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Differential significance of tumour infiltrating lymphocytes in sporadic mismatch repair deficient *versus* proficient colorectal cancers: A potential role for dysregulation of the transforming growth factor-β pathway

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ABSTRACT

The prognostic importance of tumour infiltrating lymphocytes (TILs) in colorectal cancer (CRC) remains controversial. The present study was undertaken to evaluate the role of TILs as prognostic indicators and to investigate the role of transforming growth factor-β (TGF-β) in TIL infiltration. Immunohistochemical staining for components in the TGF-β pathway was performed on a tissue microarray of 1420 unselected CRCs with complete clinico-pathological data. Statistical analyses were carried out on samples stratified by mismatch repair (MMR) proficiency status and TIL counts. TIL infiltration was found to correlate with multiple clinico-pathological features but was a prognostic marker only in MMR proficient CRCs. In all CRCs, findings indicative of insensitivity to TGF-β and increased TGF-β secretion were independent predictors of high TIL counts, suggesting that perturbations in the TGF-β signalling pathway play an important role in the recruitment and retention of TILs within CRC epithelium.

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1. Introduction

Colorectal cancer (CRC) is a heterogeneous disease that may be differentially classified according to the underlying type of genomic instability. While the majority of CRCs are chromosomally unstable, approximately 10–15% of sporadic CRCs possess high levels of microsatellite instability as a result of deficiencies in the DNA mismatch repair (MMR) system.[1] MMR deficient tumours lack the classical spectrum of mutations seen in MMR proficient CRCs and are instead characterised by the appearance of frameshift mutations in genes possessing repetitive base pair tracts.[1] Sporadic MMR deficient cancers also share particular clinico-pathological features including a predilection for the proximal colon, a later age of onset, poor histological differentiation, a generally more favourable prognosis and marked infiltration of the tumour epithelium by lymphocytes.[1,2] Tumour infiltrating lymphocytes (TILs) are located in direct contact with the tumour cells themselves and differ phenotypically and functionally from both stromal (peritumoural) lymphocytes and peripheral blood lymphocytes.[3–5] Whereas TILs are associated with improved survival, the same cannot be said for stromal lymphocytes.[6]

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Despite the diagnostic and prognostic importance of TILs, little is known about the underlying mechanisms that explain their presence or their precise role in tumour immunity. Within MMR deficient CRCs, they have been identified as predominantly CD8+ T cells frequently expressing granzyme B.[2,7] This is suggestive of an active cytotoxic phenotype which has been attributed to the inherently greater production of abnormal peptides that result from unreliable DNA repair.[8,9]

The high level of TIL infiltration characteristic of MMR deficient CRCs suggests that some unique aspect of the tumour biology is involved in their recruitment or retention. A majority of these cancers possess inactivating mutations in the type II TGF-β receptor (TGF-βRII), which contains a 10 bp polyA tract.[10] This is expected to render the cells insensitive to TGF-β, which requires both the type I and type II receptors for signal transmission.[11] Since TGF-β has the ability to negatively regulate its own production, we previously investigated a possible link between dysregulated TGF-β signalling and TIL infiltration in CRC.[12,13] The results demonstrated a link between high level TIL infiltration and both increased secretion of TGF-β and insensitivity to TGF-β's anti-proliferative effects.[13] The present study was undertaken in order to extend our previous findings using a much larger set of fully characterised CRCs.

2. Materials and methods

2.1. Tissue microarray (TMA) construction

A TMA of 1420 unselected, non-consecutive CRCs was constructed as described previously.[14] Briefly, formalin-fixed, paraffin-embedded tissue blocks of CRC resections were obtained. One tissue cylinder with a diameter of 0.6 mm was punched from morphologically representative tissue areas of each donor tissue block and brought into one recipient paraffin block (3 × 2.5 cm) using a homemade semiautomated tissue arrayer. Each punch was made from the centre of the tumour such that each TMA spot consisted of at least 50% tumour cells.

2.2. Clinico-pathological features

The criteria used to define all clinico-pathological features as well as the distribution of these features among all cases have been described elsewhere.[15,16] Additionally, treatment data were available for 479 cases, 78.7% of which were MMR proficient and 16.7% were MMR deficient. Of the MMR proficient CRCs, 76.9% were treated by surgery alone, 2.1% by adjuvant radiotherapy, 18.3% by adjuvant chemotherapy and 2.7% by adjuvant radiotherapy and chemotherapy. Within the MMR deficient subset, 92.5% were treated by surgery alone and the remaining 7.5% by adjuvant chemotherapy. This study was approved by the Institutional Review Board of the Faculty of Medicine of McGill University.

