COLORECTAL CANCER

Low level microsatellite instability may be associated with reduced cancer specific survival in sporadic stage C colorectal carcinoma

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Background: Colorectal cancers (CRCs) may be categorised according to the degree of microsatellite instability (MSI) exhibited, as MSI-high (MSI-H), MSI-low (MSI-L), or microsatellite stable (MSS). MSI-H status confers a survival advantage to patients with sporadic CRC.
Aims: To determine if low levels of MSI are related to the clinicopathological features and prognosis of sporadic stage C CRC.
Patients: A total of 255 patients who underwent resection for sporadic stage C CRC were studied. No patient received chemotherapy. Minimum follow up was five years.
Methods: DNA extracted from archival malignant and non-malignant tissue was amplified by polymerase chain reaction using a panel of 11 microsatellites. MSI-H was defined as instability at $\geq$40% of markers, MSS as no instability, and MSI-L as instability at $>$0% but $<$40% of markers. Patients with MSI-H CRC were excluded from analysis as they have previously been shown to have better survival.
Results: Thirty three MSI-L and 176 MSS CRCs were identified. There was no difference in biological characteristics or overall survival of MSI-L compared with MSS CRC but MSI-L was associated with poorer cancer specific survival (hazard ratio 2.0 (95% confidence interval 1.1–3.6)).
Conclusions: Sporadic MSI-L and MSS CRCs have comparable clinicopathological features. Further studies are required to assess the impact of MSI-L on prognosis.

Colorectal cancer (CRC) is the commonest cause of cancer related death in non-smoking men and women. In Australia, approximately one in 18 men and one in 26 women will develop the disease by the age of 75 years.[1] A variety of important prognostic factors have been identified for CRC. The most reliable of these is clinicopathological staging.[2] Patients with CRC confined to the bowel wall have the best prognosis, with virtually all patients surviving, while most patients with distant metastases at the time of surgery will die from their cancer. The most difficult group of patients to predict outcome for are those with nodal involvement, where the five year survival is approximately 50%.[3]

There has been considerable progress in the understanding of the genetic and epigenetic events that occur during colorectal carcinogenesis. Mechanisms that maintain the fidelity of the genome are targets for inactivation during malignant transformation. One of the most important of these is the DNA mismatch repair (MMR) system. This repairs base pair mismatches and short loops that arise during DNA replication.[4 5] Inactivation of DNA MMR confers a mutator phenotype on affected cells, resulting in the progressive accumulation of DNA damage.

The hallmark of defective MMR is microsatellite instability. Microsatellites are made up of multiple repeats of short sequences of DNA, 1–5 bases in length. These are scattered throughout non-coding regions of the genome. Microsatellites are prone to strand slippage during DNA replication. If this is not recognised and repaired by the MMR system, addition or deletion of the microsatellite repeat occurs, resulting in a change in the size of the microsatellite allele in daughter cells. This change is described as microsatellite instability (MSI).[6] Short mononucleotide repeat sequences are also found in the coding regions of some genes, including the transforming growth factor β receptor 2 and insulin-like growth factor II receptor.[7 8] Similar errors in microsatellite-like repeats of these genes can alter their protein product, enhancing the neoplastic potential of affected cells.

Cancers occurring in individuals with the familial cancer syndrome hereditary non-polyposis colorectal cancer (HNPCC), in which a DNA MMR gene is mutated, show high levels of MSI.[9–12] These familial MSI-high (MSI-H) CRCs appear to have a more favourable prognosis.[13] Ten to 15% of sporadic CRCs also exhibit MSI-H.[14 15] This occurs as a result of acquired hypermethylation and epigenetic silencing of the DNA MMR gene, *h*MLH1.[16 17] Several studies from our own and other centres have shown that sporadic MSI-H CRCs have unique clinicopathological characteristics and a better prognosis than microsatellite stable (MSS) CRC.[18–21]

A third group of tumours can be identified in which MSI is present at an intermediate level. The significance of this low level of MSI (MSI-L) remains controversial[22–24] but recent studies support that MSI-L is a distinct entity and a feature of specific tumour types, including CRC.[25 26] There are differences in the underlying biology of MSI-L compared with MSS CRC, in particular epigenetic silencing of the DNA enzyme O^6-methyltransferase by promoter methylation, and a greater prevalence of *K-ras* mutations.[27 28] Recent microarray studies also suggest that MSI-L CRCs have a unique profile of gene expression.[29] However, it has not been shown if the genetic and epigenetic differences between MSI-L and MSS CRC translate into differences in clinicopathological characteristics and prognosis.

