owing to the retrospective nature of the study.

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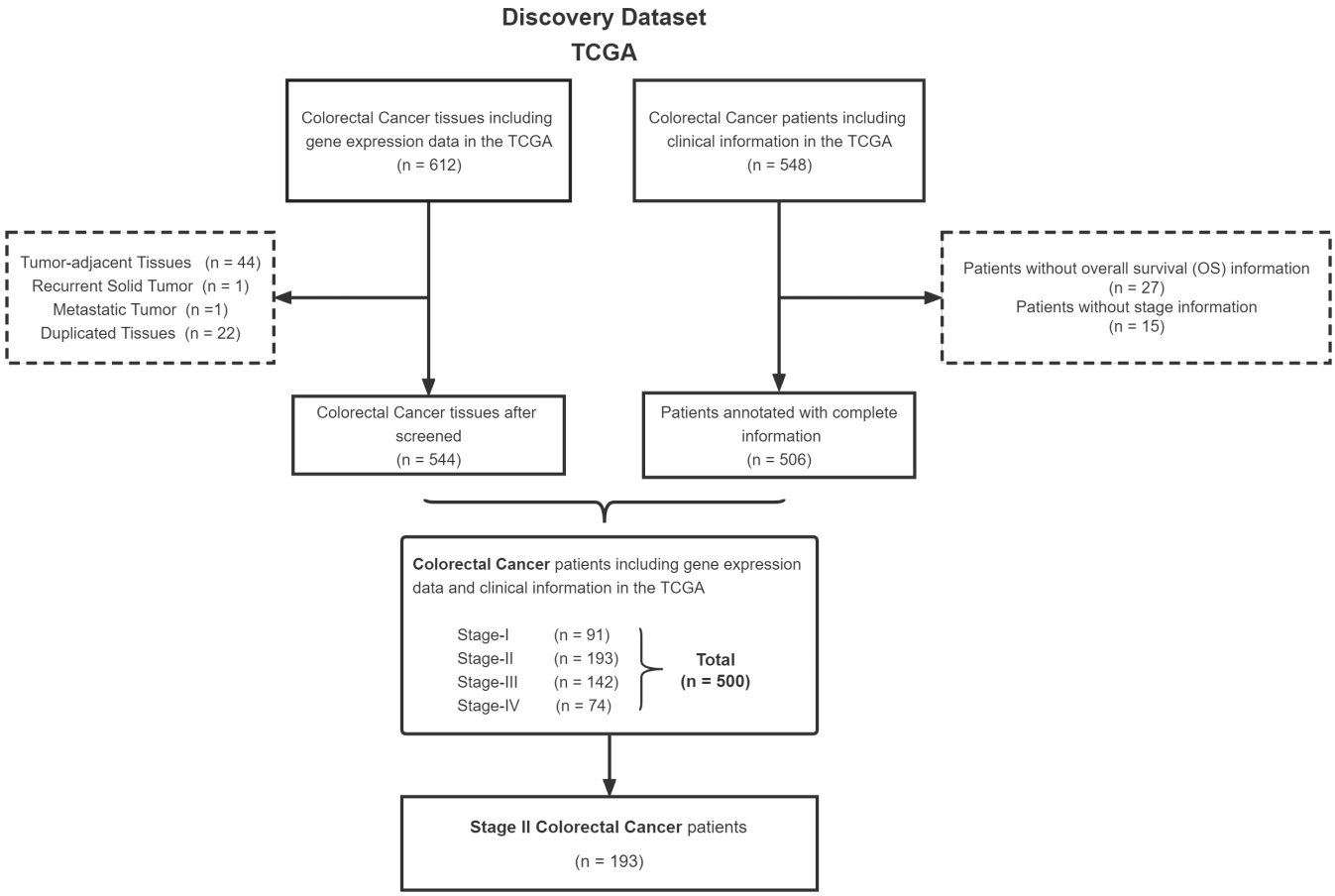

Figure S1 Patients composition of the "Discovery Dataset" TCGA.