2.3. Immunohistochemical staining

Four-micron sections of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics, Inc., Hackensack, NJ, USA) and stained with the EnVision+ System-HRP (DakoCytomation, Mississauga, Ont., Canada). Staining was performed according to the manufacturer's protocol following a pressure cooker antigen retrieval step in 1 mM EDTA (ethylenediaminetetraacetic acid) (pH 8.0). The following primary antibodies were used: MLH1 (clone MLH-1, Pharmingen, San Jose, CA, USA), MSH2 (clone MSH-2, Pharmingen), MSH6 (clone 44, Transduction Laboratories, San Jose, CA, USA), CD8 (clone C8/144B, DakoCytomation), TGF-β (clone TB21, AbCam, Cambridge, MA, USA), TGF-βRII (clone E-6, Santa Cruz Inc., Santa Cruz, CA, USA), E-cadherin (clone NCH-38, DakoCytomation), Ki-67 (clone MIB-1, DakoCytomation), phospho-Smad2 (Cell Signalling Technology, Danvers, MA, USA) and Smad4 (clone BC/B8, Biocare Medical, Concord, CA, USA). The dilution for all antibodies was 1:100 except for TGF-β, which was 1:1000, and all incubations were carried out for 2 h at room temperature. Visualisation was accomplished with the AEC+ Substrate Chromogen (3-amino-9-ethylcarbazole) and slides were counterstained with Gill's haematoxylin.

2.4. Immunohistochemical scoring

CD8 staining was used to highlight TILs and only clearly intraepithelial CD8 positive cells were counted. The total number of TILs contained within each punch, approximately the same as one high power (40×) field, was determined for each case.

MLH1, MSH2 and MSH6 were scored as negative (0%) or positive (>0%).

For all other proteins, immunoreactivity was scored semiquantitatively using the proportion of positive tumour cells over total tumour cells (%-positivity, ranging from 0% to 100% at 5% intervals).

2.5. Mismatch repair (MMR) status

The 1420 CRCs were stratified as follows according to DNA MMR status: (1) MMR-proficient tumours expressing MLH1, MSH2 and MSH6, (2) sporadic MMR deficient tumours lacking only MLH1 expression, and (3) presumed HNPCC cases demonstrating loss of MSH2 and/or MSH6 at any age, or loss of MLH1 at less than 55 years.[17] Due to their small numbers, presumed HNPCC cases were excluded from this study.

2.6. Statistical analysis

2.6.1. Receiver operating characteristic (ROC) curves

Clinico-pathologically relevant cutoffs for each protein being evaluated were determined using receiver operating characteristic (ROC) curves.[18] This allowed for the determination of the percent score with the highest specificity and sensitivity for discrimination of tumours with and without the clinical outcome under investigation.

In order to apply ROC curve analysis, the clinico-pathological features were dichotomised: T stage (early = T1 + T2, late = T3 + T4), N stage (absence of lymph node metastasis = N0, presence of lymph node metastasis = N0), tumour grade (low = G1 + G2, high = G3), vascular invasion (absence, presence), budding (absence, presence) and survival (below median, above median). The median survival time was determined to be 72 months.

The ROC curve for each feature was produced. The points on the curve correspond to the (1 – specificity) and sensitivity at each immunohistochemistry score assessed as a percent-

age of positive tumour cells. From the ROC curve, the optimal cutoff was chosen when both sensitivity and specificity were maximised. The reliability of the cutoff was determined using 100-bootstrap replications of the data.[19]

2.6.2. *Other statistical analyses*

Using the clinically important cutoffs determined by ROC curve analysis, scores for each protein were dichotomised. Subsequent univariate and multivariate binary logistic regressions using these scores were carried out within each MMR group in order to determine each protein's predictive value in the classification of CRCs as TIL positive or TIL negative. Mann Whitney testing was used to evaluate differences in protein expression between MMR groups and the χ^2-test was used to investigate differences in clinico-pathological parameters. Univariate disease specific survival analysis was carried out using the Kaplan–Meier method and log-rank test. Multivariate disease specific survival analysis was carried out using Cox proportional hazards regression.

