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Original Article

Immunohistochemical distribution of FOXP3+ regulatory T cells in colorectal cancer patients

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Abstract: *Background*: Recent studies have focused on less invasive methods for diagnosing and predicting survival outcomes for colorectal cancer (CRC) patients. *Objective*: We studied the role of the transcription factor forkhead box P3 (FOXP3) in the tumorigenesis of CRC and investigated its prognostic value in survival outcome estimates. *Methods*: FOXP3+ regulatory T (Treg) cell levels in CRC and adjacent tissues were measured by immunohistochemistry (IHC) and compared statistically. A literature search was conducted on FOXP3+ Treg cell density and survival rates, including overall survival, disease-free survival, and cancer-specific survival. Meta-analyses were then performed to determine the prognostic value of FOXP3+ Treg cell levels in CRC patients. *Results*: FOXP3+ Treg cells were increased in CRC tissues over adjacent controls according to the IHC results ($t = 14.321$; $P < 0.001$) and cell densities in cases with metastases were higher than those without metastasis ($P < 0.05$). Cases with serosal infiltration showed higher FOXP3+ Treg cell densities compared to cases without infiltration ($P < 0.05$) and cell densities in cases differentiated to a lower degree than in cases showing a medium to high degree of differentiation ($P < 0.05$). Moreover, meta-analysis found a high FOXP3+ Treg cells density in CRC tissues (standardized mean differences = 0.30 [95% CI: 0.03-0.57]), which was correlated with better overall patient survival outcome (hazard ratio = 0.749 [95% CI: 0.648-0.867]). *Conclusions*: Increased FOXP3+ Treg cells may be an effective marker for tumorigenesis and clinical prognostic evaluation for CRC patients.

Keywords: FOXP3+ Treg cell density, colorectal cancer, immunohistochemistry, meta-analysis

Introduction

Colorectal cancer (CRC) has been ranked as the second most prevalent cancer among males and the third most prevalent among females in recent years [1-3]. Three decades ago, Vogelstein B determined that four genetic alterations were responsible for the activation of the stepwise tumorigenesis of CRC [4]. CRC was therefore viewed as a disease with a long-term period of progression [5-7], especially considering that CRC is known as a polygenic disease, meaning that multiple genetic alterations are required for this progression [8]. Many factors are attributed to the tumorigenesis of CRC, including age, smoking, and immune-system regulation, etc. [9-11].

As an immune-system regulator, the transcription factor forkhead box P3 (FOXP3) is crucial for immunosuppression of CD4+CD25+ regulatory T cells (Treg) by controlling their development and function [12]. In FOXP3-positive (FOXP3+) Treg cell proteins, the forkhead domain is near the carboxyl terminus, which means that it could act as a transcriptional inhibitor for proteins lacking a *bona fide* transactivation domain [13]. A study of the stepwise induction of Treg cells in mice has shown that the demethylation of non-coding sequence 2 and its binding to runt-related transcription factor 1 promoted the stable expression of FOXP3+ Treg cells [14]. This stable expression functions as a tumor suppressor in various malignant cell types, including gastric, breast, pancreatic, prostate, ovarian, and thyroid cancer cells [15-20]. Thus, we proposed that FOXP3+ Treg cells may be a positive factor in tumor inhibition and survival prolongation.

To date, some studies have reported a correlation between expression and survival of FOXP3+

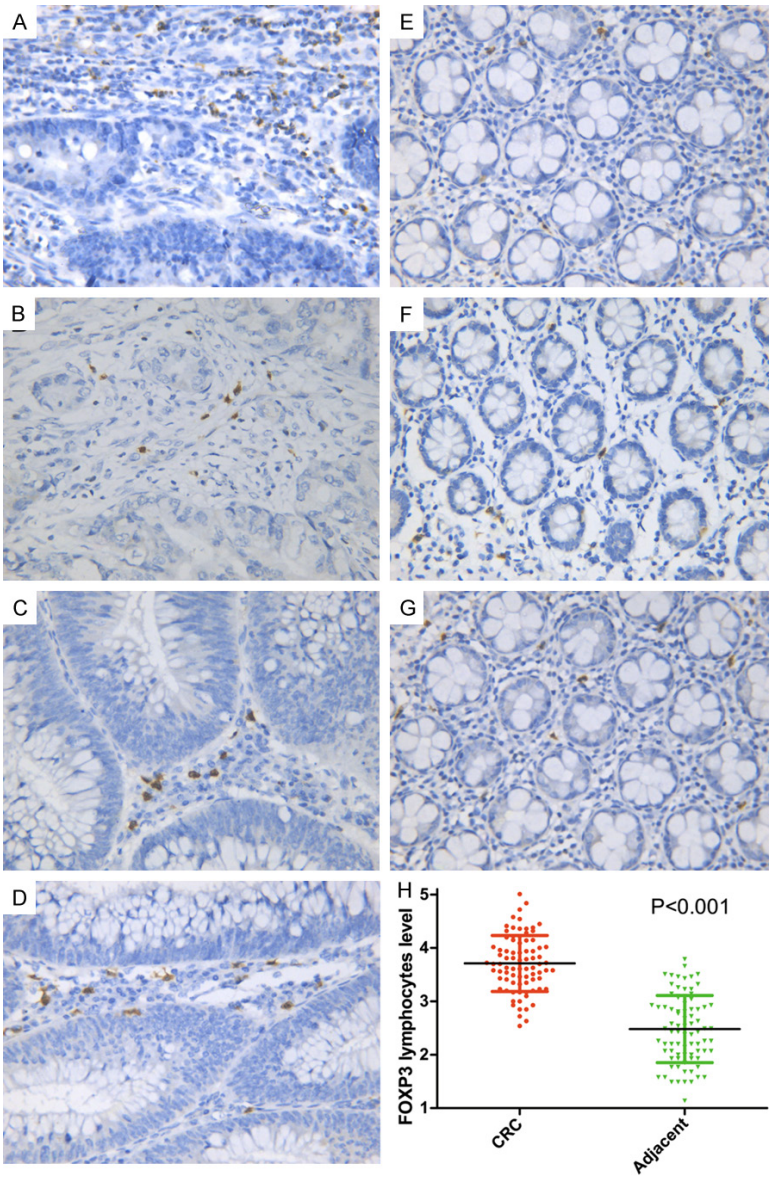

Figure 1. FOXP3+ Treg cells in CRC tissues. A-D. FOXP3+ Treg cells density in CRC tissues. E-G. FOXP3+ Treg cells density in adjacent tissues (40 × 10). H. Scatter plot for FOXP3+ Treg cells density in CRC tissues and the adjacent ones (P < 0.001).

