



Tumour infiltrating lymphocyte status is superior to histological grade, DNA mismatch repair and *BRAF* mutation for prognosis of colorectal adenocarcinomas with mucinous differentiation

David S. Williams^{1,2,3} · Dmitri Mouradov^{4,5} · Marsali R. Newman¹ · Elham Amini⁶ · David K. Nickless⁷ · Catherine G. Fang² · Michelle Palmieri^{4,5} · Anuratha Sakthianandeswaren^{4,5} · Shan Li⁴ · Robyn L. Ward^{8,9} · Nicholas J. Hawkins^{10,11} · Iain Skinner¹² · Ian Jones¹³ · Peter Gibbs^{4,5,14,15} · Oliver M. Sieber^{4,5,16,17}

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Abstract

Mucinous colorectal adenocarcinoma (CRC) is conventionally defined by extracellular mucin comprising >50% of the tumour area, while tumours with ≤50% mucin are designated as having a mucinous component. However, these definitions are largely arbitrary and comparisons of clinico-molecular features and outcomes by proportion of mucinous component are limited. A cohort of 1643 patients with stage II/III cancer was examined for tumour mucinous component, DNA mismatch repair (MMR) status, *BRAF* mutation and tumour infiltrating lymphocytes (TILs). Tumours with ≤50% mucinous component exhibited similar characteristics as mucinous tumours, including association with female gender, proximal location, high grade, TIL-high, defective MMR (dMMR) and *BRAF* mutation. Proportion of mucinous component did not stratify disease-free survival (DFS). In univariate analysis dMMR status, but not histological grade, stratified survival for mucinous and mucinous component tumours; however, in multivariate analysis dMMR status was not an independent predictor. *BRAF* mutation prognostic value depended on mucinous differentiation and MMR status, with poor prognosis limited to non-mucinous pMMR tumours (HR 2.61, 95% CI 1.69–4.03; $p < 0.001$). TIL status was a strong independent predictor of DFS in mucinous/mucinous component tumours (HR 0.40, 95% CI 0.23–0.67; $p < 0.001$), and a superior predictor of prognosis compared with histological grade, MMR and *BRAF* mutation. Mucinous component and mucinous stage II/III CRCs exhibit clinico-molecular resemblances, with histological grade and *BRAF* mutation lacking prognostic value. Prognosis for these tumours was instead strongly associated with TIL status, with the most favourable outcomes in TIL-high dMMR tumours, whilst TIL-low tumours had poor outcomes irrespective of MMR status.

Introduction

Mucinous colorectal adenocarcinoma is conventionally defined as colorectal cancer (CRC) in which more than 50% of the lesion is composed of pools of extracellular mucin [1]. Mucinous tumours exhibit variation in geographical distribution, accounting for ~5% of CRC in studies from Asian countries [2–4], and 10–20% in studies from Western countries [5–8]. Compared with non-mucinous adenocarcinoma,

mucinous tumours are more common in the proximal colon and exhibit specific molecular features including DNA mismatch repair deficiency (dMMR) [8–16], *BRAF* mutation [10, 13–16] and a high-frequency of CpG island methylator phenotype (CIMP-high) [10, 13, 16]. Mucinous CRC has further been negatively associated with p53 overexpression [12, 16–18]. The definition of mucinous CRC based on >50% mucin component, while traditional, is largely arbitrary. Limited data exist comparing the characteristics and survival of patients with mucinous tumours to those with tumours with ≤50% mucinous component [8, 11, 13, 17–22].

There are conflicting studies regarding the prognostic impact of mucinous differentiation in CRC. While some studies have reported that mucinous tumours have a worse prognosis than non-mucinous tumours [2, 6, 12, 19, 23], others have found no difference [4, 5, 7, 11, 13, 24]. The lack of consensus may be attributable to the diversity of

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✉ Oliver M. Sieber
sieber.o@wehi.edu.au

Extended author information available on the last page of the article

patients included, small cohort size because of the rarity of the mucinous subtype or limited patient follow-up. In addition, many studies have been limited by their inability to control for key molecular features of CRC, such as dMMR status and *BRAF* mutation, which are potential confounders. In early-stage CRCs, dMMR status has been associated with better survival [25, 26], while *BRAF* mutation has been associated with particularly poor survival in DNA mismatch repair proficient (pMMR) tumours [27–29]. Moreover, the prognostic significance of $\leq 50\%$ tumour mucinous component remains unclear.

