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Stroma-High Lymph Node Involvement Predicts Poor Survival More Accurately for Patients with Stage III Colon Cancer

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Abstract

Objective: The tumor microenvironment has ample impact on the behavior of the malignant process in colon cancer (CC). Patients with a high percentage of stroma within the primary tumor, determined by the tumor-stroma ratio (TSR), have a poor prognosis. In metastatic lymph nodes from patients with stage III CC, the TSR is heterogeneous, but the impact on patients' prognosis is unknown.

Methods: Haematoxylin and eosin stained tissue slides of primary tumor (PT) and associated lymph nodes (LNs) metastases from 102 patients with stage III CC were analyzed for the TSR. Stroma-high (>50% stroma) and stroma-low (≤ 50% stroma) groups were evaluated with respect to disease free survival (DFS).

Results: Of 102 analyzed primary tumors, 47 (46.1%) scored as stroma-high and 55 (53.9%) as stroma-low. In total, 33 patients had at least one stroma-high LN and 69 patients had one or more stroma-low LNs. Interestingly, 28 patients (27.5%) had both stroma-high and stroma-low LNs, but in another 44 cases the TSR between PT and LNs differed: 29 patients had a stroma-high PT with stroma-low LNs, while 15 patients displayed the opposite. As a result of the combination of the TSR analysis of the PT and the involved metastatic LNs, 62 patients (60.8%) were classified as stroma-high and 40 (39.2%) as stroma-low, restaging 14.7% of the patients to stroma-high with a significantly worse 5-year DFS compared to stroma-low patients (59% *vs.* 82%, HR=2.83 (95%CI 1.34–5.97), P=0.006). In multivariate analysis, the TSR retained its independent prognostic impact (HR=2.85 (95%CI 1.33-6.10), P=0.007).

Conclusion: The presence of abundant stroma in metastatic LNs from patients with stage III CC adds to the prognostic information learned from the primary tumor independently, and supports selective patient treatment.

Keywords Colon cancer; Disease free survival; Histology; Lymph node; Tumor-stroma ratio

Introduction

One of the primary determinants of prognosis for patients with colon cancer (CC) is lymph node involvement. For patients with a stage I or II tumor, the 5-year survival rate is more than 58% (stage IIC), but decreases to 35% (stage IIIC) when lymph nodes are involved [1].

Adjuvant chemotherapy has been shown to improve survival in patients with locoregional nodal metastases after resection, as it reduces the risk of death by an absolute 10% after 8 years [2]. Not all stage III CC patients have aggressive disease requiring treatment; however, identifying this group remains problematic.

Main factors contributing to intra-tumor heterogeneity have been well described at morphological, molecular and genomic levels. Heterogeneity between primary CC tumors and corresponding metastases has been reported on the level of biomarkers as well as genetic aberrations [3-6].

Intra-tumor heterogeneity is believed to be the origin of the selection process during metastatic progression. Tumor progression is not only driven by the malignant cells, but also by altered communication between neoplastic cells and non-malignant cell populations, including fibroblasts, endothelial and inflammatory cells in the tumor stroma. The infiltrating and surrounding fibroblasts, also known as cancer-associated fibroblasts (CAFs), also play an important role.

CAFs remodel the extracellular matrix (ECM) and secrete chemical factors, which promote the transformation process by encouraging tumor growth, angiogenesis, inflammation and metastasis and contribute to drug resistance [7].

Therefore, by ignoring the stromal compartment, valuable prognostic information is lost. The analysis of haematoxylin and eosin

(H&E) stained histologic slides reveals that the stromal compartment provides more information than previously thought.

The tumor-stroma ratio (TSR) has been shown prognostic by our group in several types of malignant epithelial neoplasms including colon cancer [8-10], breast cancer [11,12] and esophageal cancer [13,14]. The same finding has also been validated by various independent, international groups [15-18].

In the current study, patients with stage III CC were analyzed for two reasons: First, to evaluate the difference regarding the stroma between the primary tumor (PT) and metastatic lymph nodes (LNs), and second, to determine the additional prognostic value of the TSR in lymph node metastases.