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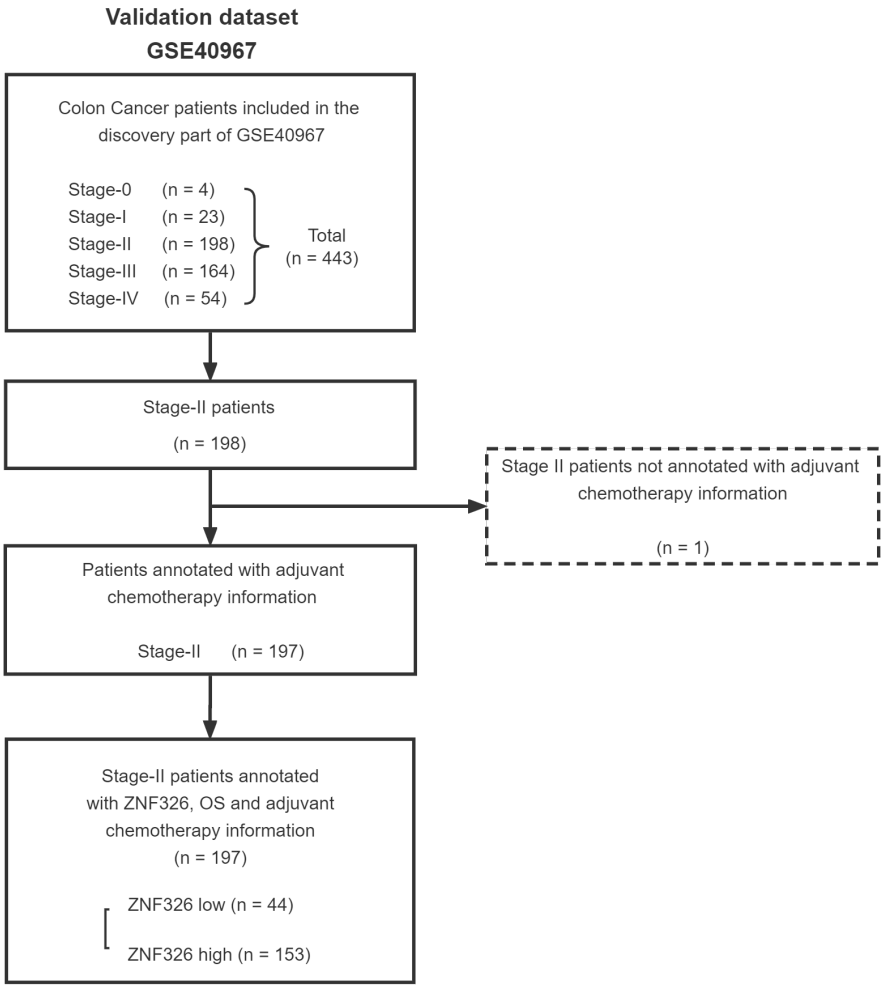

Figure S2 Patients composition of the "Validation Dataset" GSE40967.

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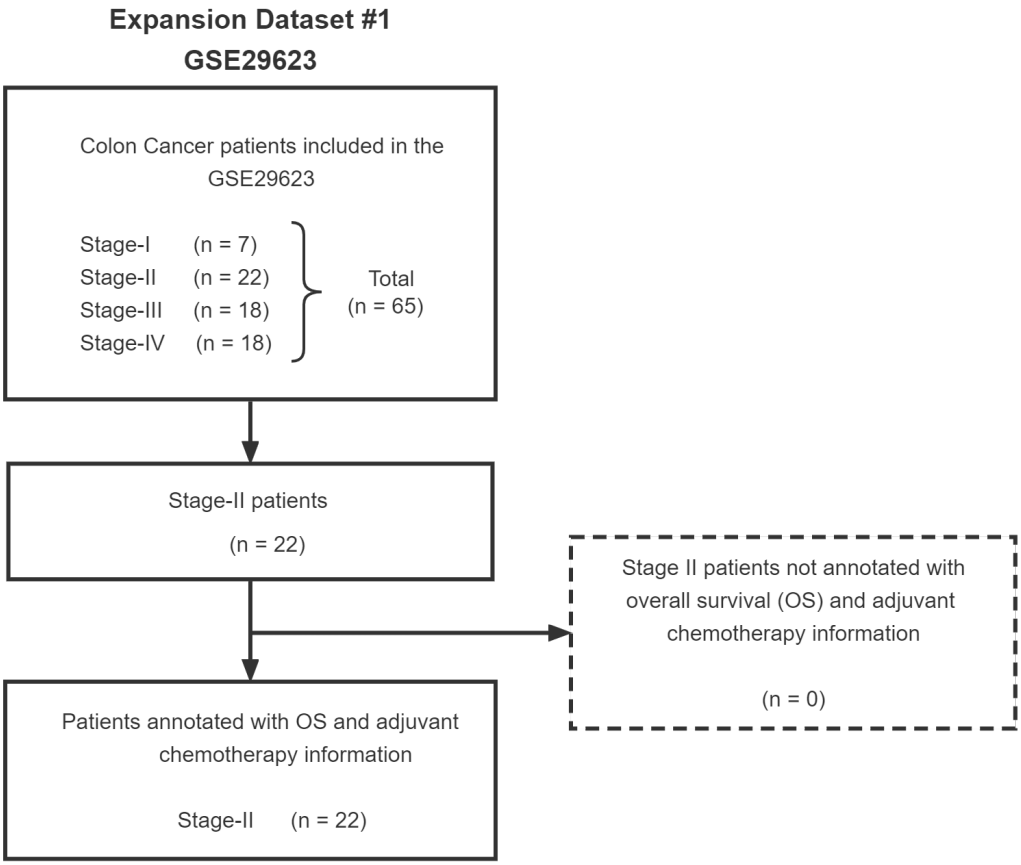