Histologic grading of mucinous CRC is challenging. Conventionally, colorectal adenocarcinoma is graded based on the degree of glandular differentiation, with cases exhibiting $\leq 50\%$ gland formation being considered as high grade [1]. The applicability of this grading scheme to mucinous carcinomas is contentious. World Health Organisation (WHO) 4th Edition guidelines (2010) recommended that mucinous CRCs should instead be graded based on MMR status, with pMMR tumours considered as high grade [1]. However, evidence that mucinous tumours can be graded exclusively based on MMR status was derived from smaller studies or subgroup analyses [11, 30, 31], and the WHO 5th Edition guidelines (2019) were revised to recommend that mucinous CRCs should be graded using the same histological criteria as conventional non-mucinous CRC [32]. Limited data exist regarding the prognostic value of histological grade and MMR status for tumours with a mucinous component [5, 12–14, 19].

Host immune response against tumour has emerged as a strong prognostic predictor for CRC. High density of tumour infiltrating lymphocytes (TILs) as determined by histopathological examination has been associated with lower rates of recurrence and longer patient survival in multiple studies [33–36]. Scoring systems such as global TIL density or assessments of T-lymphocyte populations have been proposed as prognostic classifiers [36–39]. Recent data suggest that combined consideration of TIL/MMR status provides a refined prognostic stratification for patients with stage II/III CRC [36, 39–41]. Specifically, TIL status appears to stratify both dMMR and pMMR tumours by outcome, with TIL-high tumours exhibiting significantly better outcomes. Whether TIL status refines prognostication for tumours with mucinous differentiation has not been evaluated.

The purpose of the present study was to evaluate the clinico-molecular features and oncological outcomes of CRC according to the proportion of mucinous component in a community-based cohort of 1643 patients with stage II/III disease. In particular, we evaluated the prognostic value of tumour classification by mucinous component and examined the changing WHO recommendations regarding whether mucinous CRCs should be graded on the basis of either dMMR status or glandular morphology. We explored

prognostic interactions between clinico-molecular variables and tumour mucinous component and investigated the prognostic value of TIL status.

Materials and methods

Study population

A total of 1643 patients with resected stage II or III CRC were recruited at the Royal Melbourne Hospital, Western Hospital Footscray, Austin Health and St Vincent's Hospital Sydney between 1999 and 2015. Signet-ring carcinoma or cases that had received neoadjuvant chemoradiation were excluded, as were individuals with hereditary CRC syndromes. Formalin-fixed paraffin-embedded tumour and matched normal tissue specimens were obtained at surgery. Patients were followed according to standard protocols (see Supplementary Methods). Clinicopathologic treatment and outcome data were either collected prospectively (63.5%, 1044 of 1643 patients) or retrospectively assembled from medical records (36.5%, 599 of 1643 patients). The tumour location was defined as proximal (caecum to splenic flexure) and distal (splenic flexure to rectum). Patient characteristics are summarised in Supplementary Table S1. All participants gave informed consent, and this study was approved by the medical ethics committees of all sites (WEHI HREC 12/19, Austin HREC 2013/05077).

Histopathological evaluation

A central pathology review was performed for all cases based on haematoxylin and eosin (H&E)-stained tissue sections by a gastrointestinal pathologist (DSW) unaware of other clinical and molecular data. All original H&E slides of the primary tumours (≥ 3 sections per case) were available in 880 cases and 1 tumour slide was available for the remaining 763 cases. Mucinous differentiation was scored as present for either mucinous carcinoma ($>50\%$ extracellular mucin) or CRC with a mucinous component (1–50% mucin) (Supplementary Figure S1). A subset of 325 cases with multiple slides were reviewed by a second pathologist (EA or MN). Discordant cases were resolved by a further two pathologists (MN or EA and DKN). The concordance for assessing proportion of mucinous component (trichotomized as 0 vs. 1–50 vs. $>50\%$) was good (DSW vs. MN linear weighted kappa 0.80, 95% CI 0.73–0.87, DSW vs. EA linear weighted kappa 0.73, 95% CI 0.68–0.79). Tumour grade was categorised as high or low based upon WHO grading ($\leq 50\%$ vs. $>50\%$ glandular area) (Supplementary Figure S2, see Supplementary Methods).

