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A META-ANALYSIS OF THE RELATIONSHIP BETWEEN LYMPHATIC MICROVESSEL DENSITY AND THE SURVIVAL OF PATIENT WITH COLORECTAL CANCER

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ABSTRACT

Colorectal cancer (CRC) is a frequently observed malignant neoplasm that is a leading cause of cancer death despite recent advances in treatment and research. The role of lymphangiogenesis in CRC development is a source of controversy in current research. We undertook this study to examine the relationship between lymphatic microvessel density (LMVD) and the overall survival (OS) or disease free survival (DFS) of CRC using meta-analysis of recent studies. PubMed and Embase databeases were searched and nine relevant studies including 799 total patients were included. Six studies including 575 patients focused on overall survival (OS) and 3 studies with 224 patients focused on disease free survival (DFS). We performed a meta-analysis to estimate the prognostic role of lymphatic microvessel density by combining separately estimated hazard ratios. A remarkable correlation between LMVD and DFS was observed in pooled analyses using a fixed-effect model (HR 2.29; 95% CI 1.11, 3.48). LMVD and OS showed a pooled HR value of 1.02 (95% CI 0.71,1.33), indicating no significant correlation between LMVD and OS. There was no evidence for significant heterogeneity or publishing bias in both DFS and OS (I^2=0.0%, P=0.861; I^2 =48.1%, P=0.086, respectively). A close relationship was observed between LMVD and DFS, though no correlation between LMVD and OS was apparent. The current meta-analysis suggests that LMVD may be a poor prognostic factor for CRC patients. However, these results should be regarded cautiously and future confirmatory studies are necessary.

Keywords: lymphangiogenesis, lymphatic microvessel density, colorectal cancer, prognosis, survival

Colorectal cancer (CRC) is a frequently occurring malignant neoplasm. In United States, CRC remains a second leading cause of cancer mortality (1). CRC frequently metastasizes to lymph nodes, liver, and lungs (2) and approximately 50% of patients with CRC develop synchronous or metachronous tumor metastases (3). It has been commonly accepted that neovascularization is required for the continued growth of tumor tissues to support absorption of nutrients and secretion of growth factors. Formation of new blood and lymphatic vessels from existing vascular networks is essential for tumor development (4), and growing evidence suggest that hemangiogenesis and lymphangiogenesis play critical roles in the development and metastasis of tumors (5-7). Therefore, assessment of lymphatic and blood vessels in the tumors may be very valuable for prognostic assessment and understanding of metastasis mechanisms (8,9).

Angiogenesis has been regarded as an important prognostic parameter in development and progression of many malignancies as well as crucial in dissemination of tumor cells (10). A meta-analysis of published angiogenesis studies showed that high microvessel density (MVD) predicted poor survival in patients with CRC (11). Recently, blocking tumor angiogenesis has been viewed as a potentially effective therapeutic strategy (12). Several experimental studies and clinical trials have been developed to block VEGF-A action, included blocking antibody, decoy receptor, and siRNA against VEGF-A (13). However, in contrast to (hem)angiogenesis, the role of lymphangiogenesis remains a subject of intense debate.

Lymphangiogenesis is a relatively new field of inquiry, and the prevalence of published studies describing lymphatic vessel growth and lymphangiogenesis mechanisms has only recently increased to an appreciable level (2). Notably, a major challenge facing researchers has been the absence of effective markers for the efficient identification of lymphatic endothelium (5,6). Fortunately, discovery of novel antibodies such as LYVE-1 (lymphatic endothelial hyaluronan receptor) and podoplanin or D2-40 has dramatically increased accuracy of detection methods for lymphatic invasion (2). Additionally, these novel antibodies also provide tools for different types of research associated with lymphatic vessel proliferation (14-16).