Statistical analyses were carried out using either SAS (Version 9, The SAS Institute, Cary, NC, USA) or MiniTab (Version 13.1, MiniTab Inc., State College, PA, USA). Two tailed *p*-values less than 0.05 were considered significant.

3. Results

3.1. *Clinico-pathological data*

Of the 1420 CRC punches contained on the TMAs, 1197 (84.2%) were classified as MMR proficient, 141 (9.9%) as sporadic MMR deficient and 82 (5.7%) as presumed HNPCC. Rectal tumours accounted for 37.7% and 35.5% of MMR proficient and deficient cases, respectively. Sporadic MMR deficient CRCs were associated with lower T stage ($p < 0.001$) and lower grade ($p < 0.001$) but not with lower N stage, absence of vascular invasion, absence of budding or increased survival over their MMR proficient counterparts. Five and 10 year survival rates were, respectively, 55% and 38% for the MMR proficient cases and 60% and 51% for the MMR deficient cases. Survival rates were not significantly different between these two groups at either time point.

Significantly higher expression of E-cadherin ($p = 0.014$), TGF-βRII ($p = 0.013$) and Ki-67 ($p = 0.018$) was found in MMR proficient compared to sporadic MMR deficient cases. As expected, the latter contained a significantly higher mean number of TILs (8.2 *versus* 15.5, $p = 0.008$) than the former. Differences were not found in the expression of phospho-Smad2, TGF-β, or Smad4 between these two CRC subgroups.

3.2. *ROC curve analysis*

With respect to CD8 positivity, ROC curve analysis demonstrated that the most significant cutoff for all clinico-pathological parameters was 'greater than 3' TILs. This held true for both the MMR proficient and deficient CRC groups. In order to evaluate whether differential effects were seen at different levels of TIL infiltration, the cutoffs 'greater than 9' and 'greater than 24' were also investigated.

3.3. *χ^2 and survival analysis*

As indicated in Tables 1 and 2, χ^2 analysis revealed that the presence of TILs was much more clinically significant for MMR proficient CRCs than their MMR deficient counterparts.

Table 1 – χ^2 analysis of clinico-pathological parameters in MMR proficient cases by TIL cutoff

	Cutoff > 3			Cutoff > 9			Cutoff > 24		
	Negative N (%)	Positive N (%)	*p*-Value	Negative N (%)	Positive N (%)	*p*-Value	Negative N (%)	Positive N (%)	*p*-Value
T stage									
T1	20 (3.3)	40 (7.2)	0.002*	37 (4.4)	23 (7.1)	0.001*	48 (4.9)	12 (6.7)	0.165
T2	88 (14.4)	101 (18.2)		118 (14.0)	71 (22.1)		154 (15.6)	35 (19.7)	
T3	399 (65.4)	341 (61.4)		555 (65.8)	185 (57.5)		640 (64.8)	100 (56.2)	
T4	103 (16.9)	73 (13.2)		133 (15.8)	43 (13.4)		145 (14.7)	31 (17.4)	
N stage									
N0	273 (45.9)	314 (57.4)	<0.001*	391 (47.5)	196 (61.6)	<0.001*	484 (50.1)	103 (58.9)	0.065
N1	185 (31.1)	123 (22.5)		245 (29.7)	63 (19.8)		272 (28.1)	36 (20.6)	
N2	137 (23.0)	110 (20.1)		188 (22.8)	59 (18.6)		211 (21.8)	36 (20.6)	
Grade									
G1	12 (2.0)	15 (2.7)	0.656	17 (2.0)	10 (3.1)	0.130	22 (2.2)	5 (2.8)	0.209
G2	532 (87.4)	478 (86.0)		742 (87.9)	268 (83.5)		863 (87.4)	147 (82.6)	
G3	65 (10.7)	63 (11.3)		85 (10.1)	43 (13.4)		102 (10.3)	26 (14.6)	
Vascular invasion									
Absence	412 (67.5)	422 (75.9)	0.002*	581 (68.8)	253 (78.8)	0.001*	697 (70.6)	137 (77.0)	0.081
Presence	198 (32.5)	134 (24.1)		264 (31.2)	68 (21.2)		291 (29.5)	41 (23.0)	
Budding									
Absence	194 (31.9)	236 (42.5)	<0.001*	278 (33.0)	152 (47.4)	<0.001*	346 (35.1)	84 (47.2)	0.002*
Presence	414 (68.1)	320 (57.6)		565 (67.0)	169 (52.7)		640 (64.9)	94 (52.8)	