Density of TILs for these tumours as scored using H&E-stained tissue sections has been reported previously [36].

Five consecutive random 40× fields of a BX51 microscope with 10× objective (Olympus) were counted for each H&E-stained tissue section, sampling representative different regions of each tumour (field diameter 0.55 mm, total area of five high powered fields (hpf) 1.188 mm²). For paucicellular tumours (e.g. low-grade mucinous adenocarcinomas comprising thin strips of epithelium at the periphery of the mucin pools), scoring was performed on tumour-enriched fields with the highest tumour cell density. Regions of precursor adenoma or necrosis were excluded from analysis, and only intraepithelial lymphocytes were counted, excluding those within the intervening stroma. The mean TIL per hpf for each tumour was calculated by dividing the total number of TILs by five. Tumours were classified into TIL-low (<2 TILs per hpf) and TIL-high (≥2 TILs per hpf) cases, according to a pre-determined cut-off value distinguishing dMMR and pMMR cases [36]. For tumours near the cut-point showing a heterogeneous distribution of TILs, scoring was commenced in a hot-spot region identified during low power screening, followed by random fields.

DNA mismatch repair status

MMR status for these tumours has been reported previously [36], assessed by either the Bethesda consensus panel of microsatellite markers or immunohistochemistry (IHC) (see Supplementary Methods).

BRAF mutation detection

BRAF (V600E) mutation status for these tumours has been reported previously [36], assessed either by Sanger sequencing ($n = 1044$) or by IHC ($n = 599$) (see Supplementary Methods). We have previously demonstrated high concordance of *BRAF* V600E IHC assessment with Sanger sequencing in a cohort of 477 CRCs, with sensitivity and specificity of 98.2% and 98.1%, respectively [42].

Statistical methods

Statistical analyses were conducted using the statistical computing software R (R Development Core Team, 2011). Interobserver reproducibility for determination of mucinous component between reviewers was assessed using Cohen's kappa statistic. For univariate analyses, differences between groups were assessed using the Fisher's exact test for categorical variables and the Student's *t* test for continuous variables. Outcome analyses were conducted for 5-year disease-free survival (DFS). DFS was defined as time from surgery to the first confirmed relapse, with censoring done when a patient died or was alive without recurrence at last contact. Survival

curves were generated according to the method of Kaplan and Meier. Cox proportional hazard models were used to assess the associations of tumour mucinous component with DFS in the context of patient clinico-molecular features and adjuvant treatment. Hazard ratios and 95% confidence intervals were calculated. Comparisons of Cox proportional hazard models with TIL status against models with tumour grade, MMR or *BRAF* mutation were made using the Akaike Information Criterion (AIC), a measure of how well a model fits a dataset, with a smaller AIC value indicating the better model. As described by Burnham and Anderson [43], an AIC difference >10, indicates that there is essentially no support for the model with the larger AIC of being as good a fit as the model with the lower AIC. The likelihood-ratio test, which assesses the goodness of fit of two competing models, was used to determine whether addition of TIL status improved models with tumour grade, MMR or *BRAF* status or vice versa. All statistical analyses were two-sided and considered significant if $p < 0.050$.

Results

Patient characteristics

Of the 1643 stage II/III patients, 51.6% ($n = 847$) were female, and the median age was 72 (range 22–99) years (Supplementary Table S1). 53.6% (879 of 1641) of cancers were from the proximal colon and 28.4% (467 of 1643) exhibited high grade. All patients underwent surgical resection with 42.3% (636 of 1503) receiving adjuvant chemotherapy. 22.1% (363 of 1643) of individuals experienced a disease recurrence. The median follow-up duration was 54 months.

