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The potential of EZH2 expression to facilitate treatment choice in stage II colorectal adenocarcinoma

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Summary. Background. The current selection criteria of patients with stage II colorectal carcinoma (CRC) suitable for adjuvant therapy are not satisfactory. Enhancer of zeste homolog 2 (EZH2) has been demonstrated to be over-expressed in CRC. However, data regarding the role of EZH2 in CRC survival remains controversial, and little is known about it in stage II CRC. Thus, we conducted this study to investigate the clinical significance of EZH2 expression in stage II CRC.

Methods. Cases with stage II CRC resected between 2015 and 2018 were retrospectively reviewed. EZH2 expression was analyzed by immunohistochemistry using tissue microarrays. The relationship between EZH2 expression and clinicopathological variables was analyzed. Survival curves were estimated by the Kaplan-Meier approach.

Results. We found high EZH2 expression in 134 of 221 analyzable stage II tumors (60.63%). No significant associations were observed between EZH2 expression and common clinicopathological factors. Survival analyses showed that cases receiving surgery alone had inferior overall survival (OS) than those receiving surgery and chemotherapy (P=0.0075) in stage II CRC with high EZH2 expression, however, metastasis-free survival (MFS) was similar between these two subgroups. Treatment choice had no impact on the survival of stage II CRC with low EZH2 expression.

Conclusion. The OS of stage II CRC with high EZH2 expression improved more strikingly with surgery and adjuvant chemotherapy than with surgery alone, which suggests the potential of EZH2 expression as a biomarker to help identify a subgroup of early-stage CRC benefiting from surgery and adjuvant chemotherapy. More large-scale studies are warranted to corroborate this finding and to further evaluate the predictive nature of EZH2.

Key words: EZH2, Colorectal adenocarcinoma, Immunohistochemistry, Overall survival, Surgery, Adjuvant chemotherapy

Introduction

Colorectal cancer (CRC) is one of the leading malignancies in adults worldwide (Bray et al., 2018). According to the NCCN guideline for CRC (2024 version 1), adjuvant chemotherapy is not recommended for stage I CRC patients (pT1-2N0M0) but is necessary for stages III and IV. For stage II CRC, surgical resection is still the mainstay of treatment at present, and there is a dispute about whether to apply chemotherapy or not (André et al., 2009). The current selection of patients with stage II CRC suitable for adjuvant chemotherapy mainly depends on the risk features (e.g., pMMR/MSS, poorly differentiated/undifferentiated histology, lymphatic/vascular invasion, bowel obstruction, <12 lymph nodes examined, perineural invasion (PNI), localized perforation, close, indeterminate, positive margins, or high-tier tumor budding) (Benson et al., 2004; Artac et al., 2014; Kumar et al., 2015; Park et al., 2016; Chen et al., 2017). Nevertheless, not all eligible cases under such selection principles achieve improved survival. The NCCN guideline also points out that there are no data that correlate risk features and selection of chemotherapy in patients with high-risk stage II disease.

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Abbreviations. EZH2, Enhancer of zeste homolog 2; CRC, colorectal carcinoma; PRC2, polycomb repressive complex 2; H3K27 me3, trimethylation of Lysine 27 at histone 3; TMAs, tissue microarrays; IHC, immunohistochemistry; MMR, mismatch repair; OS, overall survival; MFS, metastasis-free survival; NOS, not specific; dMMR, deficient mismatch repair.

Furthermore, a survival benefit has not been demonstrated for the addition of oxaliplatin to 5-FU/leucovorin in stage II CRC. Therefore, efforts have been currently focused on exploring novel markers that facilitate recognizing a subgroup of stage II CRC patients that would benefit from adjuvant chemotherapy the most (Lee et al., 2016).

The importance of epigenetic dysregulation involved in carcinogenesis has been recognized in recent years, especially in histone methylation abnormalities induced by histone methyltransferases and demethylases (Huang et al., 2017). A number of studies have paved the way for the recognition of epigenetic regulators as potential predictors for diagnosis and prognosis with high sensitivity and specificity in CRC (Balgkouranidou et al., 2013; Costa-Pinheiro et al., 2015). Enhancer of zeste homolog 2 (EZH2) is one of such appealing epigenetic regulators. As the crucial catalytic element of polycomb repressive complex 2 (PRC2), it leads to target gene silencing by catalyzing the trimethylation of Lysine 27 at histone 3 (H3K27me3) via its SET domain, which contributes to the oncogenic characteristics (Kirmizis et al., 2004; Kuzmichev et al., 2004; Schlesinger et al., 2007; Margueron et al., 2008).