Treg cells and CRC [21-49]. Although several previous meta-analyses have been reported, the conclusions pertaining to the prognostic value of FOXP3+ Treg cells in cancers have been controversial [50-52]. This could be due to the relatively small sample sizes reported in the previously published meta-analyses. Thus, in this study, we explored the role of FOXP3 in the tumorigenesis of CRC by applying immunohistochemistry (IHC) and conducting a comprehensive meta-analysis of the prognostic significance of the presence of FOXP3+ Treg cells in CRC.

Materials and methods

Immunohistochemistry

Patients and tissues: This study was approved by the Institutional Review Board of the First Affiliated Hospital of Guangxi Medical University. In total, 82 patients pathologically diagnosed with CRC were included (male/female = 52/30; colon cancer/rectal cancer = 41/41; median age: 56 years [27-87 years]). Tumor stoma samples (n = 82) with the corresponding adjacent tissue samples (n = 82) were collected for investigation.

Immunohistochemical staining: The main reagents used in this study included a mouse anti-human FOXP3+ monoclonal antibody (Santa Cruz Biotechnology Co. Ltd. USA) and a PowerVision™ Detection Kit (Changdao Biotechnology Co. Ltd. Shanghai, CHN). Experimental procedures were conducted according to the kit instructions. Tissue sections of 4 μm were treated at 65°C for two hours, followed by xylene dewaxing and gradient alcohol rehydration. The sections were then treated with EDTA for two minutes at a high temperature at pH = 9.0. Next, 3% H_2O_2 was used for 10 minutes to block the peroxidase. The excess reagent was removed by washing with PBS three times for five minutes each. Mouse anti-human FOXP3+ monoclonal antibody was then added to the sections and incubated at 37°C for one hour. The sections were again washed with PBS three times for five minutes each, and the secondary antibody was added for 30 minutes. Excess reagent was then washed away with PBS as described previously followed by addition of

Table 1. FOXP3+ Treg cell density in different tissues

Characteristics		N	Mean ± SD	*P* value
Sex	Male	52	14.73 ± 5.32	P > 0.05
	Female	30	12.66 ± 4.91	
Age	< 60	52	13.60 ± 5.57	P > 0.05
	≥ 60	30	14.63 ± 4.63	
Tumor	Colon cancer	41	13.26 ± 3.88	P > 0.05
	Rectal cancer	41	14.69 ± 6.29	
Metastasis	Yes	35	12.63 ± 5.33	P < 0.05
	No	47	14.98 ± 4.99	
Infiltration	Yes	45	12.18 ± 3.85	P < 0.05
	No	37	16.16 ± 5.89	
Differentiation	High	14	14.41 ± 6.28	P < 0.05
	Median	56	14.19 ± 5.23	
	Low	12	12.43 ± 4.00	
Ducks staging	A and B	31	15.61 ± 5.13	P > 0.05
	C and D	51	12.98 ± 5.07	

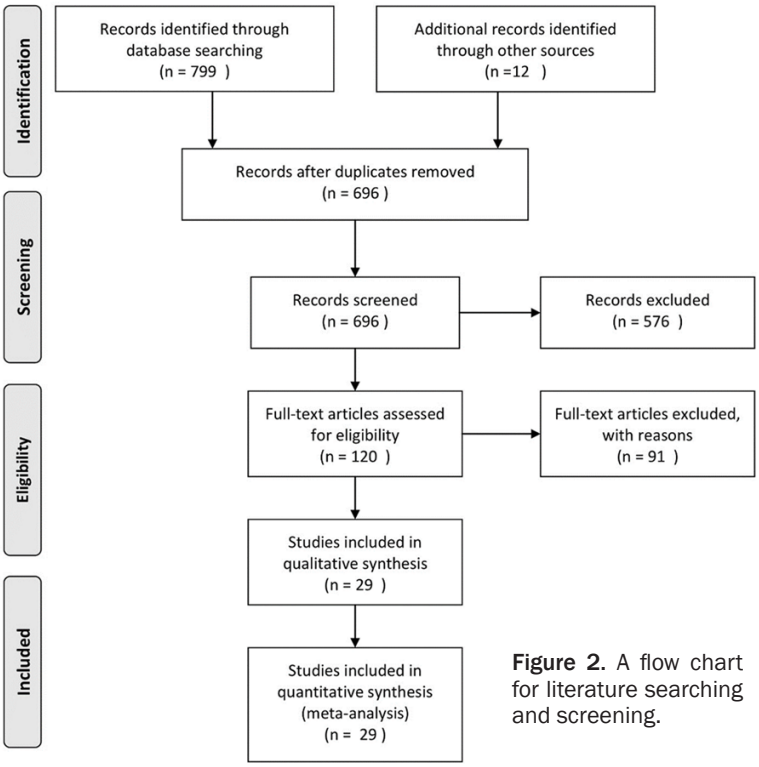

Figure 2. A flow chart for literature searching and screening.

3,3'-Diaminobenzidine tetrahydrochloride and hematoxylin, respectively, for coloring and re-dyeing the samples. Finally, the sections were dehydrated, made transparent, and sealed. These same preparations were also applied to both the positive and negative controls. Chronic amygdalitis tissue was used as the positive control, and CRC tissues without mouse anti-human FOXP3+ monoclonal antibody treatment were used as the negative control.

Evaluation of the immunohistochemical results: Double blinding was applied for reading the stained sections by two pathologists. Lymphocyte nuclei that were stained as yellow or brown were regarded as FOXP3+. The number of FOXP3+ Treg cells was counted in five high-powered fields (40 × 10) for each sample, and the mean value was regarded as the level of FOXP3+ Treg cells.

Statistical analysis: SPSS software version 22.0 (SPSS, Chicago, IL) was used for statistical analyses. The paired t test was applied for the comparison of FOXP3+ Treg cells between tumor tissues and adjacent tissues. The two-sample t test was applied for the comparison between FOXP3+ Treg cells in tumor tissues with different characteristics. ANOVA was applied for the comparison of FOXP3+ Treg cells in tumor tissues with high, medium, and low levels of cancerous infiltration.