Tumours were grouped based on mucinous component of 0%, 1–50%, and >50% into non-mucinous adenocarcinomas, adenocarcinomas with a mucinous component and mucinous adenocarcinomas, respectively. Tumour samples were analysed for MMR status and *BRAF* (V600E) mutations. 68.4% (1123 of 1643) of cancers were classified as non-mucinous, 12.2% (200 of 1643) as having a mucinous component, and 19.5% (320 of 1643) as mucinous. 26.2% (421 of 1604) of cancers were dMMR and 20.1% (321 of 1594) harboured a *BRAF* mutation. Tumour dMMR status displayed the anticipated strong direct association with *BRAF* mutation (OR = 15.3).

Relationship of tumour mucinous component with clinicopathologic and molecular characteristics

To evaluate whether tumours with a mucinous component should be clinically treated as being biologically

Table 1 Clinicopathologic and molecular characteristics of 1643 patients with stage II/III CRC according to tumour mucinous component.

	Mucinous differentiation			P value		
	Non-mucinous (CAC)	Mucinous component (AMC)	Mucinous (MAC)	MAC vs. CAC	AMC vs. CAC	AMC vs. MAC
Age (years)						
Mean ± SD	69.8 ± 12.2	72.0 ± 12.9	70.9 ± 12.1	0.157	0.032*	0.368
Median	71	74	73			
Range	24–99	22–96	28–94			
Gender						
Female	553 (49.2)	115 (57.5)	179 (55.9)	0.037*	0.032*	0.785
Male	570 (50.8)	85 (42.5)	141 (44.1)			
Tumour stage						
II	549 (48.9)	93 (46.5)	180 (56.2)	0.022*	0.540	0.038*
III	574 (51.1)	107 (53.5)	140 (43.8)			
Site						
Right	505 (45.0)	146 (73.0)	228 (71.5)	<0.001*	<0.001*	0.763
Left	617 (55.0)	54 (27.0)	91 (28.5)			
Grade						
Low	837 (74.5)	125 (62.5)	214 (66.9)	0.008*	<0.001*	0.344
High	286 (25.5)	75 (37.5)	106 (33.1)			
MMR status						
pMMR	915 (83.6)	100 (50.3)	168 (54.2)	<0.001*	<0.001*	0.413
dMMR	180 (16.4)	99 (49.7)	142 (45.8)			
<i>BRAF</i> status						
Wild-type	953 (87.5)	126 (64.0)	194 (63.0)	<0.001*	<0.001*	0.850
Mutated	136 (12.5)	71 (36.0)	114 (37.0)			

Percentages for columns are shown in round brackets.

CAC conventional adenocarcinoma, AMC adenocarcinomas with a mucinous component, MAC mucinous adenocarcinoma, dMMR DNA mismatch repair deficient, pMMR DNA mismatch repair proficient.

* $p < 0.05$.

distinct from conventional non-mucinous adenocarcinomas, we examined the relationship of mucinous differentiation with patient clinicopathologic and tumour molecular characteristics.

Tumours with a mucinous component tended to exhibit similar characteristics as mucinous carcinomas (Table 1). Compared with conventional non-mucinous adenocarcinomas, both mucinous tumours and tumours with a mucinous component were significantly associated with female gender, proximal location, high grade, dMMR, and *BRAF* mutation ($p \leq 0.037$ for all comparisons). Patients with mucinous tumours and tumours with a mucinous component appeared marginally older than patients with non-mucinous tumours. The only differentiating characteristic between mucinous tumours and tumours with a mucinous component was tumour stage, with the former associated with stage II disease ($p = 0.038$); this association was also observed when comparing mucinous to non-mucinous tumours ($p = 0.022$).

Tumour mucinous component and disease-free survival

The prognostic value of tumour classification by mucinous component remains unclear. To examine the relationship of tumour mucinous component with outcome, we focused on DFS, an appropriate measure for CRC treated in the adjuvant setting [44].

