RESEARCH ARTICLE

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Pretreatment Microvessel Density for Predicting of Tumor Responsiveness to Neoadjuvant Chemoradiotherapy of Locally Advanced Rectal Cancer

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Abstract

Objective: This study aimed to assess whether pretreatment tumor tissue microvessel density (MVD) could be a potential predictive marker for Mandard response in LARC treated with nCRT. **Methods:** A retrospective analysis was performed in pretreatment paraffin-embedded specimens of 31 pathologically confirmed rectal adenocarcinoma. All patients received nCRT and subsequent total mesorectal resection. Tumor MVD was determined by an average number of counted CD34-stained endothelial cells from two selected fields at 200x magnification in each slide and categorized into two groups: low MVD (</=60) and high MVD (> 60). The tumor response was determined using the Mandard tumor regression grading system. The subjects were grouped according to their TRG into responder (TRG 1-3) and non-responder (TRG 4–5). **Result:** Twenty out of thirty-one patients (64.5%) were defined as responders. Eleven patients (35.5%) were defined as non-responders. MVD was significantly associated with tumor responsiveness to nCRT ($p < 0.05$). High MVD was shown to be an independent risk factor associated with tumor resistance to nCRT (OR, 22.58; 95% CI, 1.943-262.34; $p = 0.013$). A strong correlation was found between MVD and TRG (correlation coefficient value of 0.642, $p <0.01$), between MVD and vascular invasion (correlation coefficient value of 0.618, $p <0.01$), and between nodal involvement and vascular invasion (correlation coefficient value of 0.521, $p <0.01$). A moderate correlation was found between nodal involvement and vascular invasion (correlation coefficient value of 0.406, $p <0.05$). **Conclusion:** High MVD in pretreatment tumor tissue was significantly associated with the tumor resistance to nCRT.

Keywords: Microvessel density- tumor regression- rectal cancer- radiotherapy- mandard

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Introduction

Colorectal cancer (CRC) is a common malignant tumor in the gastrointestinal tract and is a common cause of cancer-related death worldwide. Neoadjuvant chemoradiotherapy (nCRT) followed by a total mesorectal excision is the accepted standard therapeutic approach for locally advanced rectal cancer (LARC) (Dayde et al., 2017). Tumor response to nCRT is associated with prognosis and can be determined by various tumor regression grading (TRG) systems (Vecchio et al., 2005; Kim et al., 2006; Hermanek et al., 2013). The Mandard TRG system (Mandard et al., 1994) has been used to categorize the regressive changes after nCRT based on the proportion of therapy-induced fibrosis in relation to residual tumor. Complete tumor regression has been associated with better treatment outcomes. Accordingly, the treatment effect or TRG has now been included in the College of American Pathologists' standard protocol for the examination of LARC specimens; thereby, being reported in every case with nCRT.

Angiogenesis or neovascularization is the process of new blood vessel formation from the endothelium of the existing vasculature (Uzzan et al., 2004). Tumor angiogenesis plays a crucial role in cancer formation, metastatic potential, and survival in numerous tumors (Des et al., 2006; Svagzdys et al., 2009). In malignant tumors, the existence of abnormal angiogenesis facilitates tumor growth and enhances the risk of metastasis. When a tumor reaches the size of 1-2 mm, its further growth requires more blood supply because of an increment of metabolic needs (Uzzan et al., 2004). This will initiate new vessel formation and recruitment of blood vessels from its surrounding stroma. Tumors with a higher number of blood vessels are considered as having a high angiogenic activity and therefore categorized as an aggressive tumor with poor prognosis. It is probable that the increasing number of new vessels may lead to tumor spreading via penetration of cancer cells into the circulatory system (Uzzan et al.,2004). Angiogenesis is mediated by various angiogenic factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF),

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transforming growth factor-alpha, etc. VEGF is an essential mediator of the new vessel formation process. This factor is mainly produced by macrophage, vascular smooth muscle cells, and tumor cells. The expression of VEGF and its receptor were demonstrated in gastrointestinal adenocarcinoma (Guang-Wu et al., 2000). MVD has been used as an assessment method for quantifying intratumoral angiogenesis and has been accepted as a surrogate marker for tumoral angiogenic activity (Wang et al., 2008) that associated with aggressiveness of various types of cancer (Iakovlev et al., 2012; Wang et al., 2007).