Meta-analysis

Literature search: We systematically searched for relevant articles in PubMed, Embase, and the Web of Science databases with the language restricted to English and Chinese. This search strategy was applied to relevant articles up to December 2017 and included the key words FOXP3+ and colorectal cancer. We scrutinized the relevant references in the identified articles to broaden the search.

Inclusion and exclusion criteria: The inclusion criteria were as follows: 1. FOXP3+ Treg cell densities were measured in CRC tissues and

Table 2. Eight studies including the FOXP3+ Treg cells density data in colorectal cancer tissues and the controls

Author	Year	N1[1]	M1[2]	SD1[3]	N0	M0	SD0	Control types	Cancer types	Method
Camelia C	2010	48	4.157	2.482	69	2.716	1.501	normal	CRC[4]	IHC[5]
Beyer M	2011	6	2.433	1.270	10	1.685	0.048	normal	CRC	FC[6]
Salama P	2012	145	7.615	7.229	133	8.892	8.098	ADJ[7]	SIICC[8]	IHC
Zhu XW	2016	48	5.365	1.917	21	4.306	0.034	normal	CRC	IHC
Stanilov N	2009	12	3.445	1.287	12	3.961	1.485	ADJ	CRC	qRT-PCR
Pentherou-dakis G	2015	286	5.100	1.163	144	5.034	1.915	ADJ	CRC	IHC
Xu W	2013	90	4.007	4.062	90	2.801	3.875	ADJ	CC	IHC
IHC	NA	82	3.724	2.381	82	2.679	1.425	ADJ	CRC	IHC

NOTE: [1]Number; [2]Mean value; [3]Standard deviation; [4]Colorectal cancer; [5]Immunohistochemistry; [6]Flow cytometry; [7]Adjacent tissues; [8]Stage II colon cancer.

controls by detection assays such as immunohistochemistry, RT-PCR, western-blot, ELISA, etc. 2. The studies evaluated the correlation between FOXP3+ Treg cell densities and overall survival (OS), or/and disease-free survival (DFS), or/and cancer-specific survival (CSS). 3. The studies contained data for effectively estimating the corresponding measure of uncertainty (i.e., mean ± SD; hazard ratios [HR] with 95% CI). 4. The studies were published in English or Chinese. 5. When the data was duplicated in more than one publication, the study with the largest sample size of patients was included and the rest were excluded. The exclusion criteria were as follows: 1. The articles were about cell lines or animals. 2. The studies were about metastatic CRC. 3. The data could not be extracted or calculated from the original article. 4. Reviews, comments, duplicated studies, and irrelevant articles were excluded.

The specifics about which data should be included were extracted separately by two authors. Whenever controversy occurred, there was a discussion to reach a consensus.

Statistical analyses: The "Metan" command in the Stata 12.0 software (StataCorp, College Station, TX, USA) was applied throughout the meta-analysis. For the included studies with data presented as the mean ± SD, the standardized mean differences (SMD) were pooled in a forest plot. For the studies with survival data, including OS, DFS, and CSS, the HRs were also pooled in the forest plots. Heterogeneities were estimated throughout the meta-analysis by applying the Chi squared test and evolution of heterogeneity (I^2) tests. A random-effects model was employed when $P < 0.05$ in the Chi squared test or when I^2 was greater than 50%. Otherwise, the fixed-effect model was applied. Meta regression analysis and/or influence analysis for a single study was conducted to investigate the sources of heterogeneity whenever significant heterogeneity occurred. Moreover, Begg's test with graphs was applied to estimate the publication bias in the meta-analyses.

Results

FOXP3+ Treg cell densities were significantly increased in colorectal cancer stroma tissues

A total of 82 CRC stroma tissues and the matched 82 para-tumor tissues were used in this study (**Figure 1A-F**). The mean values with standard deviation (SD) for the FOXP3+ Treg cell levels in tumor stroma tissues and para-tumor tissues were 3.710 ± 0.524 and 2.480 ± 0.630, respectively. The paired student t test showed that these FOXP3+ Treg cell densities were significantly increased in tumor stroma tissues compared with the para-tumor tissues ($t = 14.321$; $P < 0.001$) (**Figure 1G**).

Furthermore, the FOXP3+ Treg cell densities in the CRC tissues varied between the tissues derived from different pathological factors. For instance, the cell densities in cases with metastases were higher than those without metastases ($P < 0.05$). Cases with serosal infiltration showed higher FOXP3+ Treg cell densities compared with the cases without serosal infiltration ($P < 0.05$). Moreover, the FOXP3+ Treg cell densities in cases that were differentiated to a low degree were higher than those in cases with a medium to high level of differentiation ($P < 0.05$) (**Table 1**).