Figure S3 Patients composition of the "Expansion Dataset #1" GSE29623.

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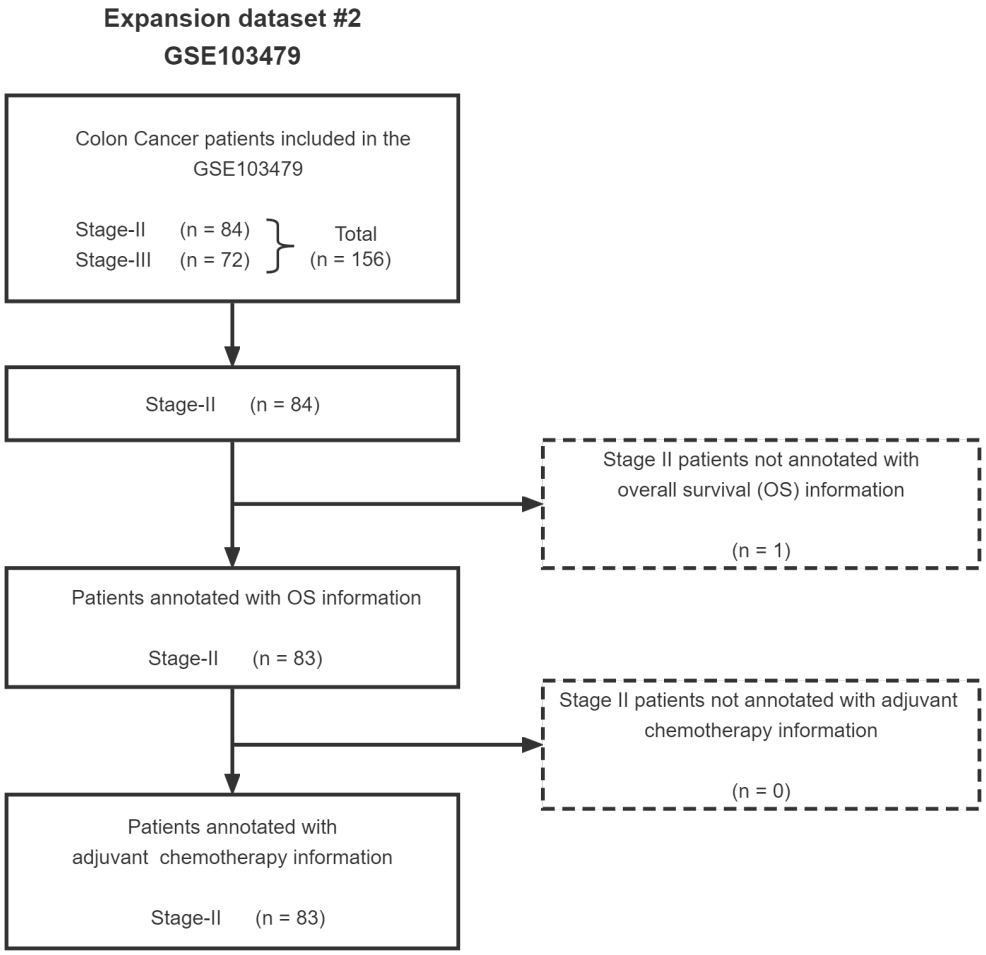

Figure S4 Patients composition of the "Expansion Dataset #2" GSE103479.