Patients with non-mucinous, mucinous component and mucinous tumours had similar 5-year DFS in our cohort. In multivariate analysis adjusting for baseline clinico-molecular variables and treatment, hazard ratios for DFS for mucinous vs. non-mucinous, mucinous component vs. non-mucinous and mucinous component vs. mucinous tumours were 0.80 (95% CI 0.59–1.09; $p = 0.163$), 0.82 (95% CI 0.58–1.16; $p = 0.260$) and 1.02 (95% CI 0.67–1.54; $p = 0.929$), respectively (Table 2). Among clinico-molecular variables, tumour stage III, high grade and *BRAF* mutation were associated with reduced DFS while dMMR status and adjuvant treatment were associated

Table 2 Tumour mucinous differentiation and disease-free survival in patients with stage II/III CRC.

	Cases/ events	HR	95% CI	<i>P</i> value
1458/336				
Mucinous vs. non-mucinous		0.80	0.59–1.09	0.163
Mucinous component vs. non-mucinous		0.82	0.58–1.16	0.260
Mucin component vs. mucinous		1.02	0.67–1.54	0.929
Age (decades)		0.97	0.88–1.07	0.559
Gender (female vs. male)		0.82	0.66–1.02	0.081
Tumour stage (III vs. II)		3.55	2.66–4.74	<0.001*
Site (right vs. left)		1.03	0.81–1.31	0.810
Grade (high vs. low)		1.73	1.37–2.19	<0.001*
MMR status (dMMR vs. pMMR)		0.53	0.38–0.74	<0.001*
<i>BRAF</i> status (mutated vs. wild-type)		1.48	1.07–2.05	0.017*
Adjuvant chemotherapy (yes vs. no)		0.65	0.49–0.86	0.003*

Data are for multivariate analysis adjusted for baseline clinico-molecular variables.

HR hazard ratio, CI confidence interval, dMMR DNA mismatch repair deficient, pMMR DNA mismatch repair proficient.

* $p < 0.05$.

with improved DFS ($p \leq 0.017$ for all variables). Age at diagnosis, gender and tumour location were not associated with survival. In subgroup analyses stratified by dMMR status and tumour location, there were also no significant differences in DFS between tumours grouped by mucinous component (Supplementary Tables S2–3).

Evaluation of the WHO colorectal cancer grading system

Under the WHO 2010 guidelines it was recommended that mucinous colorectal carcinomas should be graded on the basis of MMR status [1]. Specifically, dMMR mucinous tumours should be considered as low grade, whereas pMMR tumours should be considered as high grade regardless of histological grading.

Consistent with these guidelines, we found that MMR status stratified mucinous carcinomas according to DFS in univariate analysis with 5-year DFS rates of 85.5% for dMMR and 73.3% for pMMR tumours (HR 0.52, 95% CI 0.30–0.93; $p = 0.022$; Fig. 1a), while histological grade did not stratify mucinous tumours by survival (Fig. 1d). As observed for clinico-molecular characteristics, tumours with a mucinous component again showed similar prognostic relationships as mucinous carcinomas, with 5-year DFS

rates of 84.2% for dMMR and 69.1% for pMMR tumours (HR 0.45, 95% CI 0.24–0.86; $p = 0.013$; Fig. 1b), but similar outcomes by tumour grade (Fig. 1e). In support of the WHO 2010 tumour grading recommendations, the interaction between histological grade and mucinous component (mucinous/mucinous component vs. non-mucinous tumours) was significant in multivariate analysis adjusting for clinico-molecular features and treatment ($p_{\text{interaction}} = 0.040$), while no evidence for interaction was observed between MMR status and mucinous component ($p_{\text{interaction}} = 0.448$). However, in multivariate analyses of mucinous and non-mucinous tumours, dMMR status was not an independent prognostic predictor (Table 3A–C). This latter result is consistent with the recommendation in the WHO 2019 guidelines [32], but our findings do not support the suggestion that conventional histological grading should also be applied to mucinous tumours. In contrast, both tumour grade (HR 1.91, 95% CI 1.44–2.53; $p < 0.001$) and MMR status (HR 0.49, 95% CI 0.31–0.76; $p = 0.001$) were significant independent prognostic variables in non-mucinous tumours in multivariate analysis (Fig. 1c, f, Table 3D).

Prognostic interactions of clinico-molecular variables with tumour mucinous component

Given the evidence for differential prognostic impact of tumour grade by mucinous component, we examined whether such an interaction might also exist for other clinico-molecular variables.