Table 3. Survival data of the included studies

Author	Year	N[1]	HR[2]	LL[3]	UL[4]	Country	Method	Types
Sinicrope FA	2009	160	1.37	0.81	2.33	USA	DI[5]	OS[6]
Chen Y	2016	300	0.56	0.35	0.9	China	IHC[7]	OS
Zeestraten EC	2013	245	0.8	0.6	1.1	Holland	IHC	OS
Ganapathi S	2016	60	0.12	0.03	1.66	UK	qRT-PCR	OS
Zeestraten EC	2013	76	0.52	0.34	0.8	Holland	IHC	OS
Hanke T	2015	820	0.84	0.55	1.29	Germany	IHC	OS
Kim Y	2015	218	0.87	0.553	1.37	Korea	IHC	OS
Pentheroudakis G	2015	286	1.08	0.65	1.78	Greece	IHC	OS
Wang DL	2015	340	0.372	0.27	0.512	China	IHC	OS
Suzuki H	2010	95	1.465	0.663	3.234	Japan	IHC	OS
Weixler B	2015	657	0.77	0.66	0.88	Switzerland	IHC	OS
Yoon HH	2012	216	0.58	0.29	1.18	USA	IHC	OS
Reimers MS	2014	495	0.64	0.5	0.81	Holland	IHC	OS
Chew A	2011	120	0.58	0.36	0.94	Australia	IHC	OS
Posselt R	2016	103	0.45	0.22	0.95	Germany	IHC	OS
Xu W	2013	90	2.87	1.13	7.3	China	IHC	OS
Chen J	2014	102	0.62	0.35	1.09	China	IHC	OS
Argon A	2016	186	0.97	0.957	0.983	Turkey	IHC	OS
Teng F	2015	62	1.07	0.468	2.491	China	IHC	OS
Salama P	2009	967	0.78	0.7	0.87	Australia	IHC	OS
Sinicrope FA	2009	160	1.23	0.72	2.13	USA	DI	DFS[8]
Chen Y	2016	300	0.59	0.38	0.9	China	IHC	DFS
Zeestraten EC	2013	245	0.8	0.6	1.1	Holland	IHC	DFS
Zeestraten EC	2013	76	0.47	0.34	0.64	Holland	IHC	DFS
Pentheroudakis G	2015	286	1.41	0.83	2.39	Greece	IHC	DFS
Suzuki H	2010	95	1.335	0.639	2.788	Japan	IHC	DFS
Reimers MS	2014	495	0.62	0.49	0.79	Holland	IHC	DFS
Argon A	2016	186	0.975	0.96	0.99	Turkey	IHC	DFS
Teng F	2015	62	1.07	0.497	2.317	China	IHC	DFS
Saito T	2016	109	1.12	0.56	2.24	Japan	qRT-PCR	DFS
Salama P	2012	145	0.73	0.31	1.74	Australia	IHC	CCS[9]
McCoy MJ	2015	128	2.17	1.09	4.35	Australia	IHC	CCS
Richards CH	2014	365	1.92	1.33	2.78	UK	IHC	CCS
Märkl B	2017	60	0.3	0.1	0.9	Germany	IHC	CCS

NOTE: [1]Number; [2]Hazard ratio; [3]Lower limit; [4]Upper limit; [5]Dual immunofluorescence; [6]Overall survival; [7]Immunohistochemistry; [8]Disease-free survival; [9]Cancer-specific survival.

Meta-analyses for investigating the prognostic value of FOXP3+ Treg cell densities in colorectal cancer

Search results: The search results are shown in **Figure 2**. Seven studies that included FOXP3+ Treg cell density data in CRC tissues and the controls were chosen for the meta-analysis. Moreover, the study originating from our own results reported herein was also included, which used IHC for the analysis (**Table 2**). In total, 25 studies investigating the FOXP3+ Treg cell density in CRC and its prognostic value were included, among which, 20 studies contained OS data, 11 studies contained DFS data, and 4 studies contained CSS data (**Table 3**).

FOXP3+ Treg cells increase in patients with colorectal cancer: The pooled SMD was 0.300, with a 95% CI of 0.030-0.569 that indicated that FOXP3+ Treg cells tended to increase in

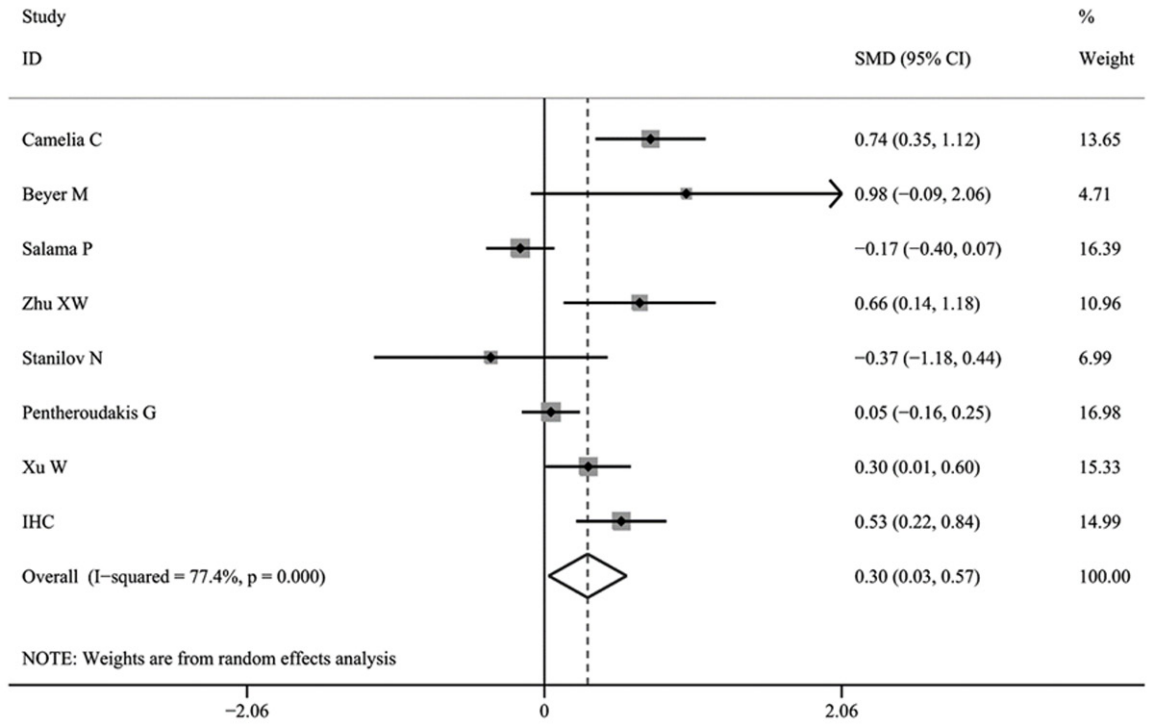

Figure 3. Pooled SMDs for FOXP3+ Treg cells in CRC tissues compared with the adjacent ones are resented in a forest plot.