The immunohistochemistry has been widely used for evaluation of VEGF expression and MVD in primary tumor specimens, both of which have been proved to be tissue biomarkers for the angiogenic activity. Several previous studies have examined the potential prognostic value of the two angiogenic markers. A study in nasopharyngeal cancer revealed that the metastatic potency of tumor tissue and the prognosis can be estimated by measuring MVD and the expression of VEGF in tumor tissue (Guang-Wu et al., 2000). A previous study found that average MVD was significantly higher in patients with vascular invasion than those with no vascular invasion and therefore suggested that angiogenesis was related to prognosis (Wang et al., 2007). Tissue biomarkers of angiogenesis for predicting tumor response to nCRT may be helpful for treatment planning. This study was therefore aimed to determine whether the pretreatment tissue MVD could be a potential predictive factor for Mandard response of LARC treated with nCRT.

Materials and Methods

The present study was reviewed and approved by the Institutional Review Board of the Royal Thai Army Medical Department before initiating. Authors conducted a retrospective analysis in pretreatment paraffin-embedded specimens of 31 pathologically confirmed rectal adenocarcinoma cases. All patients underwent nCRT followed by total mesorectal resection.

Neoadjuvant chemoradiotherapy

In our institution, patients with clinically LARC are eligible for treatment with a long course combination therapy in the neoadjuvant setting. The radiation dose was 45-50 Gy in 25-28 fractions. The chemotherapeutic agent was intravenous 5-fluorouracil (425 mg/m^2) and leucovorin (20 mg/m^2) for 5 days at weeks 1 and 5 of the radiation therapy, or capecitabine 1,650 mg twice daily throughout the radiation treatment.

Tumor tissues

All tissue specimens were obtained by endoscopic biopsy. Four to six pieces of tissues were obtained from viable areas of the cancer margin, avoiding the nearby region of central ulceration.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 5-µm tissue sections were deparaffinized with xylene, dehydrated in ethanol and incubated with 3% hydrogen peroxidase for 5 minutes. After washing with phosphate-buffered saline (PBS), tissue sections were incubated in 10% normal horse serum, followed by an overnight incubation with an anti-CD34 antibody (1:500, Dako, Denmark). On the following day, the slides were incubated with a drop of the superenhancer TM, washed with working PBS wash buffer, incubated with a drop of labelled dextran polymer conjugated horseradish peroxidase (HRP) for 30 minutes, and washed twice with cold PBS. Finally, a drop of freshly prepared DAB (32-Diamino benzidine tetra hydrochloride) a substrate chromogen was added onto the sections. Slides were then washed in running distilled water to remove excess DAB and counter stained with hematoxylin.

MVD assessment

Each tissue section was examined at 50x magnification. Three areas with the highest MVD were identified. Individual vessel counts were then performed at 200x magnification. Any single CD34-stained cell that indicated an endothelial cell was counted as a single vessel. A branching structure was counted as a single vessel unless a break was observed in the continuity of the structure. MVD was measured by average number of CD34 positive cell per tissue area analyzed from two selected fields at 200x magnification in each slide (Figure 1). The value was used to represent the average MVD in that whole tumor section.