In contemporary research, the most widely accepted technique for evaluation of lymphangiogenesis is assessment of lymphatic microvessel density (LMVD) through the application of monoclonal antibodies by immunohistochemistry (17,18). However, controversial results have been reported regarding the relationship between LMVD and clinicopathological parameters, and survival time. Omachi et al suggested there were no clear correlations between the degree of lymphangiogenesis and clinical outcome (19) while others reported positive correlations between lymphangiogenesis and clinicopathological parameters and survival rates (20-23).

Further investigation of the importance of lymphangiogenesis will be required to fully assess the prognostic role of lymphatic vessel growth in CRC patients. In the current study, the relationship between LMVD and survival of CRC patient is examined through meta-analysis. To the best of our knowledge, no published systematic review has previously evaluated the association of LMVD with survival of CRC patients.

MATERIALS AND METHODS

Publication Selection

A total of 9 studies consisting of 799 individual patients were identified by electronic search using PubMed and Embase databases without language restrictions. Included studies were required to concern CRC only, provide measurement of LMVD by immunohistochemistry, and evaluate the correlation between lymphatic microvessel count and survival. Searches were conducted using the following terms: (lymphatic microvessel destiny or lymphangiogenesis) and (colorectal or colon or rectal) and (cancer or carcinoma or tumor or neoplasia) and prognoses.

Results consisting of only abstracts were excluded from this analysis due to insufficient data for application of the scoring system and assessment of trial methodology. All eligible studies were retrieved, and each reference list was carefully scanned to identify other eligible studies and avoid duplication. Further, examination of all authors and medical centers involved was also conducted to avoid potential duplication. In order to ensure consistency of data for statistical analysis, clarification was obtained, as necessary, through direct contact with the authors of each study. The final meta-analysis data included only published data, and no contact was made for the purpose of obtaining unpublished data. The search was conducted between March 2010 and March 2012.

Study Inclusion/Exclusion Criteria

Inclusion criteria for original studies were as follows: (1) proven diagnosis of CRC in humans, (2) LMVD examination by immunohistochemical methods, (3) correlation of LMVD with overall survival (OS) or disease free survival (DFS). Quantitative aggregation of results was conducted using the LMVD measured by the hazard ratio (HR) and corresponding 95% confidence interval (CI). Studies which not directly present HR (hazard ratio) were allowed if information was available for statistical analysis. No predefined sample size was defined for studying inclusion. Both literature reviews and animal studies were excluded. For multiple studies of overlapping patient populations (cohorts), only the most recent or complete study was included.

Data Extraction

Data were extracted from each included study, including the first author's name, year of publication, study design, number of included patients, mean or median age, disease stage, grade (high, moderate or low differentiation), nodal status, number of hot spots examined, examination magnification, examination area of the field, cutoff value for LMVD, examination MLVD, HR or RR and 95% CI, median duration of follow-up, with or without anticancer treatment(s) during follow-up, results of univariate and multivariate analysis and survival curves. Disagreements were resolved by reader consensus between two independent readers, and final resolution was confirmed by expert opinion. Quality assessments was performed for each acceptable study by independent reviewers (Cheng-Guang Yang and Song Yu) using the Newcastle-Ottawa scale (24). Briefly, this scale is an nine-item instrument for describing patient population and selection, study design, MLVD counting methods, follow-up and cut-off value. Each item was assessed using an ordinal scale (possible values: 1, 0).

Statistical Methods

In each study, the relationship between LMVD and survival was considered as "significant" if the P value for the statistical test comparing survival distributions between the groups was <0.05 in the univariate analysis (two-tailed test). A study was termed "not significant" if no statistical difference between the two groups was found (P value > 0.05).