In univariate analyses for age at diagnosis, gender, tumour stage, location and *BRAF* mutation, a significant interaction was evident for *BRAF* mutation when comparing mucinous vs. non-mucinous and mucinous component vs. non-mucinous tumours ($p_{\text{interaction}} \leq 0.017$ for both comparisons). The 5-year DFS rates among mucinous and mucinous component tumours were 83.8% and 80.9% for *BRAF* mutated and 75.9% and 73.9% for *BRAF* wild-type tumours, respectively (Fig. 2a, b), while among non-mucinous tumours DFS rates were 62.3% for *BRAF* mutated and 74.2% for *BRAF* wild-type tumours (Fig. 2c). Accordingly, *BRAF* mutation was not associated with outcome in multivariate analyses for mucinous and mucinous component tumours (mucinous: HR 0.82, 95% CI 0.37–1.83; $p = 0.632$; mucinous component: HR 0.95, 95% CI 0.44–2.05; $p = 0.889$; Table 3A–B), but was an independent predictor of poor prognosis in non-mucinous tumours (HR 1.93, 95% CI 1.30–2.88; $p = 0.001$; Table 3D). In multivariate analysis adjusted for clinico-molecular variables comparing mucinous/mucinous component vs. non-mucinous tumours, the prognostic interaction with *BRAF* mutation remained significant ($p_{\text{interaction}} < 0.013$).