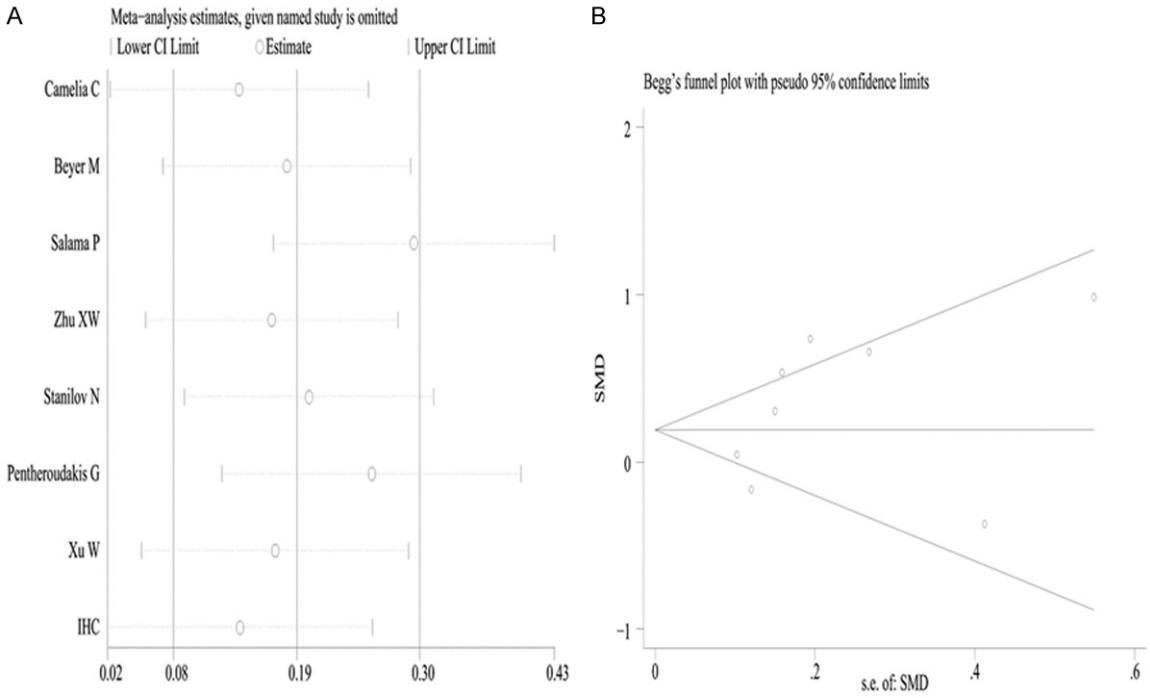

Figure 4. Meta-analysis of OXP3+ Treg cells in CRCs. A. The influence analysis graph. B. Begg's funnel plot revealed no significant publication bias in the meta-analysis.

patients with CRC (z = 2.18; *P* = 0.029) (**Figure 3**). With an I^2 of up to 77.4%, the random effect model was used in the analysis. In the influence analysis for the impact of each single study on

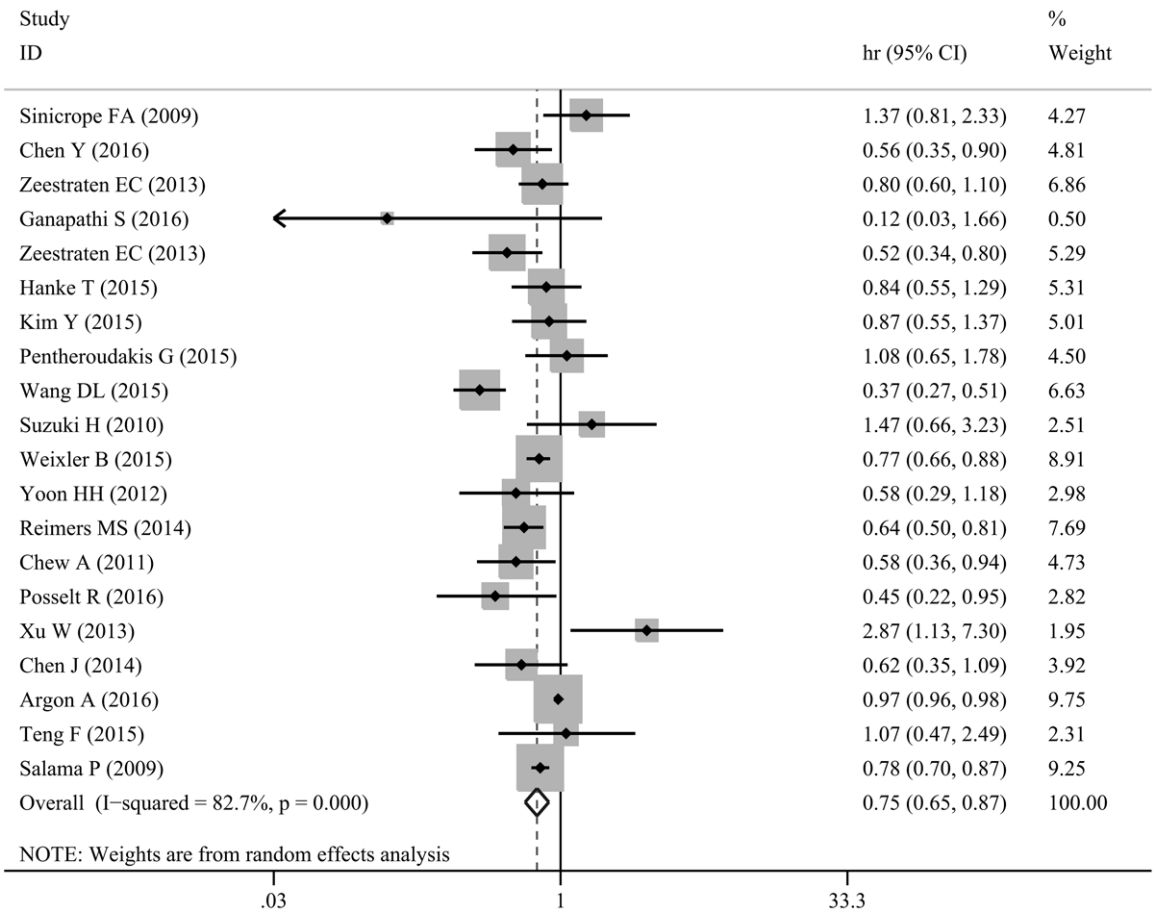

Figure 5. Summary HRs estimates of overall survival (OS) for CRC patients are resented in a forest plot.