Quantitative aggregation of survival results was conducted by measurement the impact of LMVD on survival data by estimating the hazard ratio (HR) and corresponding 95% confidence interval (CI) between the high and low LMVD groups. For each trial, this HR was assessed by a method depending on the data provided in the publication. The most accurate method consisted of calculating the HR and its standard error using the following parameters: the logrank statistic of its P value, the HR point estimate, the O-E statistics (difference between numerous of observatories and their expectative events) or its variances. When these data were not available, we looked for the total number of events, the number of patients at risk in each group, and the logrank statistics or its P value allowing us to estimate an approximation of the HR value. When data were only available in the form of graphical representations of the survival curves, the calculations were carried out at some specified time in order to reconstruct the HR estimate and its variance (25,26). If data were extracted from survival curves, three independent persons read the curves to reduce the imprecision in the reading variations.

Each individual HR estimates were combined into an overall HR, using a fixed-effect model assuming homogeneity of the individual true Hrs (27). Statistical heterogeneity among studies was evaluated by using the P value and I^2 statistics (24). If homogeneity was not assumed, a random-effects model was additionally applied. Potential publication bias was estimated using visual

funnel plots and further evaluated by Begg's adjusted rank correlation testing and Egger's regression asymmetry testing (28,29). By convention, an observed HR>1 was regarded as a worse survival event for the group with a high LMVD count. This pejorative impact of lymphangiogenesis on survival was considered as statistically significant if the 95% confidence interval for the all-inclusive HR did not overlap 1 (30,31). If 95% CI included 0, the results were considered to have no statistical significance. All statistical analyses were performed using the Stata 12.0 (Stata, College Station, Texas, USA) software. A P-value of less than 0.05 was regarded as statistically significant (P< 0.05).

RESULTS

Studies Selection and Characteristics

A total of 49 studies (2,4-8,16,19-23,32-68) were identified by preliminary searching on PubMed and Embase databases. Titles and abstracts were individually evaluated for relevance. A total of 14 studies (2,5-7,16,48-54,66,67) were excluded based on failure to meet all inclusion criteria. The most prominent reason for such exclusion was failure to assess LMVD. Additionally, 14 articles (8,20,21,37,55-63,68) were excluded due to insufficient survival data, and 8 articles (23,32,44-47,64,65) were excluded due to failure of results to be accurately described in terms of OS or DFS. Only l article (42) was excluded due to duplication of patient cohort in another included study. Three studies (19,35,43) were excluded because we could not obtain sufficient data even after communicated with authors by email.

The final meta-analysis included 9 eligible retrospective studies with a total of 799 patients, ranging from 40 to 210 patients per study (4,22,33,34,36,38-41). Among the included studies, 6 studies examined OS (4,22,33,34,38,40), and 3 studies DFS (36,39,41). A flow diagram of the search process was given in *Fig. 1*.

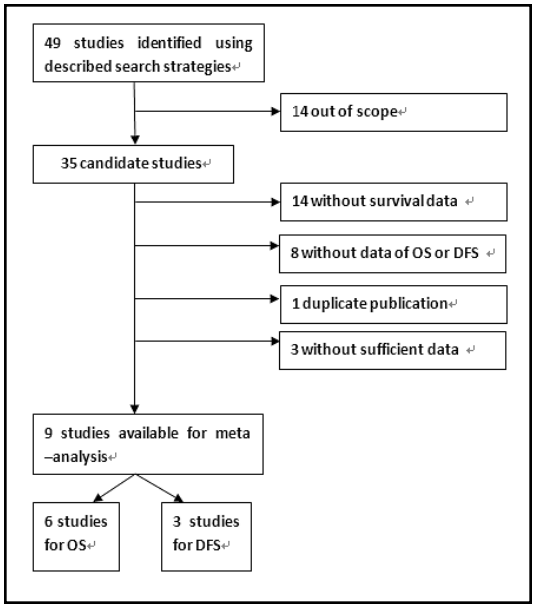

Fig. 1. Details of studies included in the meta-analysis.