Materials and Methods

Patients

The patient cohort consisted of patients with colon cancer from Leiden University Medical Center (LUMC), the Netherlands, and Vejle Hospital, Denmark. All patients were diagnosed between 1996 and 2011 and underwent complete surgical resection (R0) of stage III CC, followed by adjuvant chemotherapy.

Patients with histologically proven TNM stage III (any T, N1 or N2, M0) without gross or microscopic evidence of residual disease were included. Patients with a history of cancer other than basal cell carcinoma or cervical carcinoma in situ, or with multiple synchronous colon tumors were excluded, as well as patients who died within two months after surgery. Clinico-pathological data and outcome characteristics of these patients are shown in Table 1.

All samples were handled in a coded fashion, according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue," Dutch Federation of Medical Scientific Societies).

The Danish series of patients were included after approval from the Scientific Ethical Committee of Southern Denmark (ID#-20140117) and the Danish Data Agency according to Danish law. The tissue used for research was confirmed unregistered in the Danish Registry of Human Tissue Utilization.

Histopathological scoring

Tissue samples consisting of 5 μm H&E stained histologic sections of the PT, and the corresponding metastatic LNs were analyzed by conventional microscopy. Slides of the primary tumor were selected from the most invasive part of the tumor (i.e. the slides used in routine pathology to determine the T-status), as indicated by the pathology report. If this information was not stated, all available tumor slides were collected and analyzed [9].

Areas appearing to have the largest amount of stroma were selected using a 2.5x or 5x objectives. Hereafter, an area where both tumor and stromal tissue were present within this vision-site was selected using a 10x objective. Tumor cells were to be present at all borders of the image field to be selected.

Two investigators estimated the tumor-stroma ratio in a blinded manner. A third observer was decisive in the case of an inconclusive score and lack of consensus. Scoring percentages were given per tenfold (10%, 20%, 30% etc.) per image-field.

In case one of the metastatic LNs from a patient was stroma-high, the final score for the LNs was also considered stroma-high. When examining the four different groups (PT-low/LN-low, PT-low/LN-high, PT-high/LN-low and PT-high/LN-high), we observed that the PT-low/LN-high group had the worst outcome, supporting the large impact of TSR in the metastatic LNs (data not shown).

Therefore, when combining the lymph node TSR with the TSR of the primary tumor, we decided a patient was considered stroma-high when either the PT and/or the metastatic LNs were stroma-high. In case of a low TSR in the PT as well as in the metastatic LNs, the patient was considered stroma-low.

Statistics

Statistical analysis was performed using IBM SPSS software version 20.0. Our primary endpoint was disease free survival (DFS). This was defined as the time from the date of primary surgery until the date of:

1. Death, 2. First loco-regional or distant recurrence, or 3. Occurrence of a second primary tumor. If there was no recurrence, DFS was calculated as the time period until the date of last follow-up.

Stroma-high was defined as >50% stroma surface area, and stroma-low defined as ≤ 50% stroma surface area, as determined a priori to have maximum discriminative power [9].

Inter-observer variability was analyzed using the Cohen's kappa coefficient. Analysis of the survival curves was performed using Kaplan-Meier Survival Analysis, and differences in survival distributions were tested using log-rank statistics.

Cox regression was used for univariate and multivariate analyses. Variables with a p-value <0.1 in univariate analysis were included in the multivariate analysis. P-values <0.05 were considered statistically significant.

Results

Patients

A total of 53 LUMC patients and 55 Vejle hospital patients were included in the study. There were no significant clinicopathologic differences between the Dutch and Danish cohorts, except for tumor grade (Table 1).

Six patients (5.9%) had to be excluded due to poor quality of histological tissue, resulting in a total study population of 102 patients.

Additional patient information, including survival data, was collected after scoring all samples for the TSR. The study cohort was comprised of 58 men and 44 women, with a median age of 65 years (range 31-79 years). Of all patients, 70 (68.6%) were younger than 70 years of age, and 32 patients (31.4%) were older (Table 1).

Scoring tumor-stroma ratio

Out of 102 analyzed PTs, 47 (46.1%) were scored as stroma-high and 55 (53.9%) as stroma-low. There were no significant differences for clinicopathologic characteristics between the two groups, except for location of the primary tumor and T-status (Table1).