Abbreviations: CRC, colorectal cancer; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS, microsatellite stable; HNPCC, hereditary non-polyposis colorectal cancer; PCR, polymerase chain reaction

The aim of the current study was to address this question by comparing sporadic MSI-L CRC with a comparable group of MSS cancers in a well characterised cohort of patients. Differences between MSI-L and MSS CRC, particularly with respect to prognosis, are likely to be greatest and most clinically relevant in patients with stage C disease (that is, those with nodal involvement). To overcome potential biases related to the effect of MSI status on response to chemotherapy, only patients with stage C CRC who had been treated prior to the routine use of adjuvant chemotherapy were included. Survival data have been collected prospectively on patients in this cohort.

METHODS

Subjects

A total of 255 consecutive patients (197 males and 58 females) who had undergone resection of a clinicopathological (CP) stage C CRC between January 1986 and January 1992 were identified from the Concord Hospital Colorectal Cancer Registry. This registry was established by the Concord Hospital Departments of Surgery and Anatomical Pathology in 1971 and has prospectively collected clinicopathological and survival data on all CRC treated at Concord Hospital since this date. Since 1979, all clinical and operative data have been compiled by one surgeon (PHC). This study was approved by the Ethics Review Committee of the Central Sydney Area Health Authority and the Bancroft Centre Ethics Committee of the Queensland Institute of Medical Research.

CP stage C CRCs are cancers in which there are confirmed lymph node metastases at the time of surgery (irrespective of the depth of bowel wall invasion by the primary tumour), without pathological or operative evidence of distant metastases or local residual cancer left at the time of bowel resection.[30] Standardised surgical technique and pathology reporting were employed throughout the study period, and more than 90% of specimen dissections were performed by one pathologist (RCN) who also reviewed all of the histological specimens. All cases were deemed sporadic based on the absence of a family or clinical history suggestive of either HNPCC or familial adenomatous polyposis. No patients received adjuvant chemotherapy and the minimum follow up period was five years.

Table 1 Association between low level microsatellite instability and patient and tumour characteristics

Characteristic	n	No with MSI-L (%)	p Value
Sex			
Female	48	8 (17)	0.849
Male	161	25 (16)	
Age (y)			
20–74	155	25 (16)	0.820
75–99	54	8 (15)	
Tumour site	102	16 (16)	0.968
Colon			
Rectum	107	17 (16)	
Tumour size (cm)	127	19 (15)	0.683
<5			
⩾5	82	14 (17)	
Venous invasion			
No	146	26 (18)	0.223
Yes	63	7 (11)	
Direct spread beyond muscularis propria			
No	26	6 (23)	0.276
Yes	183	27 (15)	
Involvement of free serosal surface			
No	179	26 (15)	0.221
Yes	30	7 (23)	
Tumour grade			
Low/average	154	26 (17)	0.468
High	55	7 (13)	
No of nodes involved			
1–3	151	22 (15)	0.435
>3	58	11 (19)	
Apical node involved			
No	200	33 (17)	0.184
Yes	9	0 (0)	

MSI-low, microsatellite instability low.

Microsatellite analysis

DNA was extracted from two 10 µm sections of formalin fixed paraffin embedded tissue using a standard technique.[31] Separate blocks were used for malignant and non-malignant tissue.

Microsatellite instability was assessed using a panel of 11 microsatellites (BAT25,[7] BAT26,[7] BAT40,[7] D2S123,[32] D5S346,[33] D10S197,[32] D17S250,[34] D18S58,[35] D18S69,[32] c-myb,[36] and L-myc[37]) and included mononucleotide repeats (BAT25, BAT26, BAT40, and c-myb), dinucleotide repeats (D2S123, D5S346, D10S197, D17S250, D18S58, and D18S69), and a complex tetranucleotide repeats (L-myc). Ten of these markers had previously been recommended for the assessment of MSI,[38] and included the five microsatellites recommended for the assessment of MSI in CRC by a NCI consensus meeting on MSI.[6] An additional marker (c-myb) previously characterised in our laboratory was also included.[36]

Optimal annealing temperatures were determined for each primer pair and 1.5 µl of template DNA was amplified by polymerase chain reaction (PCR)in a final reaction volume of 15 µl. PCR product was labelled by incorporation of (α^{33}P), electrophoresed on a 5% denaturing polyacrylamide gel, and visualised by autoradiography.