To better describe the patients included in our meta-analysis, we used Dukes staging whenever possible. We found 1 study (39) only focused on Dukes' stage A, 1 study (33) only concentrated on Dukes' stage B, and the other articles used Dukes' stage A-D. Different antibodies such as LYVE-1, D2-40/podoplanin, 5'-Nase and VEGFR-3 were used to count MLVD by immunohistochemical methods. Among the 9 included studies, patient populations were examined from geographical regions of China (6), Japan (1), Italy (1), and Sweden (1). All studies were published between 2005 and 2011. A summary of the characteristics of each of the 9 included studies is provided in *Table 1*. Quality assessment of all 9 studies for meta-analysis using the Newcastle-Ottawa Scale is shown in *Table 2*. According to the score, there were 6 high-quality studies (score ≥8) and 3 low-quality studies (score <8).

Main Analysis

Meta-analysis results demonstrating

TABLE 1
Main Characteristic and Results of Eligible Studies Evaluating the LMVD

Author	Year	Stage	N	Survival Data	HR estimation	Results	Cut-off	Antibody
JIANG JB et al	2005	A-D	44	OSA	Survival curves	Negative	Mean	VEGFR-3
YANG X et al	2006	B	54	OS	Survival curves	Not significant	Arbitrary	LYVE-1
KENTARO M et al	2006	A-D	106	OS	Survival curves	Negative	Median	Podoplanin
FAN YZ et al	2006	A-D	80	OS	Survival curves	Significant	Mean	5'-Nase
GAO J et al	2008	A-D	210	OS	Logrank	Not significant	Median	D2-40
YAN G et al	2008	A-D	132	DFS	Logrank	Negative	ROC curve	D2-40
LI ZX et al	2009	A-D	40	DFS	Logrank	Significant	Arbitrary	LYVE-1
MEI L et al	2010	A-D	81	OS	HR+CI	Not significant	Arbitrary	D2-40 & LYVE-1
BARRESI V et al	2011	A	52	DFS	HR+CI	Negative	Mean	D2-40

association between LMVD and survival of CRC is shown in *Fig. 2*. LMVD and OS correlation revealed no significant association in pooled analyses using the fixed-effect (HR: 1.02; 95% CI: 0.71-1.33) with no significant heterogeneity observed (I^2=48.1%, P=0.086). LMVD and DFS, using the fixed-effect model, showed a remarkable association in pooled analyses (HR: 2.29; 95% CI: 1.11-3.48) with no significant heterogeneity observed (I^2 =0.0%, P=0.861).

Subgroup analyses were also conducted including study quality and Dukes stages (*Fig. 2*). In the OS group with the high quality score group (score ≥ 8), there was statistical heterogeneity (I^2 =68.7%, P=0.023) and the HR was 1.49 (95%:0.57-2.40) by a random-effect model. For the five studies focusing on Dukes stages (A-D) in the OS group, we also found significant statistical heterogeneity (I^2 = 58.2%, P=0.048) and the HR was 1.43 (95%:0.60-2.26) by a random-effect model.

Assessment of Publication Bias

Visual assessment of funnel plots was performed in order to assess publication bias. No significant asymmetrical distributions were observed in DFS group. Funnel plots also showed slight asymmetry in DFS group (*Fig. 3*).

DISCUSSION

Pooled data from 9 retrospective studies demonstrated a relationship between LMVD and OS or DFS in CRC. LMVD was closely correlated with DFS in CRC, although no significant association between LMVD and OS was observed.

It has been suspected that lymphatic metastasis is an important determinant of aggressive cancer phenotype which can predict poor outcome in patients with colorectal cancer (69), and recent studies have shown the importance of tumor-associated lymphangiogenesis in the processes of lymph node metastases (53). There is

TABLE 2
Assessment of Study Quality

Studies	Quality indicators from Newcastle-Ottawa scale									
	1	2	3	4	5	6	7	8	9	Score
JIANG JB et al	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes	7
LIN M et al	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	8
BARRESI V et al	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	8
YANG X et al	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes	7
LI Z et al	Yes	Yes	Yes	No	Yes	Yes	No	No	Yes	6
FAN YZ et al	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	8
KENTARO M et al	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	8
GAO J et al	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	9
YAN G et al	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	8