Tumor regression grading (TRG)

The Mandard tumor regression grading (TRG) has been proven to be a reliable system to evaluate nCRT response. In the present study, the tumor response after neoadjuvant therapy was determined using a grading system that was modified from Mandard TRG system (Mandard et al., 1994) as follows: TRG 1 (complete response with absence of residual cancer and fibrosis extending through the wall), TRG 2 (presence of residual tumor cells scattered through the fibrosis), TRG 3 (increase in the number of residual cancer cells with predominance of fibrosis), TRG 4 (residual cancer outgrowing fibrosis) and TRG 5 (absence of regressive changes). The subjects were classified according to their TRG into responder (TRG 1-3) and non-responder (TRG 4–5) groups.

Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics, Version 26.0 (IBM Corp., Armonk, NY, USA). Patients' demographic data were tested using the independent t-test for normally distributed variables or the Mann-Whitney U test for nonnormally distributed variables. The Chi-square test or Fisher's exact test was used for categorical data. Mandard TRG groups (responder/non-responder) were compared in relation to age, sex, and pathologic stage. The correlations among MVD, TRG, and various common histopathologic features were analyzed and determined using either Pearson's correlation or a Spearman rank correlation method according to types of data. A probability value of less than 0.05 was considered significant. A series of univariate analyses to determine the variables associated

with resistance to nCRT were performed. Variables associated with resistance to nCRT with a value of p <0.05 in the univariate analysis were selected for stepwise logistic regression. Potentially associated variables were then tested using multivariate logistic regression analysis with a Wald statistics backward stepwise selection. The results of the logistic regression were reported as odds ratios (ORs) with 95% confidence intervals (CIs).

Results

The clinicopathologic features of 31 patients with rectal cancer are summarized in Table 1. None manifested pathologic complete response to nCRT (TRG1/ypCR). Based on the Mandard TRG system, 20 patients (64.5%) were defined as responders (Mandard 1-3), whereas the other 11 patients (35.5%) were defined as non-responders (Mandard 4–5) as shown in Table 2. Between these two groups, no significant difference was found in age, sex, tumor stage, lymphovascular invasion, and nodal involvement. Potentially associated variables were tested using univariate and multivariate analyses. Common histopathologic features such as vascular invasion, nodal involvement, and MVD were selected for stepwise logistic regression to identify independent risk factors for tumor resistance to nCRT as shown in Table 3. MVD was found to be significantly associated with tumor resistance to nCRT (p <0.05). High MVD was shown to be an independent risk factor associated with tumor resistance to nCRT (OR, 22.58; 95% CI, 3.33-172.7; p = 0.002).

Microvessel density (MVD)

The mean MVD of 31 tissue sections was 39%, with values ranging from 15 to 80%. Patients were categorized in two groups: low MVD (</=60) and high MVD (>60). The non-responder group had a higher proportion of cases with high MVD (8/11 or 72%) than the responder group (2/20 or 10%) (p=0.01). Eighty percent of those with high MVD did not respond to nCRT (Mandard TRG of 4 or 5).

Relationship among MVD, TRG, nodal involvement, and vascular invasion

The result from the Spearman's correlation test is summarized in Table 4, indicating a positive correlation among MVD, TRG, and vascular invasion. A strong correlation was found between the MVD and TRG (correlation coefficient value of 0.642, p <0.01), between MVD and vascular invasion (correlation coefficient value of 0.618, p <0.01), and between nodal involvement and vascular invasion (correlation coefficient value of 0.521, p <0.01). A moderate correlation was found between nodal involvement and vascular invasion (correlation coefficient value of 0.406, p <0.05).