An increasing body of evidence has demonstrated EZH2 overexpression in a variety of malignancies including CRC, pulmonary adenocarcinoma, and multiple myeloma. High EZH2 expression is reported to be associated with adverse outcomes in several cancers (Bachmann et al., 2006; Bremer et al., 2021; Wu et al., 2019). However, data regarding the role of EZH2 in the survival of CRC remains controversial (Vilorio-Marqués et al., 2017; Chen et al., 2018; Bremer et al., 2021), and particularly, little is known about EZH2 expression in early-stage CRC. Thus, we conducted this study to explore the clinical value of EZH2 expression in patients with stage II CRC and the survival benefit of adjuvant chemotherapy based on EZH2 expression.

Materials and methods

Patients

Cases with stage II CRC who were completely resected at the Affiliated Hospital of Nanjing University Medical School, Nanjing Drum Tower Hospital with curative intent between 2015 and 2018 were selected following full ethical approval by the Institutional Review Boards of our hospital (Approval No. 2021-503-01). Exclusion criteria included cases without histological confirmation and where CRC was not the first primary malignancy. The medical record was retrospectively reviewed.

Tissue microarray

Tissue microarrays (TMAs) were made by TMA Master (3DHISTECH Inc, Budapest, Hungary). Three 2.0-mm cores were randomly sampled from the tumor center of each most representative tumor block. Cases with less than three evaluable cores were regarded as unanalyzable due to their not being representative of tumor heterogeneity and then be excluded for further analyses in this study.

Immunohistochemistry (IHC)

Four-micron thick TMA slides were prepared by being deparaffinized in xylene and then placed in alcohols. After target antigen retrieval by citrate buffer (pH 6.0) for 10 minutes, the slides were exposed to 3% hydrogen peroxide for another 10 minutes to block endogenous peroxidase activity. Subsequently, they were incubated with the primary antibody against EZH2 (1:400, Catalogue No. ab191080, Clone No. EPR9307, Abcam, Cambridge, UK) for half an hour at room temperature, followed by the secondary antibody (EnVision™ Detection Kit, Dako, Glostrup, Denmark).

To assess the expression of EZH2 by IHC, intensity scores were categorized into 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) (Fig. 1). The staining extent was measured by the proportion of positively stained tumor cells as follows (Abdel Raouf et al., 2021): 0 (0%), 1(0-10%], 2 (10-50%], 3 (50-80%], and 4 (80-100%]. The final point was figured by multiplying intensity and extent scores (range, 0-12). Expression of EZH2 was denoted as negative or low if the total score <4, and high if ≥ 4 according to previous studies (Kim et al., 2014; Abdel Raouf et al., 2021). The staining intensity and extent were examined by two pathologists (ZZ and LH) independently and blindly. Disagreements were later adjudicated with a multiheaded microscope to reach a final consensus.

Statistical analysis

Clinicopathological parameters were collected including tumor site, tumor differentiation, lympho-vascular and perineural invasion, mismatch repair (MMR) protein status, age, sex, year of diagnosis, use of chemotherapy, vital status, and survival (months). Tumor sites were grouped into right colon, left colon, and rectum.

Fisher's exact test was applied to compare categorical variables between the two groups (high EZH2 expression vs. low EZH2 expression). Overall survival (OS) was defined as the time from diagnosis to death from any cause or the last follow-up. Metastasis-free survival (MFS) was defined as the time from diagnosis to the date of the occurrence of distant metastases or death from any cause. Survival curves were estimated by the Kaplan-Meier approach. The Cox model was established to conduct univariate and multivariate analyses. All statistical analyses were performed using SPSS for Windows (version 18.0; IBM Corporation, Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). All P values represented were two-sided, and statistical

significance was declared at $P<0.05$.