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Table S1 Characteristics of stage II patients at baseline and follow-up in TCGA and GEO datasets

Variable	TCGA (n=193)	GSE40967 (n=197)	GSE29623 (n=22)	GSE103479 (n=83)
Age, year	68.0 [60.0, 77.0]	70.0 [61.0, 77.0]	N/A[a]	70.6 [61.9, 77.8]
Male	109 (56.5)	111 (56.3)	11 (50.0)	48 (57.8)
T stage				
T2	0 (0.0)	4 (2.0)	0 (0.0)	0 (0.0)
T3	180 (93.3)	142 (72.1)	19 (86.4)	70 (84.3)
T4	13 (6.7)	44 (22.3)	3 (13.6)	13 (15.7)
N/A[a]	0 (0.0)	7 (3.6)	0 (0.0)	0 (0.0)
N stage				
N0	193 (100.0)	190 (96.4)	21 (95.5)	83 (100.0)
NX	0 (0.0)	0 (0.0)	1 (4.5)	0 (0.0)
N/A[a]	0 (0.0)	7 (3.6)	0 (0.0)	0 (0.0)
M stage				
M0	179 (92.7)	190 (96.4)	21 (95.5)	49 (59.0)
MX	12 (6.2)	0 (0.0)	1 (4.5)	34 (41.0)
N/A[a]	0 (0.0)	7 (3.6)	0 (0.0)	0 (0.0)
Adjuvant chemotherapy	N/A[a]	40 (20.3)	9 (40.9)	25 (30.1)
Follow-up				
Overall survival	172 (89.1)	136 (69.0)	19 (86.4)	59 (71.1)
Overall survival time, year	2.0 [1.1, 3.3]	4.6 [2.8, 6.9]	4.3 [3.0, 5.1]	4.7 [3.5, 7.2]

Data are presented as median [IQR] or n (%). [a], missing value.

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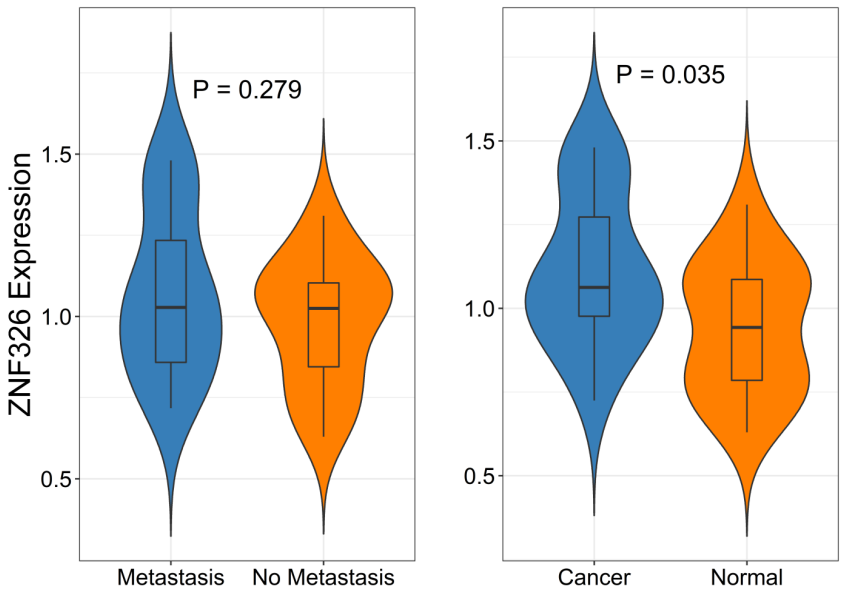
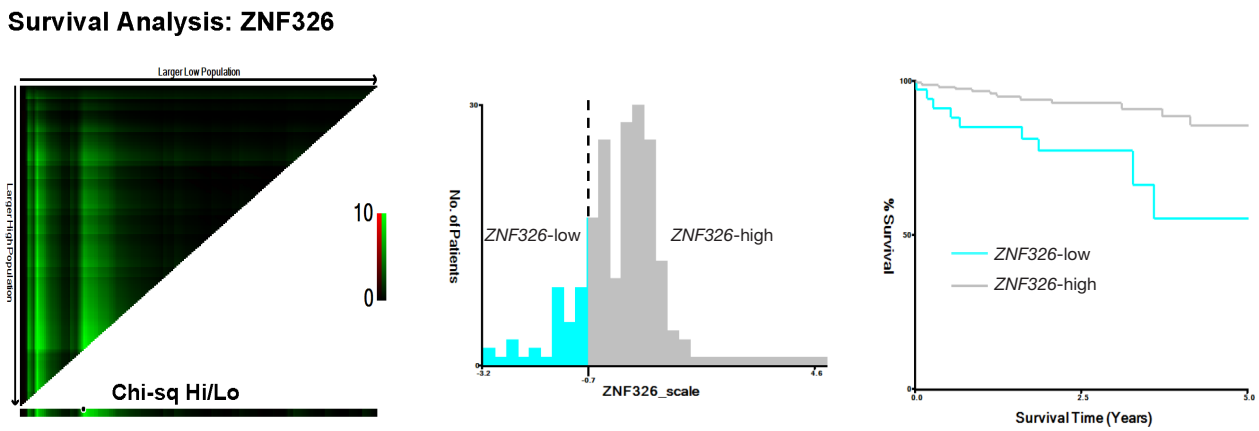