	Total		Leiden		Vejle		P-value	Stroma-low		Stroma-high		P-value
	N=102	%	N=47	%	N=55	%		N=55	%	N=47	%	
Sex												
Male	58	56.9	28	59.6	30	54.5		34	61.8	24	51.1	
Female	44	43.1	19	40.4	25	45.5	0.609	21	38.2	23	48.9	0.274
Age												
<70	70	68.6	35	74.5	35	63.6		36	65.5	34	72.3	
≥70	32	31.4	12	25.5	20	36.4	0.24	19	34.5	13	27.7	0.455
Grade												
Low	7	6.9	6	12.8	1	1.8		1	1.8	6	12.8	
Medium	60	58.8	22	46.8	38	69.1		35	63.6	25	53.2	
High	26	25.5	10	21.3	16	29.1		16	29.1	10	21.3	
Missing	9	8.8	9	19.1	0	0	**0.042***	3	5.5	6	12.8	0.067*
Histological type												
Adenocarcinoma	87	85.3	38	80.9	49	89.1		45	81.8	42	89.4	
Mucinous	13	12.7	7	14.9	6	10.9		8	14.5	5	10.6	
Signet ring cell carcinoma	2	2	2	4.3	0	0	0.24	2	3.6	0	0	0.336
Site of primary tumor†												
Left	62	60.8	28	59.6	34	61.8		28	50.9	34	72.3	
Right	40	39.2	19	40.4	21	38.2	0.817	27	49.1	13	27.7	**0.027**
T-stage												
T2/T3	90	88.2	41	87.2	49	89.1		52	94.5	38	80.9	
T4	12	11.8	6	12.8	6	10.9	0.772	3	5.5	9	19.1	**0.032**
N-stage												
N1	68	66.7	32	68.1	36	65.5		35	63.6	33	70.2	
N2	34	33.3	15	31.9	19	34.5	0.779	20	36.4	14	29.8	0.482
MSI status												
MSS	49	48	28	59.6	21	38.2		28	50.9	21	44.7	
MSI	7	6.9	5	10.6	2	3.6		6	10.9	1	2.1	
Missing	46	45.1	14	29.8	32	58.2	0.472*	21	38.2	25	53.2	0.148*
TSR Primary tumor												
Stroma-low	55	53.9	22	46.8	33	60						
Stroma-high	47	46.1	25	53.2	22	40	0.183					
TSR Lymph nodes												
Stroma-low	69	67.6	28	59.6	41	74.5		40	72.7	29	61.7	
Stroma-high	33	32.4	19	40.4	14	25.5	0.107	15	27.3	18	38.3	0.235

TSR PT+LNs								
Stroma-low	40	39.2	14	29.8	26	47.3		
Stroma-high	62	60.8	33	70.2	29	52.7	0.071	

†Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure; Bold indicates values with a significant difference P<0.05; MSI: Micro Satellite Instability; DFS: Disease Free Survival; TSR: Tumor-stroma ratio; PT:Primary tumor; LNs: Metastatic lymph nodes; *P-value excluding missing data.

Table 1: Characteristics of total patient population and stratified for each cohort, or TSR-group.

For the PT, the observers agreed on classification in 87% of all cases. In the other 13% of cases, consensus was reached or a third observer was decisive. For the metastatic LNs agreement was reached in 84% of cases, and consensus was reached or a third observer was decisive in the remaining 16%. Cohen's kappa coefficient revealed a substantial inter-observer agreement in classification for the PT as well as the LNs (κ=0.73 and 0.68 respectively).

Heterogeneity

When analyzing the TSR in the LNs, we observed that the metastasizing process of the PT to the LNs is a heterogeneous process (Figure 1). Interestingly, 28 patients (27.5%) had both stroma-high and stroma-low LNs. In 44 cases, the TSR between the PT and the LNs was different between stroma-high and stroma-low: 29 patients had a stroma-high PT but stroma-low LNs, and 15 patients *vice versa*.