Significant differences are highlighted by an asterisk (*)

Table 2 – χ^2 analysis of clinico-pathological parameters in sporadic MMR deficient cases by TIL cutoff

	Cutoff > 3			Cutoff > 9			Cutoff > 24		
	Negative N (%)	Positive N (%)	p-Value	Negative N (%)	Positive N (%)	p-Value	Negative N (%)	Positive N (%)	p-Value
T stage									
T1	0 (0)	0 (0)	0.562	0 (0)	0 (0)	0.869	0 (0)	0 (0)	0.160
T2	3 (4.4)	3 (4.2)		4 (4.4)	2 (4.2)		6 (5.6)	0 (0)	
T3	45 (65.2)	52 (73.2)		65 (70.7)	32 (66.7)		76 (71.0)	21 (63.6)	
T4	21 (30.4)	16 (22.5)		23 (25.0)	14 (29.2)		25 (23.4)	12 (36.4)	
N stage									
N0	29 (42.0)	50 (71.4)	0.002*	45 (48.9)	34 (72.3)	0.014*	55 (51.4)	24 (75.0)	0.001*
N1	21 (30.4)	8 (11.4)		25 (27.2)	4 (8.5)		26 (24.3)	3 (9.4)	
N2	19 (27.5)	12 (17.4)		22 (23.9)	9 (19.1)		26 (24.3)	5 (15.6)	
Grade									
G1	1 (1.4)	1 (1.5)	0.104	1 (1.1)	1 (2.2)	0.249	2 (1.9)	0 (0)	0.105
G2	56 (80.0)	47 (68.1)		72 (77.4)	31 (67.4)		83 (76.9)	20 (64.5)	
G3	13 (18.6)	21 (30.4)		20 (21.5)	14 (30.4)		23 (21.3)	11 (35.5)	
Vascular invasion									
Absence	54 (77.1)	50 (73.5)	0.622	70 (76.1)	34 (73.9)	0.780	83 (77.6)	21 (67.7)	0.263
Presence	16 (22.7)	18 (26.5)		22 (23.9)	12 (26.1)		24 (22.4)	10 (32.3)	
Budding									
Absence	17 (24.3)	32 (46.4)	0.006*	27 (29.0)	22 (47.8)	0.029*	34 (31.5)	15 (48.4)	0.082
Presence	53 (75.7)	37 (53.6)		66 (71.0)	24 (52.2)		74 (68.5)	16 (51.6)	

Significant differences are highlighted by an asterisk (*).

This effect was pronounced at low levels of TIL infiltration and appeared to reach a plateau after which additional lymphocytic infiltration was no longer clinically beneficial. These findings are supported by the univariate Kaplan–Meier survival curves in Fig. 1, which demonstrate a significant disease specific survival advantage at all cutoffs for patients with MMR proficient tumours containing TILs but no survival advantage for MMR deficient cancers. No significant survival difference was found between MMR proficient and deficient tumours containing greater than 3 TILs, which is likely accounted for by the survival advantage conferred by MMR deficiency on its own (data not shown). The importance of TILs in MMR proficient CRCs is further highlighted by the findings of a multivariate analysis. Cox proportional hazards regression revealed that the presence of greater than 3 TILs was significantly associated with improved disease specific survival ($p = 0.0002$; HR (95% confidence interval (CI)) = 0.699 (0.58–0.84)) independently of T stage, N stage, tumour grade, vascu-