For the included studies, 1, indicates case independently validated; 2, cases are representative of population; 3, ascertainment of exposure (Proof of CRC and LMVD measurement) 4, detect and compare both intratumoral MLVD and peritumoral MLVD; 5, same method and location of counting used for cases and controls; 6, study controls for age, gender, and stages; 7, cutoff value for LMVD by median or mean; 8, ascertainment of exposure by blinded interview of record; 9, adequacy of follow-up of cohorts and follow-up long enough for outcomes to occur (at least 2 years). Yes = 1 score, No = 0 score.

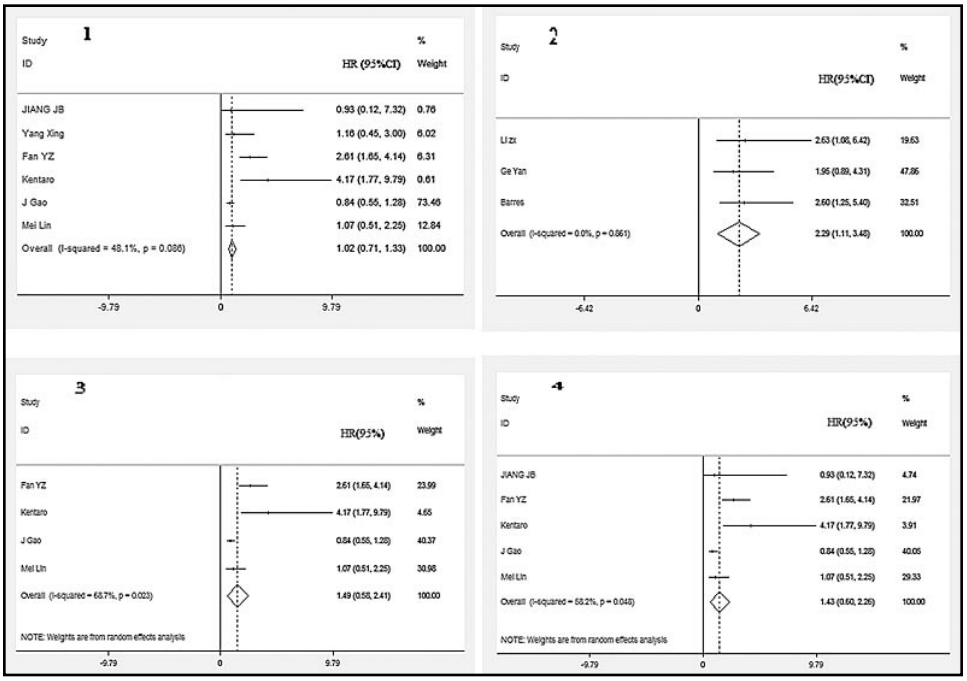

Fig. 2. Evaluating HR of high LMVD as compared to low LMVD: (1) a total of 6 articles for OS group, (2) a total of 3 articles for DFS group, (3) a total of 4 high quality articles in OS group, (4) a total of 5 studies focusing on Dukes stages in OS group.

great interest in identifying new prognostic markers for patients with colorectal cancer because these markers may help improve clinical or therapeutic management (43,70). Tsirlis et al reported that lymphangiogenesis assessment might be a valuable prognostic