Relation with Outcome

Primary tumor

The stroma-high population had a significantly worse DFS compared to the stroma-low patients (HR=1.89 (95%CI 1.00-3.56), P=0.046) (Figure 2A and Table 2), with a 5-year DFS of 61% versus 74% (stroma-high versus stroma-low, respectively). In multivariate analysis the TSR remained a significant prognostic variable (HR=1.98 (95%CI 1.04-3.77), P=0.038) (Table 2).

Lymph node involvement and combined analysis

A total of 1398 LNs were examined (median 13 per patient; range 3-49), of which 348 (median 2 per patient; range 1-17) contained metastasis from the PT, and 68 patients had stage N1 and 34 had N2. In total, 33 patients had at least one metastatic LN with a high amount of stroma, and were, therefore, considered stroma-high. The remaining 69 patients had one or more metastatic LNs with only a low TSR.

As a result of combining the stroma analysis of the PT and the involved LNs, 62 patients (60.8%) were classified as stroma-high and 40 (39.2%) as stroma-low. This resulted in restaging of 14.7% of stroma-low patients to the stroma-high group, which increased the DFS of the remaining stroma-low patients from 74% to 81% for 5-year DFS. In the stroma-high population, patients had a worse 5-year DFS compared to the stroma-low population (60% versus 81%, HR=2.83 (95%CI 1.34–5.97), P=0.004) (Figure 2B and Table 2). In multivariate analysis the combined TSR remained a significant prognostic variable (HR=2.85 (95%CI 1.33-6.10), P=0.007) (Table 2).

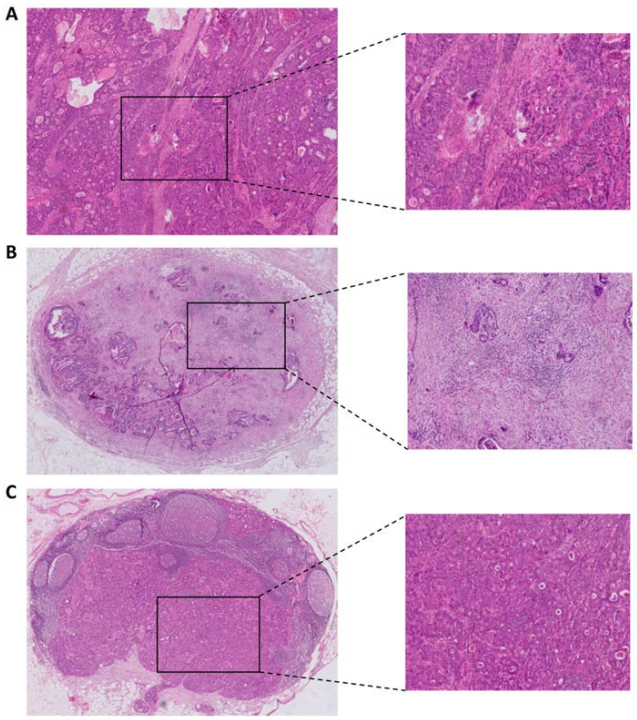


Figure 1: A) Haematoxylin and eosin stained section of a stroma-low primary tumor, with B) a corresponding stroma-high metastatic lymph node, and C) a corresponding stroma-low metastatic lymph node, showing the heterogeneity of the tumor-stroma ratio.