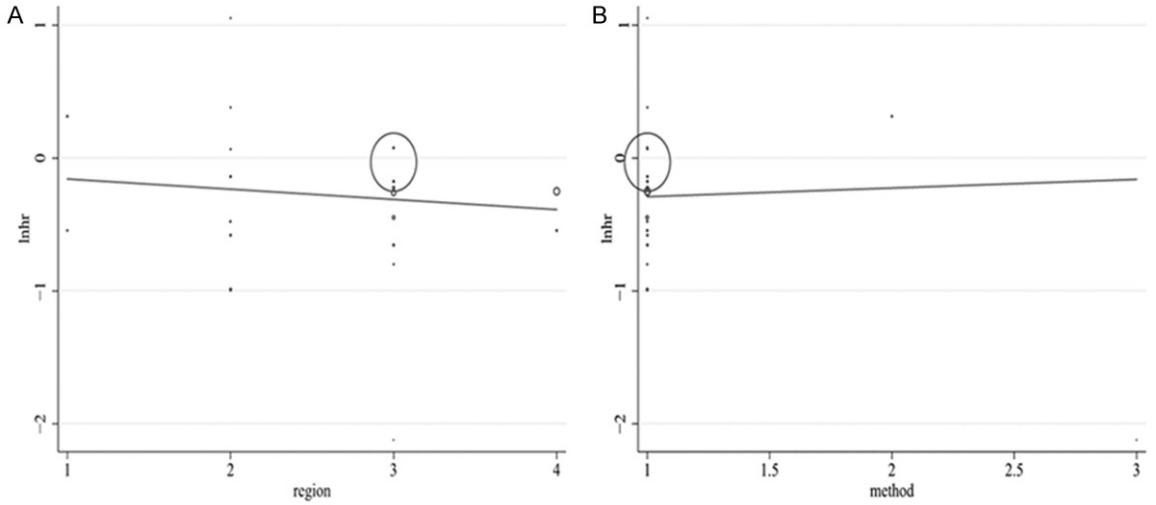

Figure 6. Regression analysis for the sources of heterogeneity exploration. Factors: (A) regions (B) methods.

the overall meta-analysis estimate, no statistically significant study was shown to impact the certain heterogeneity (**Figure 4A**). The funnel plots that were made to demonstrate publication bias estimating showed no significant publication bias in this part of analysis (**Figure 4B**).

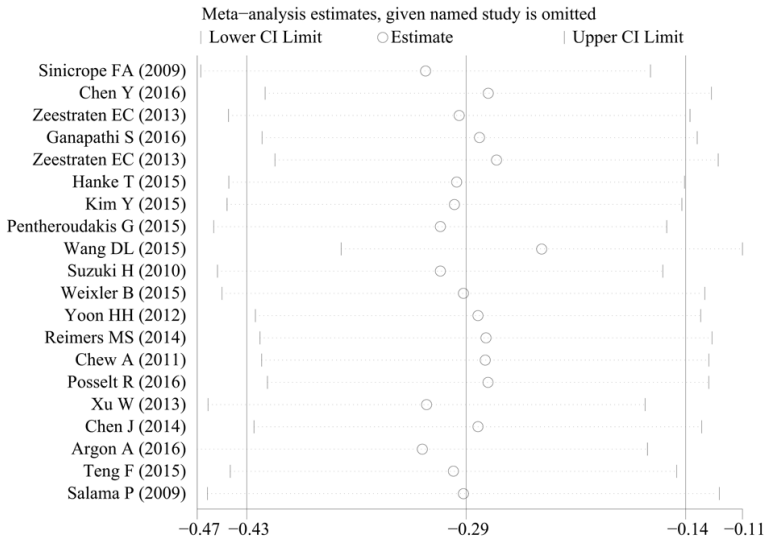

Figure 7. The influence analysis graph. No statistically significant evidence to suspect any of the 20 studies as the sources of the significant heterogeneity.

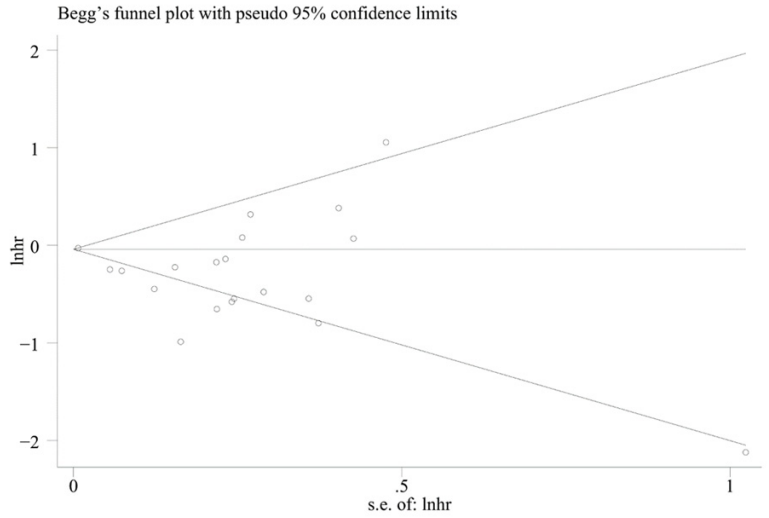

Figure 8. Begg's funnel plot revealed significant publication bias in the meta-analysis for overall survival estimates (P = 0.041).

Increased FOXP3 + Treg cell levels correlate with better overall survival in colorectal cancer: The pooled HR of the OS analysis for the included studies revealed that CRC patients with relatively increased FOXP3+ Treg cell levels had a better overall survival time than those with lower levels of FOXP3+ Treg cells (HR = 0.749 [95% CI: 0.648-0.867]; z = 3.89, *P* < 0.001) (**Figure 5**). The I^2 for estimating the heterogeneity was 0.827, thus indicating that the random effect model was applied throughout the analysis. Neither variations in regions, including Europe, Asia, Australia, and America, nor measuring methods were the sources of the heterogeneity according to the regression analysis (*P* = 0.816; 0.395, respectively) (**Figure 6**). The influence analysis that estimated the effect of any single study on the overall meta-analysis revealed that no statistically significant study influenced the heterogeneity (**Figure 7**). Begg's test showed a significant publication bias in this part of analysis (*P* = 0.041) (**Figure 8**). By adopting a nonparametric "trim and fill" analysis of the publication bias, it turned out that the pooled HR was 0.749 (0.648-0.867), as was shown previously. The funnel plot for estimating this publication bias is shown in **Figure 9**.

FOXP3+ Treg cell levels did not impact the disease-free or cancer-specific survival for colorectal cancer: The pooled HR for DFS and CSS revealed no significance in terms of patient survival (*P* = 0.159; 0.801, respectively) (**Figure 10**). The I^2 estimates for DFS and CSS were 0.775 and 0.781, respectively, and the random effect model was adopted in both analyses. No significant publication bias appeared in either of these analyses (*P* = 0.815; 0.089, respectively) (**Figure 11**).