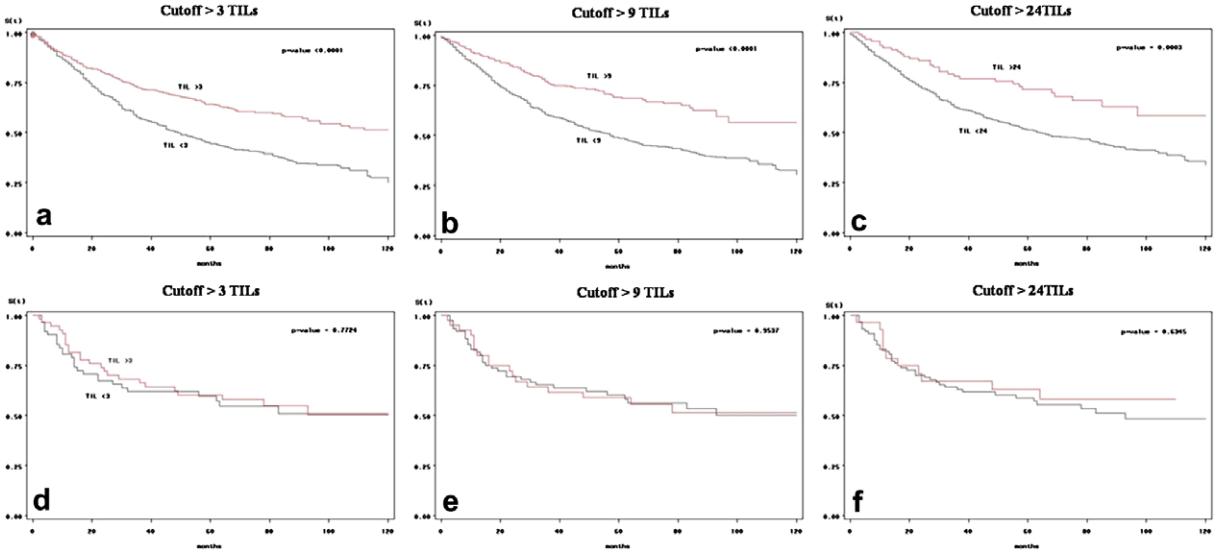

Fig. 1 – Kaplan–Meier disease specific survival curves at different TIL cutoffs for MMR proficient (a, b, c) and MMR deficient (d, e, f) CRCs.

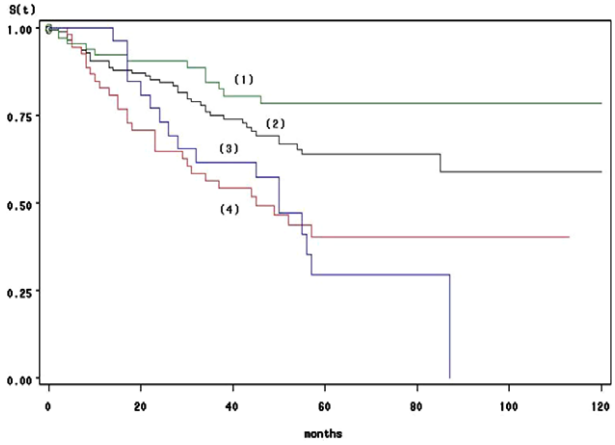

Fig. 2 – Kaplan–Meier survival curves for patients having greater than or less than 3 TILs who underwent only surgery or post-operative radio- and/or chemotherapy. *p*-Value <0.001 between TIL positive and negative cases for each treatment condition. (1) >3 TILs, surgery alone, (2) ⩽3 TILs, surgery alone, (3) >3 TILs, adjuvant therapy, (4) ⩽3 TILs, adjuvant therapy.

lar invasion and age in MMR proficient CRCs (data not shown). TIL positivity, however, was not found to have independent prognostic value in MMR deficient CRCs ($p = 0.1898$).

Since survival is affected by treatment, Kaplan–Meier survival analysis was carried out separately according to TIL status on the group of MMR proficient cancers for which the course of treatment was known. As illustrated in Fig. 2, the presence of greater than 3 TILs was significantly associated with better disease specific survival in patients having undergone surgery alone as well as in those treated with either post-operative radiotherapy, chemotherapy, or a combination of the two ($p < 0.001$).

3.4. *Logistic regression analysis*

Univariate logistic regression revealed increased Ki-67 and Smad4 to be universal predictors of high TIL infiltration (Table 3). Additionally, decreased phospho-Smad2 was associated with elevated TIL infiltration in MMR proficient cancers and increased TGF-β secretion was linked to high TIL status in MMR deficient CRCs. Multivariate analysis confirmed the importance of Ki-67 as a universal and independent indicator of elevated T cell infiltration, whereas increased Smad4 was only significant in MMR proficient cancers (Table 4). Interestingly, adjustment for the level of TGF-βRII within MMR proficient tumours rendered increased TGF-β secretion an independent predictor of clinically important TIL levels in MMR proficient CRCs.