PCR was repeated if a microsatellite locus failed to amplify with the initial PCR. In an attempt to reduce the failure rate of PCR for these cases, PCRs were also performed using a 1/10 dilution of template DNA, and with 3.0 µl of template DNA solution. Only cases with PCR product from at least 10 of 11 microsatellite loci using this strategy were included in the subsequent analysis.

Scoring of microsatellite instability

MSI was identified by the presence of novel bands in the PCR product from malignant colorectal tissue compared with the pattern from non-malignant tissue. Each gel was scored independently by two experienced study personnel (CMW, MB). If there was discordance in their interpretation, the gels were reviewed by a third investigator (JPY) to provide a consensus result. If a clear consensus was then not achieved the marker was scored as non-informative for that locus.

A case was designated MSI-H if microsatellite instability was present at ⩾40% of microsatellite markers, while MSS CRCs had no evidence of MSI. These criteria were proposed by a NCI consensus meeting on MSI in CRC.[6] Using these criteria we have previously demonstrated improved survival in MSI-H sporadic CP stage C CRC in this patient cohort.[18] A CRC was classified as having MSI-L if there was MSI present that fell short of the threshold required for a diagnosis of MSI-H.

Statistical analysis

Overall survival time was measured from the date of resection to the date of death from any cause, with patients surviving to at least February 1997 and those lost to follow up being censored in survival analyses. Cancer specific survival time was measured from the date of resection to the date of death due to CRC, with censoring as above but also including those who died from causes other than CRC. MSI-H cases were excluded from the analyses.

Table 2 Bivariate association between survival and patient and pathology characteristics

Characteristic	Overall survival Hazard ratio (95% CI)	p Value	Cancer specific survival Hazard ratio (95% CI)	p Value
MSI-low	0.9 (0.6–1.4)	0.627	1.3 (0.7–2.2)	0.426
Male	1.5 (1.0–2.3)	0.049	1.6 (0.9–2.9)	0.107
Age 75–99 y	2.0 (1.4–2.9)	0.000	1.5 (0.9–2.5)	0.148
Rectal tumour	1.4 (1.0–1.9)	0.057	1.3 (0.8–2.1)	0.230
Tumour size ⩾5 cm	1.2 (0.8–1.6)	0.404	1.1 (0.7–1.8)	0.634
Venous invasion present	1.9 (1.3–2.6)	0.000	2.9 (1.9–4.6)	0.000
Direct spread beyond muscularis propria	1.6 (0.9–2.7)	0.094	2.3 (1.0–5.2)	0.052
Free serosal surface involved	1.0 (0.6–1.6)	0.995	1.6 (0.9–2.8)	0.102
High grade tumour	1.7 (1.2–2.4)	0.003	2.5 (1.6–4.0)	0.000
More than 3 nodes involved	1.4 (1.0–2.0)	0.063	2.2 (1.4–3.4)	0.001
Apical node involved	2.4 (1.2–4.8)	0.019	2.7 (1.0–7.3)	0.058

MSI-low, microsatellite instability low.
95% CI, 95% confidence interval.

The χ^2 test was used to evaluate the significance of differences in contingency tables. Comparisons of overall survival time between strata of categorical variables were made by the Kaplan-Meier method[39] (SPPS[40]) and log rank test. Hazard ratios and their confidence intervals, for both overall survival and cancer specific survival, were obtained by proportional hazards regression. The assumption of proportional hazards was checked by examination of log cumulative hazard plots for parallelism and in no instance was this materially violated. In multivariate proportional hazards modelling, all variables having an association with survival with a p value of <0.1 were entered into an initial model which was then reduced by successive elimination of variables with a p value of >0.05. The provisional final model resulting from this procedure was then re-examined by introducing each previously excluded variable separately; this did not result in any material change in the model in any instance.