Figure S5 Violin diagram showing the distribution of ZNF326 protein expression. Left: the distribution of ZNF326 protein expression between metastatic and non-metastatic groups. Right: tumor and adjacent tissues.



Subpopulation Cutpoints:

Pt No	% Total	Events	Rate	Rank	Range
35	18.13	9	25.71	0 to 34	-3.18 thru -0.72
158	81.87	12	7.59	35 to 192	-0.72 thru 4.62
193	100.00	21	10.88	0 to 192	-3.18 thru 4.62

Figure S6 X-tile plots of *ZNF326* gene expression in stage II patients, using TCGA dataset.

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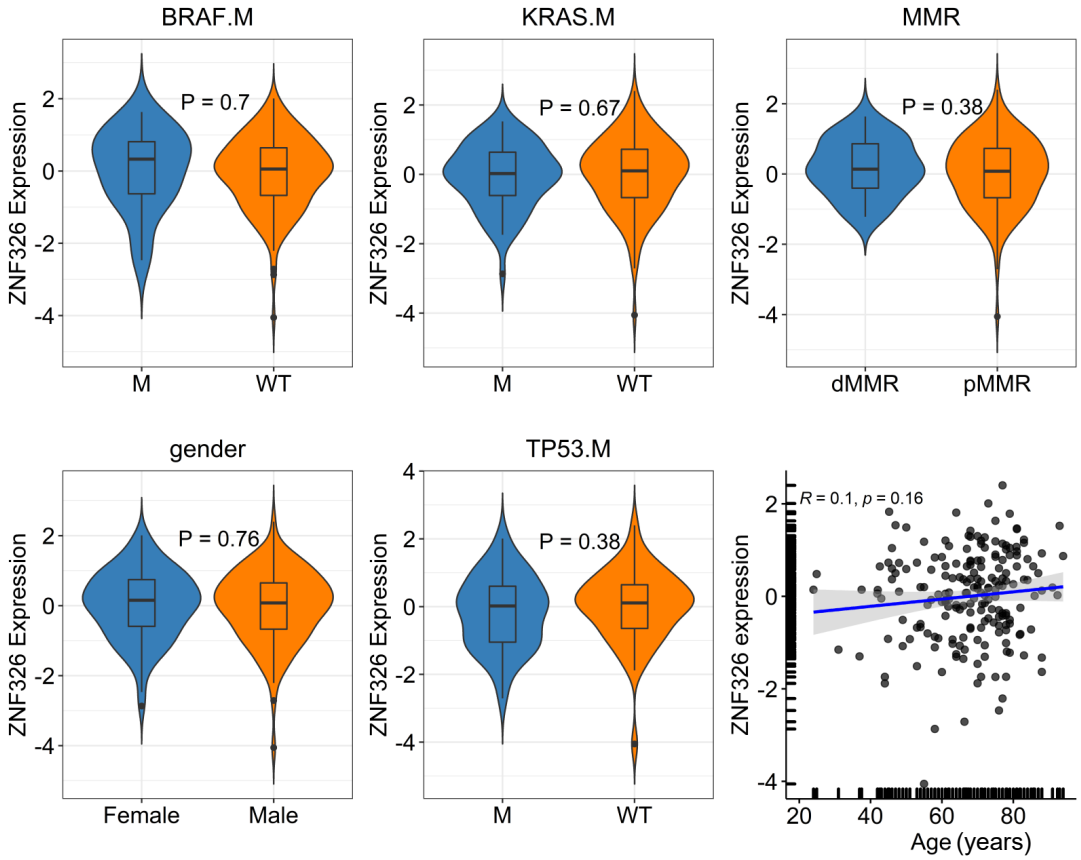