4. Discussion

Anti-tumour immune responses may be one of the most important weapons in the arsenal against cancer. The present study confirms that intraepithelial TILs are indeed an important clinical and prognostic indicator in CRC, but suggests that these benefits may be confined mainly to the MMR proficient subset. Furthermore, the results support and expand upon those from our previous study which, using an entirely different scoring system, implicated disruptions to TGF-β signalling as one mechanism responsible for increased TIL pres-

Table 3 – Univariate logistic regression analysis for different TIL cutoffs

Marker (cutoff)	TIL cutoff	MMR proficient cases			Sporadic MMR deficient cases		
		p-Value	OR[a]	95% CI[b]	*p*-Value	OR	95% CI
E-cadherin (95%)	>3 TILs	0.847			0.494		
	>9 TILs	0.432			0.014*[c]	0.38	0.18–0.82
	>24 TILs	0.933			0.509		
Phospho-Smad2 (60%)	>3 TILs	0.005*	0.69	0.54–0.89	0.297		
	>9 TILs	0.047*	0.74	0.55–1.00	0.214		
	>24 TILs	0.081			0.466		
TGF-β (15%)	>3 TILs	0.467			0.215		
	>9 TILs	0.690			0.048*	2.19	1.01–4.75
	>24 TILs	0.943			0.014*	3.30	1.28–8.55
TGF-βRII (95%)	>3 TILs	0.904			0.277		
	>9 TILs	0.998			0.785		
	>24 TILs	0.507			0.521		
Ki67 (10%)	>3 TILs	<0.001*	1.94	1.48–2.54	<0.001*	9.23	4.04–21.10
	>9 TILs	0.014*	1.50	1.08–2.07	<0.001*	9.17	3.43–24.50
	>24 TILs	0.005*	2.06	1.24–3.42	0.001*	7.12	2.26–22.44
Smad4 (0%)	>3 TILs	<0.001*	1.76	1.35–2.30	0.025*	2.38	1.12–5.05
	>9 TILs	0.001*	1.71	1.25–2.35	0.013*	2.83	1.25–6.42
	>24 TILs	0.003*	2.10	1.30–3.39	0.009*	3.64	1.37–9.67

a OR, odds ratio. ORs are only given for significant variables.
b CI, confidence interval.
c Significant associations are highlighted by an asterisk (*).

Table 4 – Multivariate logistic regression analysis for different TIL cutoffs

	Cutoff (%)	MMR proficient			Sporadic MMR deficient		
		p-Value	OR[a]	95% CI[b]	*p*-Value	OR	95% CI
>3 TILs							
E-cadherin	95	0.316			0.201		
Phospho-Smad2	60	0.114			0.847		
TGF-β	15	0.048*[c]	1.49	1.06–2.86	0.550		
TGF-βRII	95	0.438			0.389		
Ki67	10	<0.001*	2.10	1.55–2.85	<0.001*	10.99	4.20–28.71
Smad4	0	0.001*	1.66	1.23–2.23	0.296		
>9 TILs							
E-cadherin	95	0.992			0.001*	0.09	0.02–0.35
Phospho-Smad2	60	0.103			0.435		
TGF-β	15	0.611			0.038*	3.29	1.07–10.13
TGF-βRII	95	0.724			0.590		
Ki67	10	0.061			<0.001*	31.40	6.54–150.87
Smad4	0	0.006*	1.66	1.16–2.37	0.093		
>24 TILs							
E-cadherin	95	0.425			0.126		
Phospho-Smad2	60	0.580			0.395		
TGF-β	15	0.636			0.008*	4.81	1.49–15.49
TGF-βRII	95	0.786			0.811		
Ki67	10	0.035*	1.84	1.04–3.22	0.002*	8.03	2.13–30.31
Smad4	0	0.005*	2.15	1.26–3.67	0.036*	3.47	1.09–11.11

a OR, odds ratio. ORs are only given for significant variables.
b CI, confidence interval.
c Significant associations are highlighted by an asterisk (*).

ence.[13] Univariate logistic regression in the present study identified increased Smad4 and Ki-67 as universal predictors of elevated TILs. Additionally, increased TGF-β secretion emerged as a predictor of TILs in MMR deficient CRCs. The universal predictive value of Ki-67 was maintained in multivariate analysis, while Smad4 only retained independent significance in MMR proficient cases. Interestingly, adjustment for the expression of TGF-βRII rendered increased TGF-β an independent TIL predictor in MMR proficient CRCs, thereby supporting the notion that it is the combination of increased TGF-β secretion and decreased tumour TGF-β sensitivity that is linked to elevated TIL presence.