The level for statistical significance was taken as $p \leqslant 0.05$ and confidence intervals (CI) were set at 95%.

RESULTS

Twenty one of 255 cases (8.2%) were MSI-H and were excluded from further analysis. Twenty five cases amplified at <10 loci and were considered not informative for analysis.

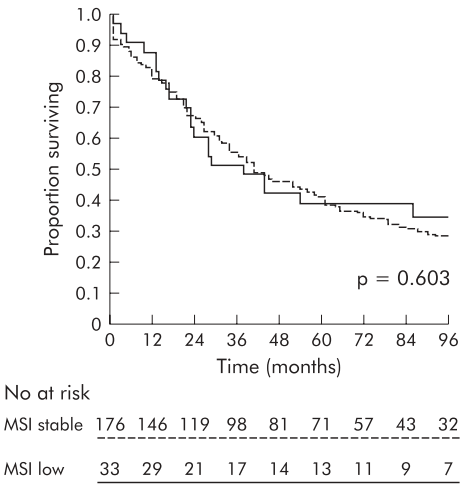

Figure 1 Kaplan-Meier survival curves (overall survival) for sporadic clinicopathological stage C microsatellite instability (MSI) low and MSI stable colorectal carcinomas.

This left a study group of 209 cases of which 33 (15.8%) were MSI-L and 176 MSS CRC.

There was no significant association between MSI-L status and any of 10 patient and tumour pathology characteristics previously shown to be associated with survival in patients with stage C CRC (table 1).[41] Unlike MSI-H cancers, MSI-L cancers show no predilection for anatomical site, and were comparable in size and their histology to MSS cancers.

Follow up time in 56 patients surviving at the close of the study ranged from 52 months to 168 months (median 106 months). Median survival time of 78 patients who died of CRC was 28 months and that of 37 patients who died of other causes 29 months. Thirteen patients died postoperatively (within one month of resection) and four were lost to follow up. Twenty one patients died of unknown causes (four MSI-L patients and 17 MSS patients) and these patients were excluded from the analyses of cancer specific survival.

Bivariate analysis showed no significant difference in overall survival between MSI-L and MSS (table 2, fig 1). Among the other patient and tumour characteristics previously shown to be associated with diminished survival in stage C patients, male sex, age 75–99 years, venous invasion, high grade tumour, and apical node involvement were again significantly associated with poorer overall survival in this study, although tumour site, size, direct spread beyond the muscularis propria, involvement of a free serosal surface, and the presence of more than three involved nodes were not (table 2). All of the variables having a significant bivariate association with overall survival remained significant in the multivariate model (table 3). MSI-L was not found to be independently associated with overall survival.

MSI-L CRCs showed poorer cancer specific survival than MSS (fig 2) although this difference was not statistically significant. Bivariate analyses for cancer specific survival showed significant effects only for venous invasion, high grade tumour, and involvement of more than three nodes; direct spread beyond the muscularis propria and apical node involvement just failed to reach statistical significance (table 2). As would be expected, advanced age was not significant, the effect of normal mortality largely being eliminated by the censoring of patients who died of causes other than CRC. The multivariate model for cancer specific survival showed significantly diminished survival was associated with male sex, venous invasion, high grade tumour, apical node involvement, and also MSI-L (table 3).

A summary of the marker profile of the MSI-L cases is shown in table 4. The majority of the 33 MSI-L tumours showed instability at only one microsatellite (fig 3). Only four of the 33 MSI-L cases showed instability at a mononucleotide

Table 3 Proportional hazards regression of survival on patient and tumour characteristics

Characteristic	Overall survival		Cancer specific survival	
	Hazard ratio (95% CI)	p Value	Hazard ratio (95% CI)	p Value
MSI-low	1.1 (0.7–1.8)	0.583	2.0 (1.1–3.6)	0.022
Male	1.7 (1.1–2.7)	0.018	1.9 (1.0–3.6)	0.036
Age ⩾75 y	2.1 (1.5–3.0)	0.001	1.5 (0.8–2.5)	0.176
Venous invasion present	1.7 (1.2–2.4)	0.003	3.1 (1.9–4.9)	0.000
High grade tumour	1.5 (1.1–2.2)	0.022	2.1 (1.3–3.4)	0.002
Apical node involved	3.2 (1.4–7.3)	0.005	4.6 (1.5–14.2)	0.008

MSI-low, microsatellite instability low.
95% CI, 95% confidence interval.