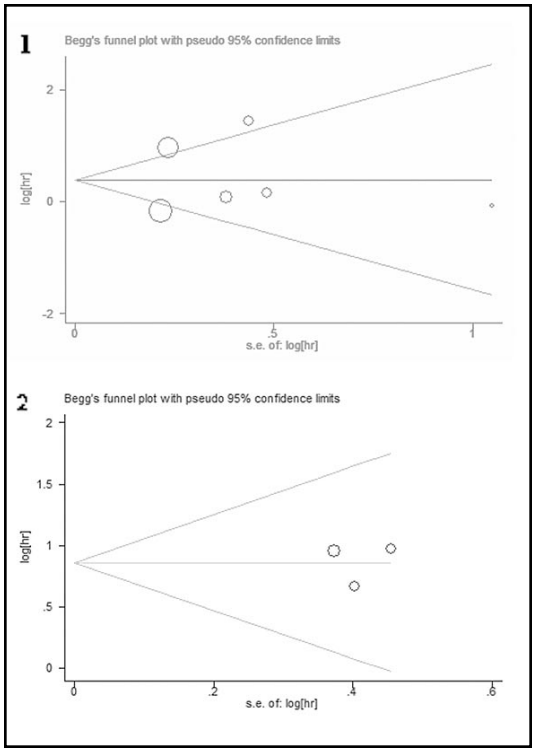

Fig. 3. Bias assessment plots for OR and DFS studies: (1) a total 6 articles in OS group, (2) a total of 3 articles in DFS group.

factor in colorectal cancer and a determinant of the success of combined therapy involving adjuvant as well as neoadjuvant treatment strategies (49). However, in contrast to hemangiogenesis, the role of lymphangiogenesis remains a subject of intense debate.

The current study provides a novel meta-analysis focused on the association between LMVD and survival data in CRC. The findings suggest that presence of LMVD may be a poor prognosis factor for DFS in colorectal cancer patients. Although LMVD may have potential as a prognostic factor, other factors, such as TNM classification and MVD, may be stronger.

Several potential limitations affect the results of this meta-analysis. First, since the sample size was small, we could not detect publication bias and significant heterogeneity in the DFS group. We also could not develop the Begg' or Egger' tests for publication bias and subgroup analysis according to different antibodies. Second, these findings may be limited by the weight assigned to each study by quality scoring, and no standard is widely accepted for such weighting in meta-analysis (71). The Newcastle-Ottawa Quality Assessment Scale is a new assessment tool. Additionally, insufficient information exists as to which scores constitute high-quality or low-quality studies, which may add additional limitations of this method (72). Third, because of the natural heterogeneous distribution of lymphatic vessels, lymphatic vessel quantification is much more challenging than quantifying blood vessels. As a result, subtle increases in LMVD may not be apparent in immunohistochemical analysis of tumor sections. Because the current methodology only utilized data available in full publications, significant data that may have been provided in unpublished trials and abstracts were not assessed. Thus, the conclusions of this analysis should be applied cautiously in the development and assessment of further research.

In conclusion, LMVD exhibits a close relationship with DFS of CRC patients. This observation supports the hypothesis that LMVD can predict CRC patient survival and that it has potential importance for treatment strategies and prognostic assessments in colorectal cancer. These results are based on an aggregation of information obtained from independently conducted retrospective trials. In order to further investigate the role of lymphangiogenesis in CRC, standardization of methods for quantification of lymphangiogenesis and study quality score assessment will be required. Additionally, the inclusion of adequately designed prospective studies will be necessary in future studies to provide thorough analysis of the role of lymphangiogenesis in CRC.

Abbreviations:
LMVD (lymphatic microvessel density); MVD (microvessel l density); CRC (colorectal

cancer); OS (overall survival); DFS (disease free survival); HR (hazard ratio); CI (95%confidence interval).

Conflicts of Interest:
The authors are fully responsible for all content and editorial decisions. No financial support or other form of compensation was provided for the development of this article. The authors declare no conflict of interest.

Author Roles:
Electronic database searches were conducted by Yi-Gang Chen, Jun Yan and Zi-Ming Yuan. Data extraction was conducted by Yi-Gang Chen and Jun Yan. Data read by consensus of Yi-Gang Chen and Jun Yan, and final data extraction was confirmed by the expert opinions of Zhi-Gang Wang and Qi Zheng. Quality assessments for each study were performed by Cheng-Guang Yang and Song Yu. All authors contributed to and approved the final manuscript.

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