Recurrence

Among a total of 31 patients, 10 had tumor recurrence. The high MVD group had a higher 5-year recurrence rate (40 %) than the low MVD group (28.6%). Regarding types of recurrence, one patient had local recurrence and three patients had distant metastases (two with liver metastasis and one with lung metastasis), whereas the

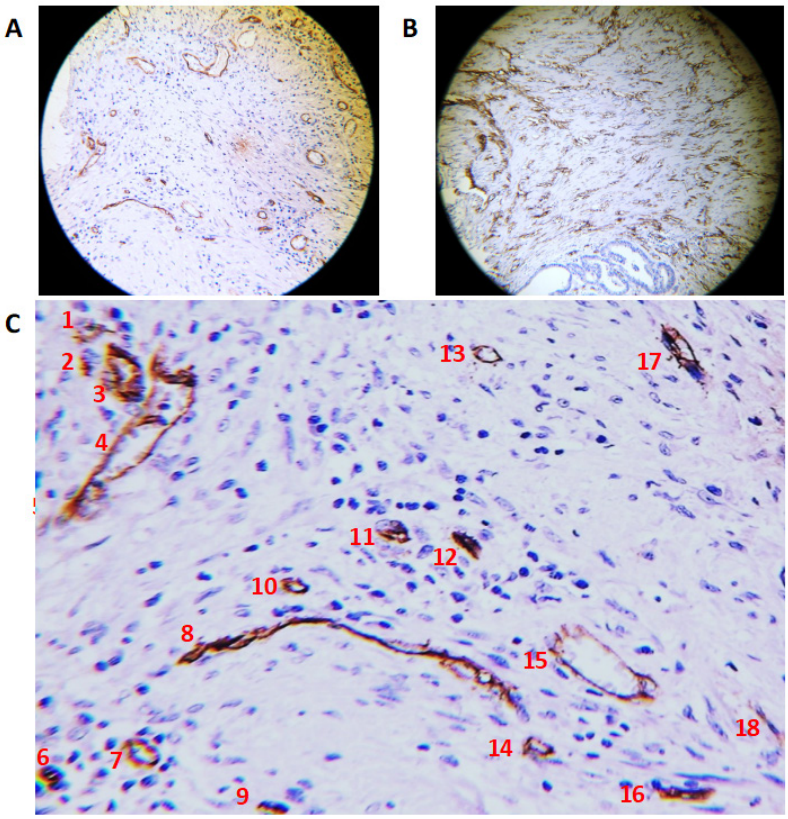

Figure 1. CD34 Immunostaining and Microvessel Counting. Microvessels in a colorectal cancer tissue prior to neoadjuvant treatments were highlighted by CD34 immunohistochemical staining. Representative images at X200 magnification of a case with low microvessel density (A) and a case with high microvessel density (B) are shown. An area of the tissue in Figure 1A is selected to demonstrate microvessel counting.

Table 1. Patients' Demographic Data

Variables	Number (%)
Gender	
Male	22 (71)
Female	9 (29)
Age (Years)	
Mean ± SD	60.4 ± 14.2
Median (Min, Max)	63 (23, 80)
Pathological tumor staging	
ypTMN tumor stage	
Stage 1	5 (16.1)
Stage 2	14 (45.2)
Stage 3	12 (38.7)
ypT1-T2	7 (22.6)
ypT3-T4	24 (77.4)
Pathological lymph node	
Number of lymph node retrieved (Mean ± SD)	10.97 ± 5.61
Negative	19 (61.3)
Positive	12 (38.7)
Lymphatic/ Vascular Invasion	13 (41.9)
Microvessel density (CD34)	
Low (</= 60)	21 (67.7)
High (>60)	10 (32.3)
Mandard tumor regression grading	
Responder (TRG1-3)	20 (64.5)
Non-responder (TRG4-5)	11 (35.5)
Disease recurrence	10 (32.3)