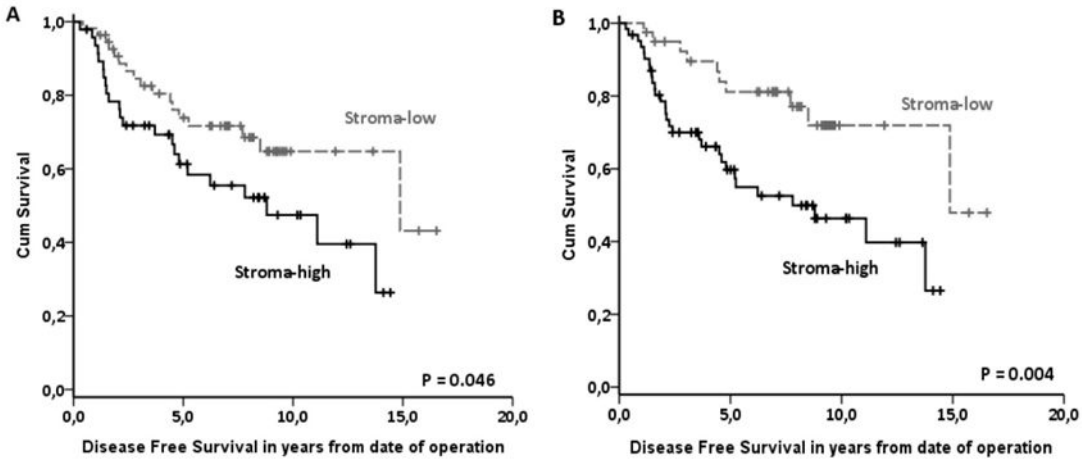
Discussion

In this study we analyzed the TSR, primarily used for analysis of the PT, in metastatic LNs from patients with stage III CC. The number of metastatic LNs evaluated in surgical specimens of CC has risen significantly over the past two decades. However, according to a study of Parsons et al., this improvement has not been associated with an increase in higher-staged cancers [19], raising the question whether the absolute number of metastatic LNs should be evaluated as the primary basis for estimating prognosis, or if a different approach should be considered. As we have shown in this study, the analysis of the TSR of metastatic LNs adds value with respect to the disease free survival of adjuvantly treated patients with stage III CC.

	N	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
Sex							
Male	58	1	0.435-1.555	0.547			
Female	44	0.822					
Age							
<70	70	1	0.912-3.293	0.093	1	1.007-3.641	**0.047**
≥70	32	1.733			1.915		
Grade							
Low	7	1		0.891			
Medium	60	1.106	0.261-4.696				
High	26	1.331	0.291-6.085	0.712			
Histological type							
Adenocarcinoma	87	1					
Mucinous	13	0.945	0.368-2.425	0.907			
Signet ring cell carcinoma	2	0.553	0.072-4.243	0.569			
Site of primary tumor†							
Left	62	1	0.734-2.557	0.323			
Right	40	1.37					
T-stage							
T2/T3	90	1	0.843-4.361	0.12			
T4	12	1.918					
N-stage							
N1	68	1	1.059-3.798	**0.033**	1	1.051-3.778	**0.035**
N2	34	2.006			1.992		
MSI status							
MSS	49	1	0.160-3.115	0.645			
MSI	7	0.705					
TSR PT‡							
Stroma-low	55	1	0.999-3.588	**0.046**	1	1.037-3.774	**0.038**
Stroma-high	47	1.893			1.978		
TSR PT+LNs‡							
Stroma-low	40	1	1.338-5.965	**0.006**	1	1.331-6.104	**0.007**
Stroma-high	62	2.825			2.85		

†Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure; ‡TSR PT and TSR PT+LNs have been analyzed in two separate models, both adjusted for age and N-stage; Bold indicates values with a significant difference P<0.05; MSI: Micro Satellite Instability; DFS: Disease Free Survival; TSR: Tumor-stroma Ratio; PT: Primary Tumor; LNs: Metastatic lymph nodes.

Table 2: Uni and multivariate analysis regarding DFS.



Numbers at risk								
Stroma-low	51	21	4	1	38	18	4	1
Stroma-high	43	18	6		56	21	6	
Survival %								
Stroma-low	100	75	64	48	100	82	72	52
Stroma-high	100	60	47	30	100	59	45	30

Figure 2: A) Kaplan-Meier disease free survival curves regarding stroma score of primary tumor. B) Combined analysis of primary tumor and associated metastatic lymph nodes.

Although the metastasizing process to the LNs is very heterogeneous, the presence of just one metastatic LN with a high amount of stroma is enough to predict a worse DFS. This might indicate that a different treatment approach is necessary for patients classified as stroma-high compared to patients in the stroma-low group.

Cancer research for the development of targeted therapies has focused largely on genetic and epigenetic abnormalities of the epithelial component of solid tumors. Recent approaches focus on gene signature profiles using microarray gene analysis to predict recurrence or benefit from therapy. New colorectal cancer (CRC) subtypes have been identified by three independent research groups [20-22]. All groups identified one subtype associated with poor prognosis, and more importantly, this subtype was recently observed to associate with a high stromal content [23]. This finding is in line with our observation that patients with a stroma-high tumor have a worse prognosis. Moreover, recently identified mechanisms of therapeutic resistance, which were mainly conferred by changes in the tumor microenvironment, indicate the importance of the development of therapies targeting the non-cancer stromal cells, like fibroblasts and extracellular matrix components [24].