Evidence remains inconclusive over the exact role played by TILs in predicting CRC prognosis.[2,6] It is likely that much of this controversy arises from inconsistent definition of the term TILs, some studies including and some excluding stromal lymphocytes in this category, as well as from the lack of segregation of cases along MMR lines. Two recent studies using the same large tumour population determined that TILs were associated with the absence of metastases and prolonged survival but did not state how TILs were defined nor did they stratify their samples according to MMR status.[20,21] The explicit disclosure of both of these details in future studies is likely to clarify the role played by TILs within different tumour compartments and among different tumour subtypes.

The present results clearly demonstrate that intraepithelial TILs are an important predictor of favourable clinico-pathological parameters and prognosis in MMR proficient CRCs. These cancers typically have very little lymphocytic infiltration, yet even the presence of low numbers of intraepithelial TILs is a positive sign. In contrast, MMR deficient cancers are typically characterised by high intraepithelial TIL levels and this was not found to be associated with a more favourable prognosis within this subset. In terms of clinico-pathological parameters, only N stage and tumour budding were associated with TIL infiltration in these cancers and the N stage effect disappeared at higher TIL levels. Several scenarios may explain the lack of association found between TILs and the outcome in MMR deficient CRCs. TIL distribution within a tumour may be focal and, as such, the availability of only a single punch from each tumour may have led to an underestimation of the true extent of TIL infiltration in these tumours. Such an effect would be magnified in MMR deficient tumours due to their smaller numbers. Furthermore, activation status of the TILs was not examined and this may have led to an underestimation of the number of cytotoxically active TILs.[2,7]

Alternatively, it is possible that the function of TILs differs between the two cancer subgroups. The normally high prevalence of TILs in MMR deficient cancers suggests that this feature may be inherent in the biology of the tumour. The innateness of TILs to MMR deficient cancers is consistent with the theory of tumour immuno-editing, which dictates that tumours reaching the stage of clinical detection have been antigenically shaped by the initial immune responses mounted against them to a point where they are no longer recognised as foreign to the body.[22] In MMR proficient cancers, which arise in a much more immunologically sparse environment, the tumours are less likely to have antigenically adapted and may thus be more sensitive to late stage immune attacks. Thus, even though MMR deficient cancers are ex-