Table 2. Comparison of Clinicopathologic Features between Groups

Variables	Responder group (TRG 1-3) N (%)	Non-responder group (TRG 4-5) N (%)	p-value
Gender			
Male	15 (75)	7 (63.6)	0.683
Female	5 (25)	4 (36.4)	
Age (year)			
Mean ± SD	59.3 ± 15.8	60.4 ± 11.2	0.57
Duration between nCRT and surgery			
Median (Min,Max)	39.5(36,46)	43(38,47)	0.208
Pathological tumor staging			
Stage I-II	13 (65)	6 (54.5)	0.572
Stage III	7 (35)	5 (45.5)	
Lymphatic/Vascular invasion			
No invasion	14 (70)	4 (36.4)	0.128
Invasion	6 (30)	7 (63.6)	
Nodal involvement			
No	13 (65)	6 (54.5)	0.572
Yes	7 (35)	5 (45.5)	
MVD (CD34)			
Low </=60	18 (90)	3 (27.3)	0.01*
High >60	2 (10)	8 (72.7)	
Recurrence			
No	13 (65)	8 (72.3)	1
Yes	7 (35)	3 (27.7)	

*Fisher's exact test

other six patients developed both local recurrence and distant metastases. High MVD tended to be associated with poorer recurrence-free survival than low MVD but without statistical significance (49.1 months versus 58.3 months; Log-rank p-value = 0.54) as shown in Table 5.

Discussion

Neoadjuvant chemoradiation (nCRT) has been demonstrated to decrease the local recurrence rate and toxicity compared to postoperative chemoradiation among patients with LARC (Sauer et al., 2004; Bosset et al., 2006; Roh et al., 2009; Sauer et al., 2012). The direct cell-killing effect of ionizing radiation is primarily attributed to damages of nuclear DNA and plasma membranes affecting cell integrity (Yarnold, 1997; Haboubi et al., 2000; Wachsberger et al., 2003). Cancer cells are more sensitive to DNA-damaging effects than normal cells due to the higher proliferation rate, but responses to ionizing radiation varies among patients. Given the role of oxygen as a radio-sensitizer. Oxygen is essential for the radiation-induced DNA damages that are mediated by the free radicals produced through ionization of the water component of the cells (Wachsberger, 2003; Baskar, 2014). The ability to predict tumor response to nCRT may have a significant impact on treatment planning for patients with rectal cancer. Related studies have revealed several clinicopathologic factors associated with response to nCRT in rectal cancer, including clinical tumor size, nodal involvement, distance from anal verge, tumor differentiation, mucinous histology, and gross tumor ulceration (Qiu, 2011; Huh, 2013; Bitterman, 2015; Ferrari,

Table 3. Correlation among MVD (CD34), Mandard TRG, Vascular Invasion and Nodal Involvement

Variables	Correlation coefficients			
	MVD (CD34)	Mandard TRG	Vascular invasion	Nodal involvement
MVD(CD34)	1	0.642**	0.618**	0.16
Mandard TRG	0.642**	1	0.406*	0.103
Vascular invasion	0.618**	0.406*	1	0.521**
Nodal involvement	0.16	0.103	0.521**	1

*, Spearman's correlation test is significant at the 0.05 level (2 tailed); **, Spearman's correlation test is significant at the 0.01 level (2 tailed)

Table 4. Univariate and Multivariate Analyses of Variables Associated with TRG

Variables	Number	Univariate analysis	Multivariate analysis		p-value
		Crude OR	Adjusted OR	95% CI	
MVD(CD34)					
Low (</= 60)	21	Ref	Ref		
High (> 60)	10	24	22.58	1.943-262.34	0.013*
Vascular invasion					
No	24	Ref	Ref		
Yes	7	4.08	1.12	0.073-17.24	0.936
Nodal involvement					
No	19	Ref	Ref		
Yes	12	1.548	0.235	0.035-1.564	0.174

*Fisher's exact test

Table 5. Recurrence Rate Categorized According to MVD and TRG

Variables	Recurrence N (%)		Recurrence free survival (months)
	No	Yes	Mean (SE)
MVD (N=31)	No	Yes	Mean (SE)
Low (</= 60)	15 (71.4)	6 (28.6)	58.3 (7.74)
High (> 60)	6 (60)	4 (40)	49.1 (11.37)
Mandard TRG (N=31)			Mean (SE)
Responder group (TRG: 1-3)	4 (66.7)	2 (33.3)	45 (15.89)
Non-Responder group (TRG: 4-5)	17 (68)	8 (32)	57.8 (6.95)