Results

Patient characteristics

A total of 232 CRC specimens from 232 patients with stage II CRC were included in this study, of which 11 cases (4.74%) had less than three analyzable sample cores, leading to 221 cases for the final analyses of EZH2 expression. The median age of patients at diagnosis was 66 years. Overall, there was a male predominance (146 males and 75 females). Microscopically, all patients had basic morphologic characteristics of adenocarcinoma with 212 not specific (NOS), 8 NOS with mucinous features, and 1 NOS with micropapillary features. The majority (82.20%) had moderately differentiated tumors. Eighty-four (38.01%) patients presented right colon involvements, 74 (33.48%) left colon involvements, and 63 (28.51%) with rectum involved. Most patients (95.93%) were pathologically grouped as stage T3. In addition, all 221 cases underwent an R0 resection. The clinicopathological characteristics of the entire cohort are summarized in Table 1.

Association of EZH2 expression with clinicopathological variables

Inter-observer analyses throughout the entire cohort demonstrated a high concordance of EZH2 staining (Cohen κ value: 0.80). Of the 221 stage II CRCs with EZH2 expression analyzed, 134 cases (60.63%) displayed high expression of EZH2 in tumor cell nuclei, and 87 cases (39.37%) low expression of EZH2. No remarkable clinicopathological differences were observed between the two groups regarding sex, tumor differentiation, lymphovascular invasion,