Discussion

We have identified a higher level of FOXP3+ Treg cell densities in CRC and have measured the prognostic value of this finding. This study confirmed that the density of FOXP3+ Treg cells was higher in CRC tissues than in the adjacent controls. Moreover, by adopting meta-analyses, high FOXP3+ Treg cell densities were investigated in CRC tissues and proved to be correlated with a better outcome for CRC patient OS, although no significant prognostic value was shown for DFS and CSS.

This study investigated the higher FOXP3+ Treg cell densities in CRC tissues than the adjacent

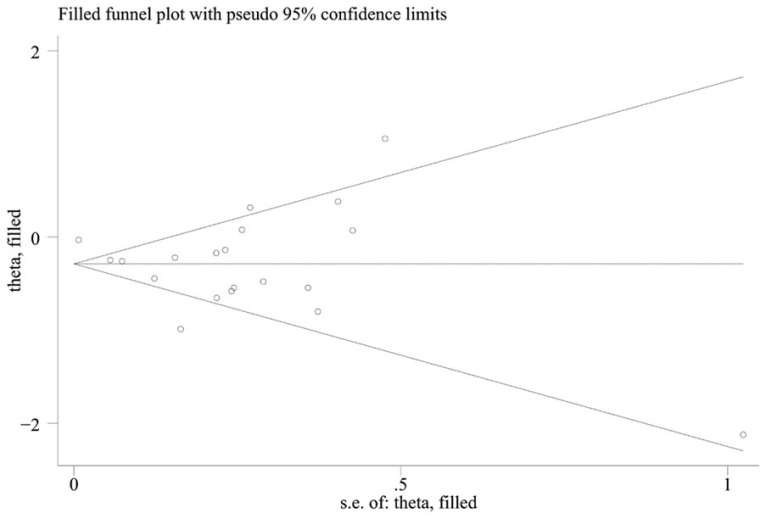

Figure 9. The funnel plot for publication bias estimates after "trim and fill" analysis, which showed no significant publication bias with symmetric dots in the plot.

tissues, which was also seen in various previous studies [26, 30, 43]. Furthermore, this study also discovered that CRC tissues with higher pathological characteristics, such as metastases, infiltration, and low differentiation, tended to have higher FOXP3+ Treg cell densities, which indicated that increased densities were correlated to the malignancy of CRC. The exact FOXP3+ Treg cell densities in CRC compared with the controls, however, remains contradictory because several studies have reported a decreased cell density in CRC tissues [29, 31]. Thus, in this study, a meta-analysis was conducted to comprehensively determine whether CRC tissues tended to have higher FOXP3+ Treg cell densities than the controls. It confirmed the need to investigate the impact of high FOXP3+ Treg cell densities in CRC patients.

Since many studies have shed light on the clinical value of FOXP3+ Treg cell densities in CRC, this study used a meta-analysis to uncover the prognostic value of FOXP3+ Treg cells for CRC patients. A total 25 studies with 6,379 total CRC patients were included in the analysis. The results showed that higher FOXP3+ Treg cell densities were related to better overall survival outcomes, which could be an effective means for making a relatively accurate prognostic diagnosis for CRC patients clinically. In fact, several studies have conducted similar meta-analyses [53, 54], although the previous studies excluded the studies which merely contained Kaplan-Meier survival curves [53]. It should be noted that in our study, studies containing only Kaplan-Meier curves were also included in order to extract the essential data using Engauge tools [55]. Moreover, another previously reported study only searched for the data in the PubMed and EBSCO databases, which may omit a certain amount of studies present in other databases [54]. Thus, our study may be more comprehensive.

Notably, this study suggested that a high FOXP3+ Treg cell density in CRC patients was associated with a better OS, which was inconsistent with what the previous studies have reported [43]. Numerous studies have reported that FOXP3+ Treg cells could mediate the immune suppression of the organism, which could lead to the progression of various tumor types, including hepatocellular carcinoma, breast cancer, lung cancer, etc. [56-58]. Accordingly, a high FOXP3+ Treg cell density in CRC should lead to a worse survival outcome for the patients. The colon and rectum are hollow tracts with a large quantity of bacteria living on the corresponding mucosal surfaces, however, many previous studies have determined that the homeostasis brought about by the bacteria living in the gastrointestinal tract could be an anti-cancer factor [59, 60]. Also, studies have reported that FOXP3+ Treg cells are tolerant of the bacteria in gastrointestinal tract, which could lead to a good microenvironment for bacteria to develop anti-cancer abilities [61]. Thus, a high FOXP3+ Treg cell density in CRC patients is a favorable factor for overall survival outcome.

However, limitations still exist in our study, which include the significant heterogeneity that cannot be explained by regression analysis or influence analysis, owing to the limitations of meta-analysis. The number of studies for the