Figure S7 Violin-plots and *t*-test, scatterplot and Pearson's correlation analysis showing the relationships between *ZNF326* gene expression and existing prognostic factors (age, gender, genetic mutations in the *BRAF*, *KRAS*, and *TP53* genes, and MMR), using GSE40967 dataset.

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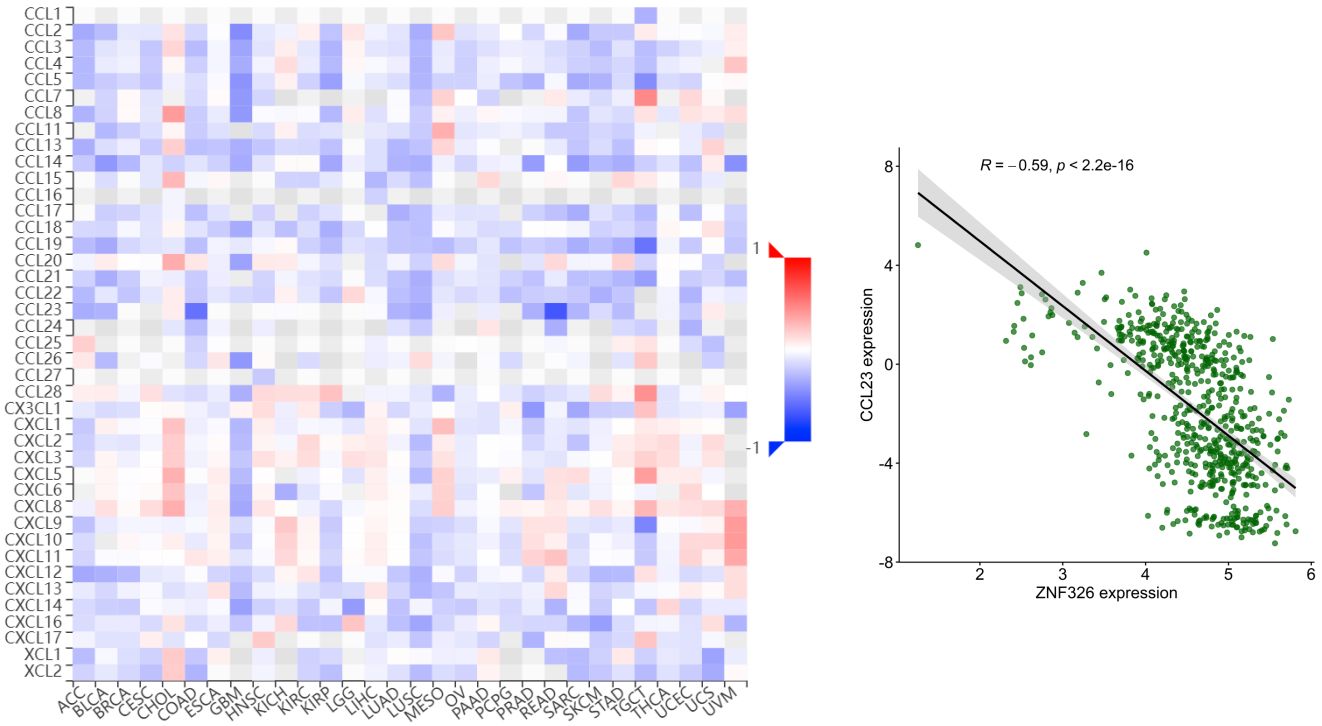

Figure S8 Spearman correlations between *ZNF326* expression and chemokines (left) across human cancers (X axis) and CCL23 in human colorectal cancer (right).

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