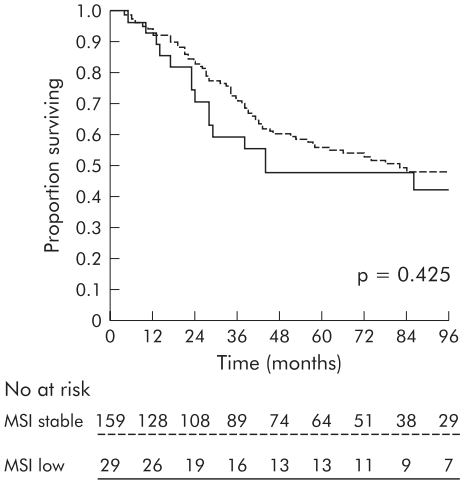

Figure 2 Kaplan-Meier survival curves (cancer specific survival) for sporadic clinicopathological stage C microsatellite instability (MSI) low and MSI stable colorectal carcinomas.

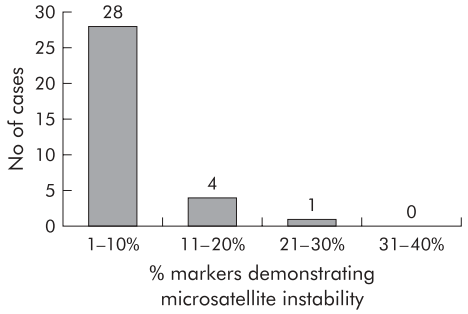

Figure 3 Histogram showing the degree of microsatellite instability in the 33 microsatellite instability low colorectal carcinomas.

repeat, most showing instability of loci containing dinucleotide or tetranucleotide repeats, in particular D2S123 (a dinucleotide (CA) repeat) and L-myc (a complex tetranucleotide repeat). This contrasts with the pattern seen in MSI-H cancers. The microsatellite profile data for the MSI-H cases from the same patient cohort has previously been reported,[18] but is presented in table 5 for comparison.

DISCUSSION

The relationship of MSI-L to MSS and MSI-H cancers is currently under debate,[22–25 42] particularly as MSI-L CRCs share characteristics of both MSS and MSI-H cancer. The current study confirms that MSI-L CRCs are comparable with MSS cancers in terms of clinicopathological features and overall survival. A similar effect of MSI-L status on overall prognosis has previously been suggested but in a significantly smaller cohort of 90 subjects with MSS or MSI-L stage C CRCs.[24]

In light of the similarities for overall survival, the finding on multivariate analysis that MSI-L status is an independent predictor of poorer cancer specific survival for sporadic CRC was unexpected. The Concord Hospital Colorectal Cancer Registry prospectively collects data on overall survival and cancer specific survival, these being relatively robust outcome measures. Data on locoregional recurrence is only collected for rectal cancers. This prevents direct analysis of recurrence free survival in the present study of both colon and rectal cancer patients. Time to death due to CRC (cancer specific survival) can be considered a surrogate for recurrence free survival but it is not an ideal substitute as it inflates the event free survival time by including time from recurrence to death. Additionally, it excludes patients who have had locoregional recurrence treated successfully, patients who had recurrence but died from another cause, and patients with recurrence who had not died by the close of the study. However, the number of patients in each of these categories is likely to be very small.

In view of these limitations, care needs to be taken in asserting the cause of the observation that MSI-L independently predicted poorer cancer specific survival. However, it is our opinion that MSI-L CRC patients may be more likely to develop recurrence and MSI-L may predict duration of survival to recurrence. Why this effect is not seen when

Table 4 Summary of the microsatellite instability of 33 sporadic clinicopathological stage C microsatellite instability low (MSI-L) colorectal cancers with a panel of 11 microsatellite markers*

C-myc	BAT26	D2S123	BAT25	D5S346	D17S250	L-myc	BAT40	D10S197	D18S69	D18S58
1/33	0/33	10/32	1/33	5/32	5/32	11/33	2/32	3/30	1/31	0/32

*No of cases showing MSI/total informative cases at each microsatellite locus.
Mononucleotide markers: C-myc, BAT26, BAT25, and BAT40.
Dinucleotide markers: D2S123, D5S346, D17S250, D10S197, D18S69, and D18S58.
Complex tetranucleotide repeat: myc-L.