2015; Zeng, 2015; McCawley, 2016). Tissue biomarkers such as survivin, p53, and Ki-67 were examined for their ability to predict rectal cancer response to chemoradiation. An inverse correlation between survivin expression and the level of spontaneous apoptosis in pretreatment biopsies was found in one study, and this protein was therefore suggested to be a strong inhibitor of tumor cell apoptosis in rectal cancer (Rödel, 2002). Yoshikawa (2018) revealed that the 5-year disease-free survival rate and the overall survival rate of patients with rectal cancer with low Ki-67 index were significantly higher than those of patients with high Ki-67 index. Although the finding for survivin was not significant in the study, the authors concluded that the Ki-67 index and survivin may be useful biomarkers for rectal cancer with preoperative chemoradiation. MVD has been accepted as a surrogate marker for tumor angiogenesis and used as a prognostic marker in numerous tumors (Duff, 2007). Related studies reported that high MVD predicted poor clinical outcomes and metastasis (Des Guetz, 2006; Cho, 2017). High levels of angiogenesis increase tumor cell proliferation and tumor cell resistance to radiation therapy. Svagzdys (2009) and colleagues examined post-radiation changes in rectal cancer tissues after a long course of radiotherapy. MVD was found to be significantly decreased in the tissues after radiotherapy, and patients with lower MVD were shown to have a better overall survival. Similar to the present study, the authors found that high MVD was significantly associated with tumor resistance to nCRT. This phenomenon may suggest the tumor vascular endothelial lining differs from its normal counterpart and possesses different phenotypic properties (Eberhard, 2000). High MVD in tumor specimens represents a high level of angiogenesis, and the radiation effect on angiogenesis produces undesired upregulation and intensification of angiogenic pathways. These effects consequence increased vascular permeability, decreased tumor perfusion, and enhanced tumor stem cell proliferation (Hendry, 1992; Hansen-Algenstaedt, 2000; Lee, 2000; Jain, 2001). The radiation effect on angiogenesis could therefore lead to resistance to radiation therapy in high MVD tumor.

The biopsy specimens were obtained from the peripheral region of each tumor. Given that MVD may differ between the central and peripheral areas, MVD in such tissues may not truly represent the MVD status of the whole tumor. Thus, further determination of the consistency of MVD between different regions of a tumor would be essential. With a limited number of samples in this study, a strong positive correlation between the MVD and TRG was demonstrated. Based on the results of this study, measurement of pretreatment rectal cancer tissue MVD may be helpful for treatment planning and selection of an appropriate therapeutic approach. High MVD in pretreatment LARC specimens may indicate tumor resistance. A vascular targeting agent or oxaliplatin may be selected instead of conventional regimen of nCRT as a concurrent treatment regimen for patients with high MVD LARC. To confirm the potential usefulness and clinical implication of the pretreatment MVD, further studies with a larger number of LARC samples are crucial for optimizing the future therapeutic approach.

In conclusion, Pretreatment tissue MVD could be a potential predictive factor for Mandard response of LARC treated with nCRT. High MVD was significantly associated with the tumor resistance to nCRT.

Author Contribution Statement

Dr. Sahaphol reviewed literature for this manuscript, collected data, prepared manuscript, discussed of study findings, provided description of the introduction, results and discussion. Dr. Thirayost reviewed literature,

reviewed tissue specimens, collected data, provided a description of the results, and participated in preparing the manuscript. Dr. Chinakrit reviewed literature, participated in collecting data, provided a description of the results and participated in preparing the manuscript. the manuscript. All authors read and approved the final manuscript.

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Ethics statement

This study was reviewed and approved by the Institutional Review Board of The Royal Thai Army, Medical Department prior to initiation.

Statement of Conflict of Interest

Authors declared no conflicts of interest concerning the content of the present study.

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