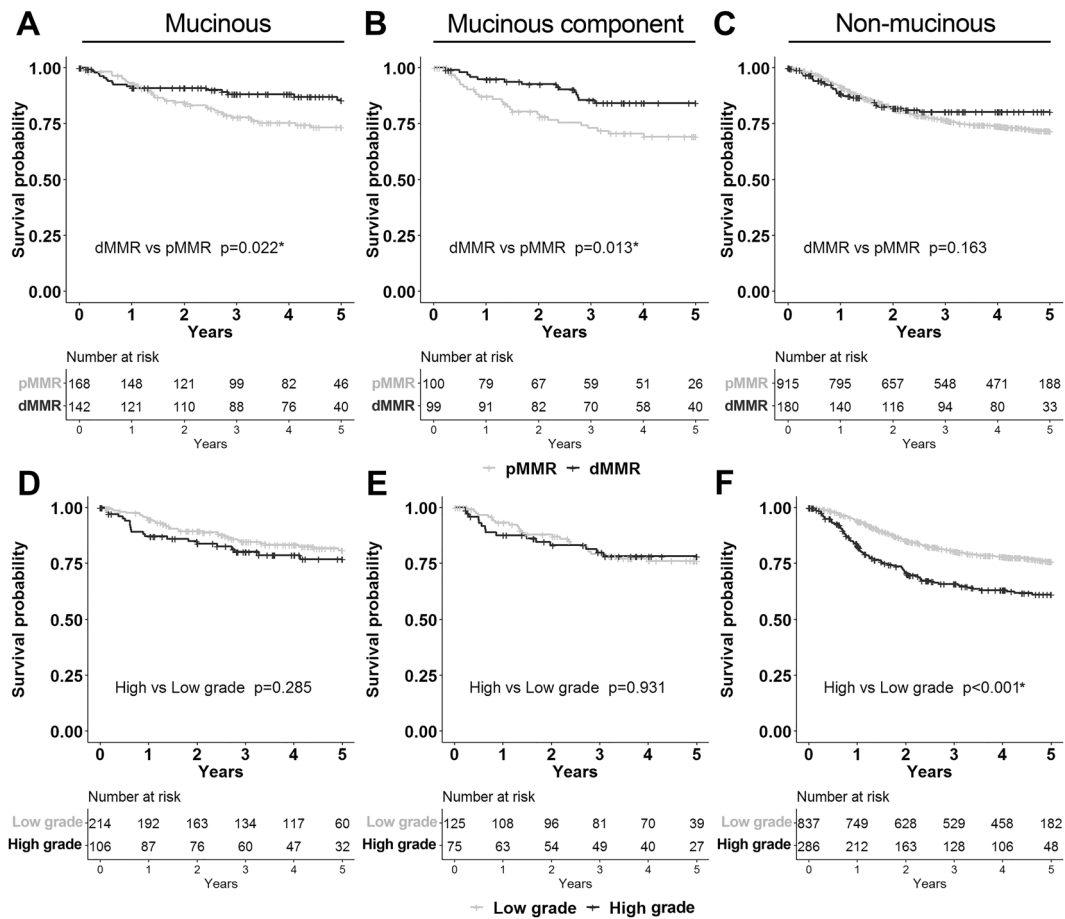


Fig. 1 Kaplan-Meier plots for disease-free survival in patients with stage II/III CRC. Patients are grouped according to tumour (a-c) MMR status and (d-f) histological grade for (a, d) individuals with mucinous tumours, (b, e) individuals with tumours with a

mucinous component, (c, f) individuals with non-mucinous tumours. dMMR DNA mismatch repair deficient, pMMR DNA mismatch repair proficient; $*p < 0.050$.

Considering *BRAF* prognostic value by tumour MMR status, *BRAF* mutation was associated with the anticipated inferior outcomes for pMMR but not dMMR cases among non-mucinous tumours in multivariate analysis (pMMR: HR 2.61, 95% CI 1.69–4.03; $p < 0.001$; dMMR: HR 1.03, 95% CI 0.48–2.21; $p = 0.945$; Supplementary Figure S3C–D). In contrast, among mucinous/mucinous component tumours *BRAF* mutation was not associated with prognosis irrespective of tumour MMR status (pMMR: HR 1.26, 95% CI 0.65–2.45; $p = 0.489$; dMMR: HR 0.70, 95% CI 0.32–1.54; $p = 0.372$; Supplementary Figure S3A–B;).

No significant interactions for clinico-molecular variables were observed when comparing mucinous component and mucinous tumours ($p_{\text{interaction}} > 0.083$ for all comparisons).

Prognostic value of TIL status

Emerging findings indicate that consideration of TIL density provides a refined prognostic stratification for

patients with stage II/III CRC irrespective of MMR status [36, 39–41].

In our cohort, TIL status data were available for 1636 patients as described previously [36], with 31.9% (522 of 1636) of cancers classified as TIL-high (≥ 2 TILs/hpf). Compared with non-mucinous tumours, both mucinous component and mucinous tumours were significantly associated with TIL-high (non-mucinous: 25.3% (283/1119), mucinous component: 51.5% (102/198), mucinous: 42.9% (137/319), $p < 0.001$ for both comparisons); this was not due to the higher rate of dMMR in mucinous/mucinous component tumours, with the association maintained when restricting the analysis to pMMR tumours ($p \leq 0.006$ for both comparisons).

Among both mucinous/mucinous component and non-mucinous tumours, TIL status stratified DFS, with TIL-high tumours exhibiting significantly better outcomes as compared with TIL-low tumours (univariate $p = 0.001$ for both comparisons; Fig. 3a, d). Five-year DFS rates among mucinous/mucinous component and non-mucinous,

Table 3 Clinico-molecular variables and disease-free survival in patients with stage II/III CRC for (A) mucinous tumours, (B) tumours with a mucinous component, (C) mucinous/mucinous component tumours and (D) non-mucinous tumours.