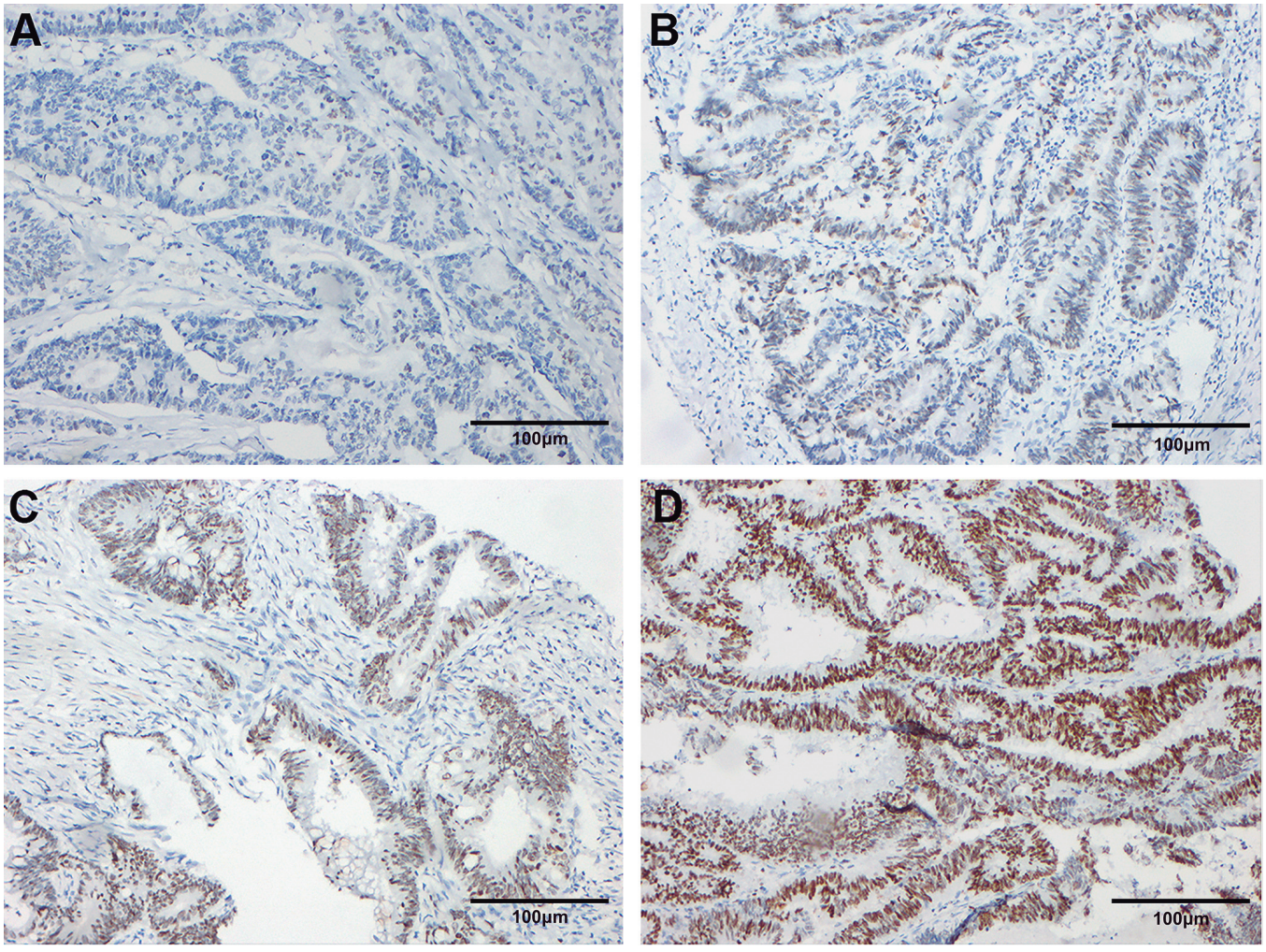

Fig. 1. Immunohistochemical staining for EZH2 in colorectal carcinoma. **A.** No nuclear expression of EZH2 (score 0). **B.** Weak nuclear expression of EZH2 (score 1). **C.** Moderate nuclear expression of EZH2 (score 2). **D.** Strong nuclear expression of EZH2 (score 3).

perineural invasion, tumor location, and MMR status (Table 1).

Survival analysis

A total of 177 cases with complete follow-up were studied for survival analyses, of which 95 cases received adjuvant chemotherapy, including capecitabine or with 5-fluorouracil (5-FU)/leucovorin alone, oxaliplatin-based FOLFOX, or CAPEOX regimens. Until May 2021, there were 20 (11.30%) deaths recorded with a median follow-up of 56 months. The median OS and MFS were not reached (Fig. 2A,B). For the entire cohort, high EZH2 expression was associated with prolonged OS compared with the low EZH2 expression group (P=0.0225) (Fig. 2C). There was no significant

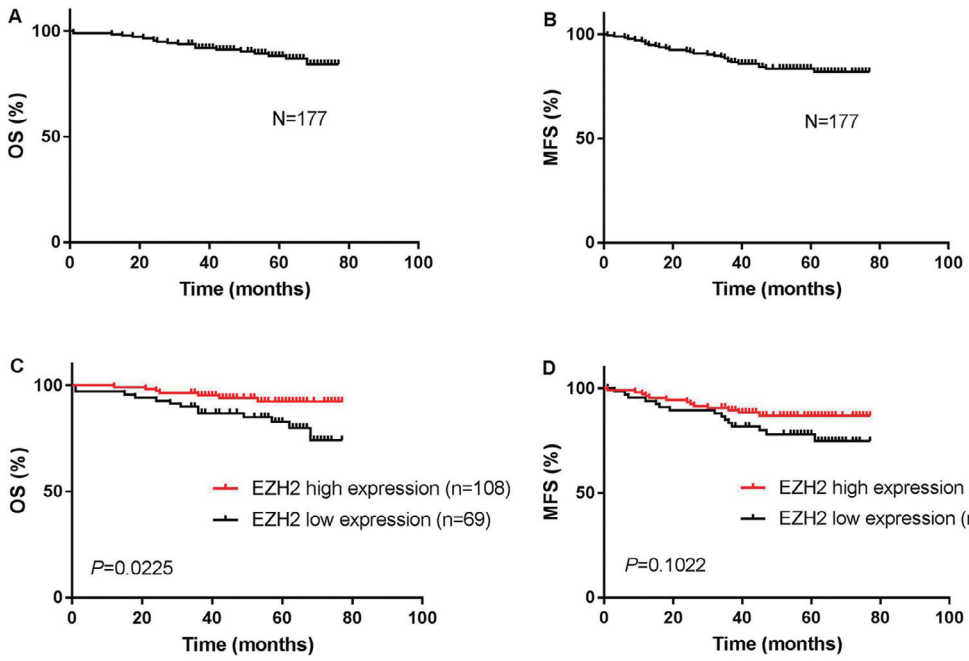

Fig. 2. Survival of all patients with stage II CRC. **A.** The overall survival (OS) and metastasis-free survival (MFS) **(B)** curves for patients with stage II CRC. **C.** OS and MFS **(D)** of patients with stage II CRC by EZH2 expression.

Table 1. Clinicopathological characteristics stratified by EZH2 expression for patients with stage II CRC (N=221).