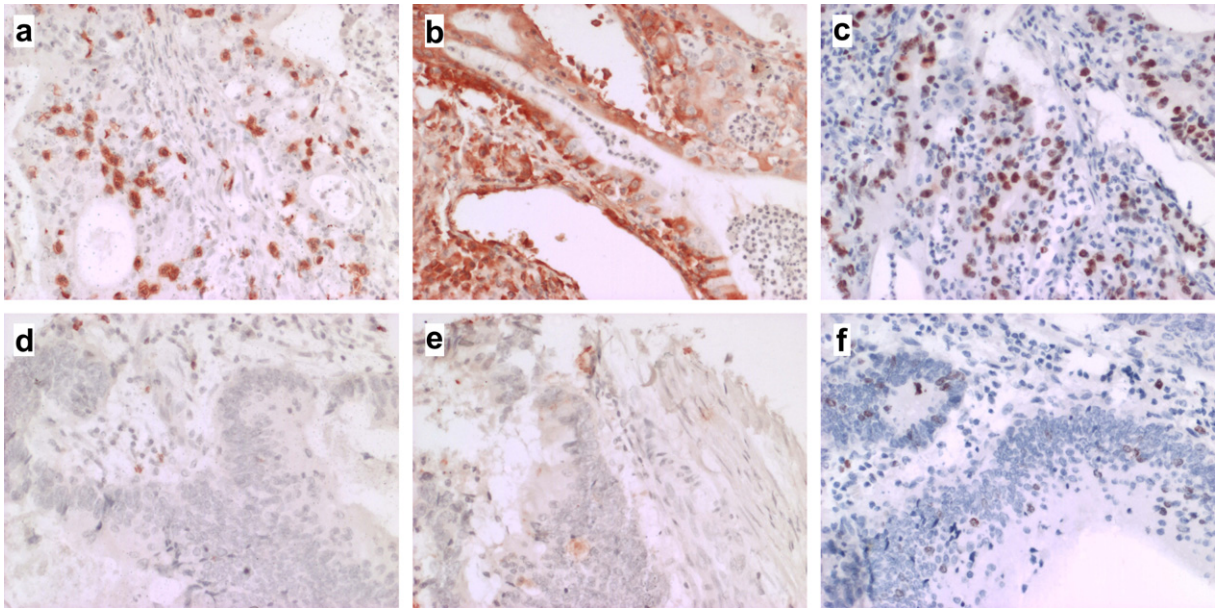

Fig. 3 – Representative immunohistochemical staining of one TIL high (a, b, c) and one TIL low (d, e, f) MMR proficient CRC. Comparative expressions of TILs (a and d), TGF-β (b and e) and Ki-67 (c and f) are shown. Original magnification 20×.

pected to produce a greater number of immunologically stimulating tumour specific antigens, the elevated levels of these coupled with the lack of appropriate costimulatory molecules on the tumour cells may generate a microenvironment which leads to a state of TIL anergy, thereby preempting any beneficial effect on survival.[8,9] Such an effect would be a local one and so would not contradict previous findings of peripheral blood derived T cell responses to frame-shift mutated peptides.[8]

The question thus arises as to why MMR deficient CRCs have such large levels of TIL infiltration if these cells are not immunologically active. Findings from the present study provide a potential explanation. The majority of MMR deficient CRCs possess inactivating mutations in TGF-βRII that render them insensitive to signalling by TGF-β.[1,10–12] A major negative autoregulatory feedback pathway is thus inactivated such that TGF-β no longer negatively regulates its own synthesis.[23,24] Univariate analysis revealed that increased TGF-β secretion was an important predictor of high TIL infiltration in MMR deficient CRCs. The role of TGF-β in TIL presence may be mediated by its ability to upregulate CD103, an E-cadherin binding adhesion molecule found almost exclusively on intraepithelial mucosal CD8+ T cells and which has been found in 27-fold excess in MMR deficient CRCs.[3,25,26] The lack of a formalin-compatible CD103 antibody precluded further study of this pathway with the available material although E-cadherin proved to be independently predictive of moderate TIL status in MMR deficient CRCs. Multivariate analysis in MMR proficient CRCs further validates the proposed hypothesis since adjustment for the effect of decreased TGF-βRII rendered increased TGF-β, a significant predictor of high TIL infiltration in MMR proficient CRCs (Table 4). This pattern mimics that found in MMR deficient cancers and suggests that it is a combination of increased TGF-β production and decreased TGF-β sensitivity that is crucial to TIL accumulation. The importance of TGF-β insensitivity is highlighted by the finding that increased proliferation and Smad4 expression, both of which are decreased by TGF-β, emerged as universal predictors of intraepithelial lymphocytic infiltration (Fig. 3).[27,28] While the need for decreased TGF-β sensitivity is not clear, it may implicate the need for a reduction in TGF-β-induced expression of other adhesion molecules on the CRC cells which would, in turn, facilitate interaction between CD103 and E-cadherin.[29] Since TGF-β is known to promote cancer progression in late stage disease, it is possible that the more favourable prognosis associated with MMR deficiency is linked to an insensitivity to these oncogenic effects.[30]

Findings from the present study validate the notion that CRC infiltration by intraepithelial TILs is a favourable clinico-pathological and prognostic indicator but suggest for the first time that this effect is limited mainly to MMR proficient cancers. Additionally, they implicate the combination of tumour TGF-β insensitivity with increased TGF-β secretion as an important component of high level TIL infiltration in both MMR proficient and MMR deficient CRCs. While this may be an inherent biological property of most MMR deficient tumours, this pathway is likely to be involved predominantly in the initial retention of TILs in MMR proficient cancers before further immune stimulation induces an anti-tumour immune response.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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