Table 5 Summary of the microsatellite instability of 21 sporadic clinicopathological stage C microsatellite instability high (MSI-H) colorectal cancers with a panel of 11 microsatellite markers*

C-myb	BAT 26	D2S123	BAT 25	D5S346	D17S250	L-myc	BAT 40	D10S197	D18S69	D18S58
19/21	18/21	13/18	15/21	10/21	6/19	17/21	16/21	18/20	12/17	14/18

*No of cases showing MSI/total informative cases at each microsatellite locus.
Mononucleotide markers: C-myb, BAT26, BAT25, and BAT40.
Dinucleotide markers: D2S123, D5S346, D17S250, D10S197, D18S69, and D18S58.
Complex tetranucleotide repeat: myc-L.

overall survival is compared and why it only emerges in the multivariate model is hard to explain but may be related to our sample size and the statistical power of the study.

At the molecular level, characteristics shared by MSS and MSI-L but not MSI-H CRCs are a relatively high degree of chromosomal instability[43] and the presence of *K-ras* mutations, although the targets of allelic imbalance and prevalence of *K-ras* mutations are significantly different in the two groups.[28] Of clinical relevance, the response of MSI-L and MSS CRC to fluorouracil based adjuvant chemotherapy has also recently been shown to be similar and superior to the response of MSI-H CRC.[44]

As an alternative view, there are a number of shared features of MSI-L and MSI-H CRCs, in particular the presence of microsatellite instability and that both undergo gene silencing by promoter hypermethylation, although the targets of inactivation differ, these including the DNA repair enzyme *O^6-methylguanine methyltransferase* in MSI-L[27] and *hMLH1* in MSI-H[16 17] cancers. In addition, there is evidence that both MSI-L and MSI-H CRC evolve from serrated adenomas.[28]

The evidence increasingly supports the fact that MSI-L is a distinct biological entity.[25 26] Overall, MSI-L CRCs share features of both MSS and MSI-H cancers and the issue for clinical practice is whether the biological behaviour of MSI-L CRCs is unique. There is the potential for a differential effect of adjuvant chemotherapy therapy on MSS and MSI-L CRCs. The only study to examine this issue to date found no difference in the response of MSI-L and MSS CRCs to adjuvant chemotherapy but was probably underpowered to detect a significant difference.[19] Importantly, in the current study, there was no potential confounding effect on survival from adjuvant chemotherapy.

To clarify the relationship of MSI-L CRC to MSS and MSI-H CRC, more precise characterisation of MSI-L status is required. In addition to the number of microsatellites studied, the type of microsatellite loci tested is also important.[38 45] In the current study, the MSI-L cancers predominantly showed instability of dinucleotide and complex repeats and were less likely to demonstrate instability in mononucleotide repeat loci. There was disproportionate MSI of four of the 11 microsatellites utilised (χ^2, $p<0.00001$) in the MSI-L cases compared with MSI-H CRCs. In order of prevalence of MSI these were: L-myc, D2S123, D5S346, and D17S250. The last three of these markers were from the NCI reference panel for the detection of microsatellite instability in CRC.[6] MSI of the BAT mononucleotide repeats used (BAT25, 26 and 40) was virtually confined to MSI-H CRC. Instability at BAT 26 was not detected in any of the MSI-L cases whereas it has previously been shown to be highly sensitive and specific for identifying MSI-H CRC.[46] Using the criteria for MSI-L of MSI at one or more of the four microsatellites, L-myc, D2S123, D5S346, and D17S2504, but not one of the mononucleotide markers, would have identified 27 of the 33 (82%) MSI-L cancers in the current study.

In summary, the current study showed no difference in the clinicopathological features or overall survival of MSI-L versus MSS cancers in a large prospectively evaluated cohort of sporadic stage C CRCs. However, in multivariate analysis, patients with MSI-L CRCs had significantly poorer cancer specific survival. This observation needs to be confirmed but if real, is presumably due to an increased tendency of MSI-L CRCs to develop recurrent disease. The underlying biological mechanism for this is unclear and should continue to be investigated in a research setting.

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