Clinicopathological characteristics	Number (%)	EZH2 high expression (n=134)	EZH2 low expression (n=87)	P-value
Age (mean, range)	65 (27-92)	63 (27-92)	67 (43-88)	0.06
Sex				0.67
Male	146 (66.06)	90 (67.16)	56 (64.37)	
Female	75 (33.94)	44 (32.84)	31 (35.63)	
Tumor differentiation				0.19
Well-differentiated	4 (1.81)	2 (1.49)	2 (2.30)	
Moderately differentiated	182 (82.35)	106 (79.10)	76 (87.36)	
Poorly differentiated	35 (15.84)	26 (19.40)	9 (10.35)	
Lymphovascular invasion				0.54
Yes	194 (87.78)	116 (86.57)	78 (89.66)	
No	27 (12.22)	18 (13.43)	9 (10.34)	
Perineural invasion				0.93
Yes	138 (62.44)	84 (62.69)	54 (62.07)	
No	83 (37.56)	50 (37.31)	33 (37.93)	
Tumor location				0.42
Right colon	84 (38.01)	47 (35.08)	37 (42.53)	
Left colon	74 (33.48)	45 (33.58)	29 (33.33)	
Rectal	63 (28.51)	42 (31.34)	21 (24.14)	
MMR status				0.22
Proficient	192 (86.88)	113 (84.33)	79 (90.80)	
Deficient	29 (13.12)	21 (15.67)	8 (9.20)	

difference in MFS for stage II CRC between the two groups according to EZH2 expression (Fig. 2D). In cases receiving surgery only (n=82), no survival benefit was observed in those with high EZH2 expression for either OS (Fig. 3A) or MFS (Fig. 3B). In cases receiving surgery and adjuvant chemotherapy (n=95), however, high EZH2 expression imparted a better OS (Fig. 3C) but not MFS (Fig. 3D).

For stage II CRC with high EZH2 expression (n=108), cases receiving surgery alone had inferior OS than cases receiving surgery and chemotherapy (*P*=0.0075) (Fig. 4A), but MFS was similar between these two subgroups (Fig. 4B). Treatment had no impact on survival of stage II CRC with low EZH2 expression (n=69, Fig. 4C,D).

Univariate analysis showed that left colon tumor location, high EZH2 expression, and treatment combining surgery and chemotherapy were significantly related to longer OS (Table 2). Then, these variables were selected to construct multivariate models, revealing that all maintained their prognostic value for OS (Table 3). Unfortunately, no variables were linked to MFS by univariate analysis (Table 2), and multivariate analysis was therefore inapplicable.

Discussion

Aberrant histone methylation has been indicated to play vital roles in CRC. As a methyltransferase to induce H3K27 methylation, EZH2 participates in regulating