Many prognostic and predictive biomarkers have been, or are currently, under investigation for possible implementation in routine clinical diagnostics. Markers such as BRAF, KRAS and NRAS are well-known prognostic (BRAF) and predictive (RAS, for metastatic CRC) markers used in the clinic, whereas serial measurement of carcinoembryonic antigen is the standard for disease monitoring. Also, multiple markers have been associated with resistance or sensitivity to therapy. RAS mutations and BRAF mutations are already known to cause resistance to anti-EGFR therapy, but recently also PTEN- and PI3K-mutations, miR-181a and IGF2 overexpression have been found to be predictive for response to anti-EGFR therapy [25]. Although these markers might contribute to further characterization of the tumor and, therefore, facilitate the selection of treatment for the individual patient, the techniques used to determine these markers, like gene expression arrays or next generation sequencing, are time consuming and costly. Moreover, for gene expression array analyses, it is common practice to select those parts of the tissue in which tumor cells form the major component, as admixtures of stroma and inflammatory cells will lead to masking of amplifications and deletions. This may lead to exclusion of stroma-high tumors, which may form a selection bias for patients with a better prognosis. On the contrary, determining the TSR is easy, has high reproducibility, has low

inter-observer variation, and is not associated with extra costs. The TSR has also been discussed by the TNM Evaluation Committee and the College of American Pathologists, who stated that our observations are important, novel, and have the potential to be included in the TNM staging algorithm. They advocated validation in a prospective, multi-center study, development of a consensus agreement, and a quality assessment program. Therefore, a reliability and reproducibility study will be conducted among national and international pathologists. An e-learning module will be developed with a quality assessment program in the framework of the European Society of Pathology EQA program. At the same time, an automated method is currently being developed to obtain an even more robust measurement, which is essential for estimating the cut-off threshold, as well as an even higher reproducibility.

Although the extent of nodal involvement (i.e. N1 versus N2) is a known predictor for survival amongst stage III CC patients [26,27], in this study we found no correlation between N-status and TSR (Table 1), and both variables were found to be independent prognostic parameters. Furthermore, we analyzed the association between the lymph node ratio (LNR; in quartiles) and the TSR in metastatic LNs (TSR-LN) to see if these parameters, when combined, would have additional impact on prognosis. This analysis showed that stroma-high LNs indeed were more likely to have a higher LNR (χ^2-test P=0.004). However, the combination of LNR and TSR-LN was not a prognostic factor in multivariable analysis (P=0.115). This might be explained by a small sample size of subgroups, and should be further investigated in a larger study.

Also the MSI status has proved to be a predictive marker for the survival of colon cancer patients. In the current study, this was not found and may be due to the fact that for almost half of the study cohort, the MSI status was unknown. However, in previous studies we have already shown that the TSR is a prognostic parameter, independent of MSI status [8,9].

In this study we also found a strong heterogeneity within the metastasizing process of the stroma based on visual evaluation, whereas several studies have investigated the expression levels of different prognostic markers in CRC and corresponding LN metastases on the molecular level [28-30]. In concordance with our data, the expression patterns of some of these markers also showed to be heterogeneous between the PT and LN metastases. For example, the expression of p53 has been documented to be similar between PT and LN metastases [28,30], whereas EGFR expression differed. This difference in EGFR expression indicates that the PT does not reflect the situation in LN metastases, which might have important clinical implications [29].

Although there have been studies published which describe the expression of biomarkers in the stroma of metastatic LNs, as discussed above, to our knowledge, this is the first study investigating the amount of stroma present in LN metastases from patients with CC. In this study we have shown that the analysis of the TSR in metastatic lymph nodes has an additional value with respect to disease free survival in patients with stage III CC. Taking tumor heterogeneity into consideration, this parameter might be used as a marker to select patients for therapy targeting the stromal compartment of the tumor.

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Conflicts of Interest and Source of Funding

The authors have declared no conflicts of interest. No funding has been received.

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