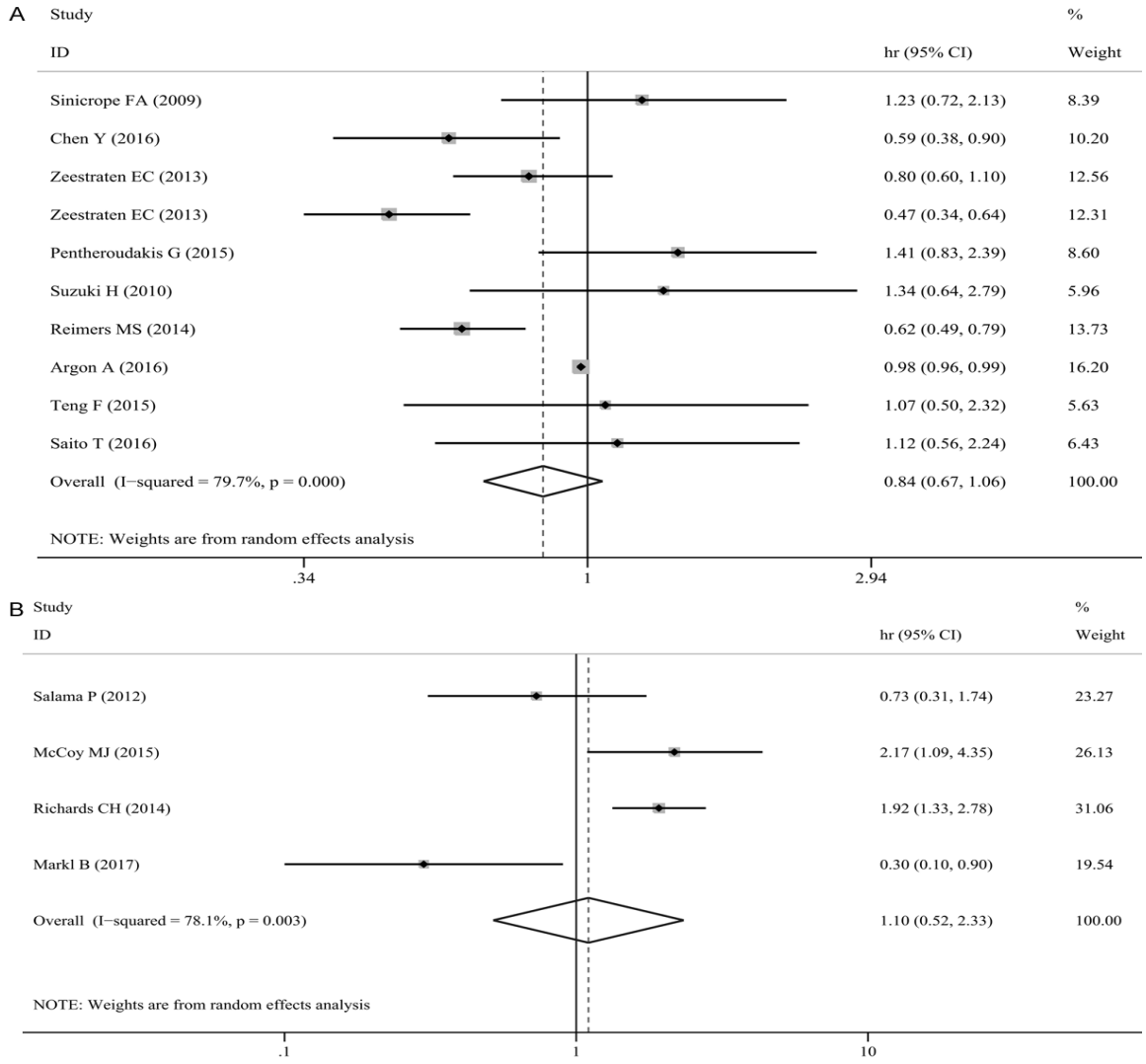

Figure 10. Meta-analysis of the survival implication of OXP3+ Treg cells in CRCs. A. Summary HR estimates of disease-free survival (DFS) for CRC patients are resented in a forest plot. B. Summary HR estimates of cancer-specific survival (CSS) for CRC patients are resented in a forest plot.

cancer-specific survival estimate was small, meaning that more studies are needed to enhance our study. Furthermore, the mechanism of how FOXP3+ Treg cell affect CRC patients remained unclear in this study. Therefore, our next steps involve exploring this mechanism.

In conclusion, this study proves that a higher FOXP3+ Treg cell density in CRC tissues leads to a better prognostic factor for CRC patient OS, which indicates that FOXP3+ Treg cells may be an effective marker for the clinical prognosis and treatment of CRC patients.

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Disclosure of conflict of interest

None.

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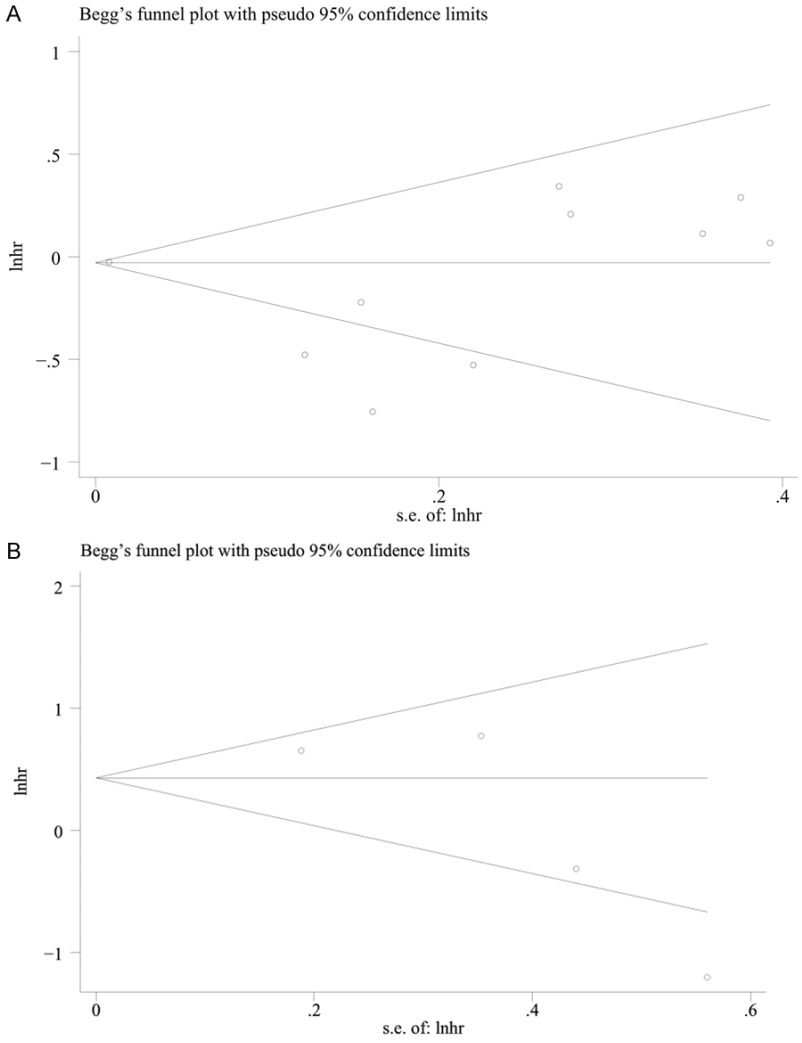

Figure 11. Begg's funnel plots of meta-analysis of the survival implication of OXP3+ Treg cells in CRCs. A. Begg's funnel plot revealed no significant publication bias in the meta-analysis for disease-free survival (DFS) estimates. B. Begg's funnel plot revealed no significant publication bias in the meta-analysis for cancer-specific survival (CSS) estimates.

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