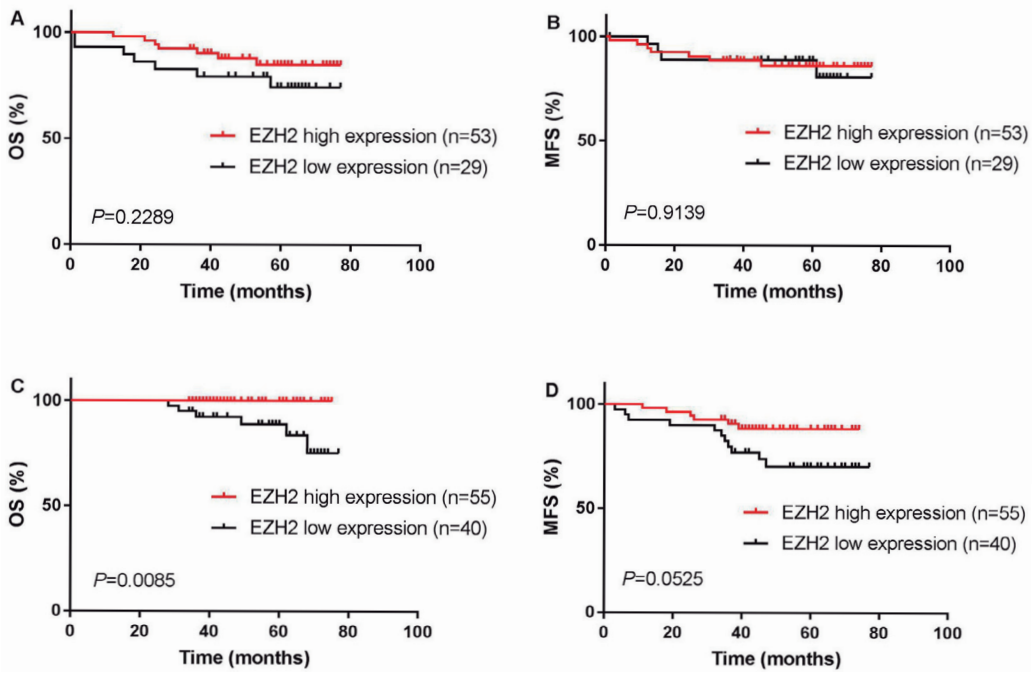

Fig. 3. Subgroup analyses. **A.** OS and MFS **(B)** of the cases receiving surgery only based on EZH2 expression. **C.** OS and MFS **(D)** of the cases receiving surgery and adjuvant chemotherapy based on EZH2 expression.

Table 2. Univariate Cox regression analysis of the clinicopathological characteristics for OS and MFS in 177 patients with stage II CRC.

Clinicopathological characteristics	Univariate analysis (OS)		Univariate analysis (MFS)	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≤60 vs >60)	1.626 (0.590-4.480)	0.348	0.593 (0.282-1.248)	0.169
Sex (male vs female)	0.998 (0.382-2.607)	0.997	1.434 (0.671-3.065)	0.352
Tumor differentiation (well-differentiated vs moderately differentiated vs poorly differentiated)	0.527 (0.140-1.976)	0.342	0.550 (0.169-1.784)	0.319
Lymphovascular invasion (yes vs no)	0.402 (0.054-3.009)	0.375	0.259 (0.035-1.905)	0.184
Perineural invasion (yes vs no)	1.494 (0.618-3.614)	0.373	1.107 (0.518-2.364)	0.793
Tumor location (left colon vs right colon vs rectum)	0.502 (0.275-0.917)	0.025	1.003 (0.630-1.597)	0.989
MMR status (proficient vs deficient)	0.744 (0.173-3.207)	0.691	0.494 (0.117-2.082)	0.337
EZH2 expression (high vs low)	0.359 (0.143.-0.901)	0.029	0.544 (0.259-1.143)	0.108
Treatment (surgery and chemotherapy vs surgery alone)	0.358 (0.138-0.933)	0.035	1.348 (0.631-2.881)	0.441

OS, overall survival; MFS, metastasis-free survival; HR, hazard ratio; CI, confidence interval. *P*-value<0.05 in bold font is statistically significant.

transcription and cell proliferation (Segovia et al., 2017). The oncogenic activities of EZH2 result in silencing tumor suppressor genes or inhibiting apoptosis of tumor cells.

Higher expression of EZH2 has been documented in tumor tissues than in adjacent normal mucosa in CRC (Takawa et al., 2011; Liu et al., 2015). It may have an impact on the progression from non-neoplastic colonic tissues to carcinoma (Ohuchi et al., 2018; Abdel Raouf et al., 2021). As a result, it was regarded as an early event in CRC tumorigenesis. Previous studies have probed into the prognostic significance of EZH2 expression in CRC with inconsistent data (Vilorio-Marqués et al., 2017; Chen et al., 2018; Bremer et al., 2021). For example, Vilorio-Marqués et al. (2017) concluded that high EZH2 expression may serve as a predictor of superior survival according to a meta-analysis of eight studies including 1059 CRC patients in total, similar to the findings by two independent cohorts with over eight years of follow-up. Nevertheless, Chen et al. (2018) put forward that overexpression of EZH2 was associated with adverse outcomes in CRC. We attribute the discrepancy in results partly to the heterogeneity of the inclusion criteria of patients with CRC in different studies, and partly to the dual-faced property of EZH2 itself. For the former, the conclusions of some studies may be biased due to the lack of a control for confounding such as treatment modalities, and the distribution of baseline clinicopathological factors (e.g., tumor type and stage). For the latter, EZH2 has been recently proposed to act not only as a transcription repressor but also as an activator (Deb et al., 2014; Sashida and Iwama, 2017; Wassef and Margueron, 2017). Current hypotheses for the double prognostic properties of EZH2 in cancers lie in post-translational modifications, variations in its interaction with other PRC2 subunits, PRC2-independent activities of EZH2, and so on (Sauvageau and Sauvageau, 2010; Crea et al., 2012a,b; Xu et al., 2012; Lu et al., 2016; Wen et al., 2017). For the moment, there has been limited data regarding EZH2 expression in early-stage CRC. To this end, we focused on resected stage II CRC to investigate the clinical significance of EZH2 expression and to translate the survival benefit with adjuvant

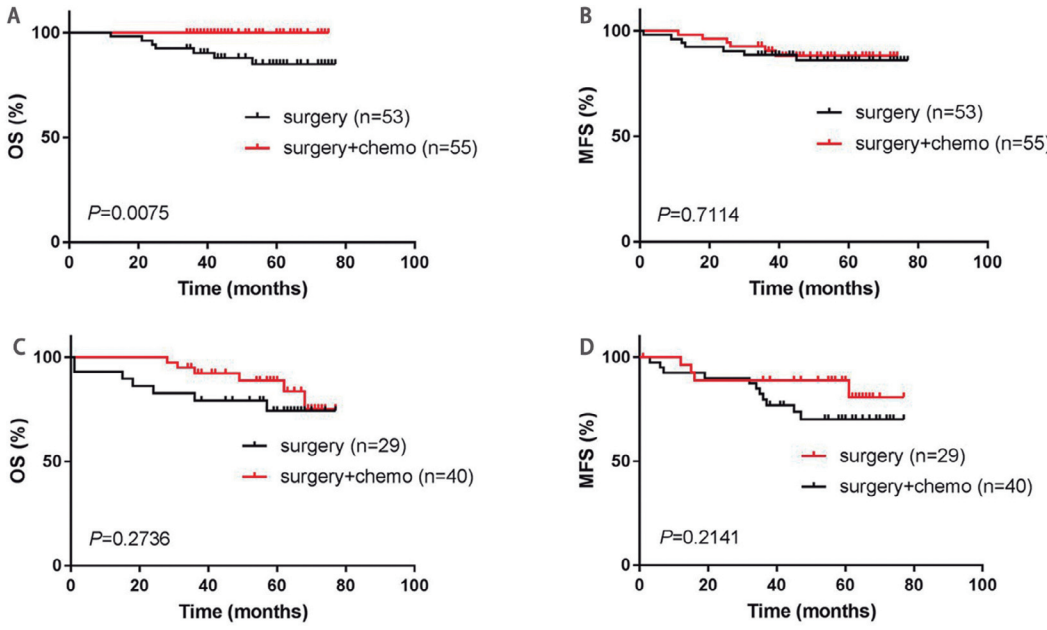

Fig. 4. Survival of patients with stage II CRC by treatment modalities. **A.** OS and MFS **(B)** of stage II CRC patients with high EZH2 expression. **C.** OS and MFS **(D)** of stage II CRC patients with low EZH2 expression.

Table 3. Multivariate Cox regression analysis of the clinicopathological characteristics for OS in 177 patients with stage II CRC.

Clinicopathological characteristics	Multivariate analysis (OS)	
	HR (95% CI)	P-value
Tumor location (left colon vs right colon vs rectum)	0.521 (0.275-0.989)	0.046
EZH2 expression (high vs low)	0.365 (0.144-0.922)	0.033
Treatment (surgery and chemotherapy vs surgery alone)	0.324 (0.124-0.848)	0.022

OS, overall survival; CSS, cancer-specific survival; HR, hazard ratio; CI, confidence interval. *P*-value<0.05 in bold font is statistically significant.

chemotherapy conferred by EZH2 expression.

In our research, survival analyses showed that high EZH2 expression was associated with favorable OS for the entire stage II CRC cohort (P=0.0225), which was predominantly attributed to the survival benefit of high EZH2 cases in the cohort receiving surgery and chemotherapy. Moreover, for patients with high EZH2 expression, further subgroup analyses revealed that patients treated with surgery and adjuvant chemotherapy had better OS than those with surgery alone (P=0.0075); while treatment had no impact on the OS of patients with low EZH2 expression. Therefore, we propose that it is stage II CRC with high EZH2 expression that most likely benefits from surgery and adjuvant chemotherapy, which suggests that EZH2 expression could be a potential biomarker to help decide between surgery or surgery combining chemotherapy. Additionally, neither EZH2 expression nor treatment demonstrated any impact on MFS in the entire cohort and subgroup analyses. Similarly, Fluge et al. also studied the association between EZH2 expression and prognosis in 409 patients with CRC treated with surgery alone or surgery followed by adjuvant chemotherapy. They reported that strong EZH2 expression was associated with better relapse-free survival (RFS) in stage II and III colon cancer (high EZH2 staining index 4-9 vs. low EZH2 index 0-3, P=0.041) and tended toward significance in stage II and III colon cancer receiving adjuvant chemotherapy (high EZH2 staining index 4-9 vs. low EZH2 index 0-3, P=0.077) (Fluge et al., 2009). However, they did not explore the impact of EZH2 expression on stage II CRC separately, which differs from our study. Masashi et al. examined the association of EZH2 expression with clinical outcomes in surgically treated CRC patients and found that EZH2 overexpression significantly indicates better tumor-specific 5-year survival after resection of primary tumors (P=0.014) (Takawa et al., 2011). Another *in vitro* work revealed that elevating H3K27me3 levels sensitizes CRC cells to oxaliplatin, and inhibiting H3K27me3 expression with an EZH2 inhibitor (EPZ-6438) caused a remarkably decreased proportion of apoptotic cells in CRC, which may indirectly reflect the potential impact of EZH2 overexpression on chemotherapy response (Wang et al., 2020). More *in vitro* evidence illustrating the association of EZH2 expression with therapy response is warranted.

Some studies also focused on the differences in EZH2 expression between the tumor center and invasion front. For example, Böhm et al. showed a significant decrease in EZH2 expression at the tumor invasion front in 105 specimens from colon cancer patients. They also demonstrated that it was the loss of EZH2 at the tumor invasion front, not in the tumor center, that was correlated with an unfavorable prognosis and more advanced tumor stages (Böhm et al., 2019). Unfortunately, we only sampled the tumor center to make tissue microarrays without focusing on the invasion front. We therefore were not able to analyze EZH2 expression in stage II CRC separately for the tumor center and invasion front, which warrants further investigation in the future.

As to clinicopathological characteristics, no correlation was observed between high EZH2 expression and tumor grade, size, sex, or age as recorded by the literature (Crea et al., 2012a,b; Kurihara et al., 2016). Although high EZH2 expression was reported to be significantly associated with poor differentiation in previous studies (Chen et al., 2018; Abdel Raouf et al., 2021), and the poorly differentiated tumors seemed to have a higher percentage of EZH2-overexpressed tumors (26/35, 74.29%), in the present study, the chi-square test did not find any statistical significance (P=0.187), which may be partly attributable to the relatively small sample size, or to the diverse scoring methods and cutoff values.

There are several limitations to this study. First, the study is limited by its retrospective nature. Second, the number of samples with complete follow-up was relatively small for survival analysis. A calculation of the predictive performance of EZH2 expression with a validation cohort was also lacking. Third, since only stage II patients were included in this cohort, it is unclear whether EZH2 has a similar performance in advanced CRC or not.

Conclusions

In conclusion, we found that the OS of stage II CRC with high EZH2 expression improved more strikingly with surgery and adjuvant chemotherapy than with surgery alone, which suggests the potential of EZH2 expression as a biomarker to help identify a subgroup of early-stage CRCs benefiting from surgery and adjuvant chemotherapy. More large-scale studies are warranted to corroborate this finding and to further evaluate the predictive nature of EZH2.

Acknowledgements. None.

Ethical Statement. This study was approved by the Institutional Review Boards of Nanjing Drum Tower Hospital, China (Approval No.2021-503-01; Date: 2021/10/31). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee(s) and with the Helsinki Declaration (as revised in 2013).

Data Availability Statement. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest. The authors declare that they have no competing interests.

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Authors' contributions. LH and XQZ analyzed the data and wrote the manuscript. ZZ and LH performed the histological examination of the tissue microarray. ZZ, YW, and CSW made the tissue microarray. JY and BZ performed the immunostaining. XQZ collected the patients' data. ZWL and LH instructed the study. LH and ZWL revised the manuscript. All authors read and approved the final manuscript.

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