	Cases/ events	HR	95% CI	P value
A. Mucinous				
	259/52			
Age (decades)		0.96	0.74–1.24	0.745
Gender (female vs. male)		1.22	0.69–2.16	0.487
Tumour stage (III vs. II)		1.91	0.95–3.84	0.070
Site (right vs. left)		1.19	0.64–2.22	0.579
Grade (high vs. low)		1.27	0.70–2.31	0.425
MMR status (dMMR vs. pMMR)		0.77	0.36–1.67	0.509
<i>BRAF</i> status (mutated vs. wild-type)		0.82	0.37–1.83	0.632
Adjuvant chemotherapy (yes vs. no)		1.14	0.56–2.31	0.721
B. Mucinous component				
	187/40			
Age (decades)		1.06	0.79–1.42	0.704
Gender (female vs. male)		0.56	0.30–1.07	0.078
Tumour stage (III vs. II)		2.40	1.05–5.49	0.037*
Site (right vs. left)		1.14	0.56–2.31	0.726
Grade (high vs. low)		1.41	0.68–2.95	0.359
MMR status (dMMR vs. pMMR)		0.52	0.24–1.12	0.094
<i>BRAF</i> status (mutated vs. wild-type)		0.95	0.44–2.05	0.889
Adjuvant chemotherapy (yes vs. no)		0.80	0.34–1.89	0.616
C. Mucinous/mucinous component				
	446/92			
Age (decades)		1.02	0.85–1.23	0.833
Gender (female vs. male)		0.87	0.57–1.32	0.512
Tumour stage (III vs. II)		2.03	1.20–3.44	0.008*
Site (right vs. left)		1.20	0.76–1.91	0.440
Grade (high vs. low)		1.29	0.83–2.00	0.261
MMR status (dMMR vs. pMMR)		0.63	0.37–1.07	0.085
<i>BRAF</i> status (mutated vs. wild-type)		0.93	0.55–1.58	0.789
Adjuvant chemotherapy (yes vs. no)		1.03	0.61–1.76	0.908
D. Non-mucinous				
	1012/244			
Age (decades)		0.96	0.85–1.08	0.467
Gender (female vs. male)		0.80	0.62–1.04	0.095
Tumour stage (III vs. II)		4.42	3.12–6.27	<0.001*
Site (right vs. left)		0.94	0.71–1.24	0.649

Table 3 (continued)

	Cases/ events	HR	95% CI	P value
Grade (high vs. low)		1.91	1.44–2.53	<0.001*
MMR status (dMMR vs. pMMR)		0.49	0.31–0.76	0.001*
<i>BRAF</i> status (mutated vs. wild-type)		1.93	1.30–2.88	0.001*
Adjuvant chemotherapy (yes vs. no)		0.54	0.39–0.76	<0.001*

Data are for multivariate analysis adjusted for baseline clinico-molecular variables.

HR hazard ratio, CI confidence interval, dMMR DNA mismatch repair deficient, pMMR DNA mismatch repair proficient.

* $p < 0.05$.

tumours were 87.9% (95% CI 83.5–92.5%) and 86.2% (95% CI 81.9–90.7%) for TIL-high tumours, and 70.8% (95% CI 64.4–76.3%) and 67.7% (95% CI 64.3–71.3%) for TIL-low tumours, respectively. The prognostic value of TIL status remained when considering mucinous and mucinous component tumours separately (Supplementary Figure S4). Moreover, in multivariate analyses TIL-high remained a strong independent predictor of good prognosis in both groups (mucinous/mucinous component: HR 0.40, 95% CI 0.23–0.67; $p < 0.001$; non-mucinous: HR 0.37, 95% CI 0.24–0.56; $p < 0.001$).

Considering dMMR tumours, TIL-high status maintained its association with good prognosis among mucinous/mucinous component and non-mucinous tumours (univariate $p = 0.001$ for both comparisons; Fig. 3b, e). Similar prognostic trends were observed for pMMR tumours, with TIL status exhibiting borderline statistical significance for mucinous/mucinous component tumours (univariate $p = 0.060$; Fig. 3c) and maintaining significance for non-mucinous tumours (univariate $p < 0.001$; Fig. 3f). Five year DFS rates for mucinous/mucinous component CRCs were 90.5% (95% CI 85.7–95.6%) for TIL-high/dMMR tumours, 81.1% (95% CI 72.0–91.4%) for TIL-high/pMMR tumours, 73.1% (95% CI 62.9–84.9%) for TIL-low/dMMR tumours and 68.0% (95% CI 61.1–75.7%) for TIL-low/pMMR tumours, respectively. The univariate results for dMMR and pMMR tumours were reflected in multivariable analysis adjusting for clinico-molecular variables and treatment (Supplementary Table S4). Similarly, TIL-high status maintained its association with good prognosis among mucinous/mucinous component and non-mucinous tumours when stratified by grade (Supplementary Figure S5) or *BRAF* mutation status (Supplementary Figure S6).

In a direct comparison of the prognostic value of TIL status among mucinous/mucinous component tumours against histological grade, MMR and *BRAF* mutation status

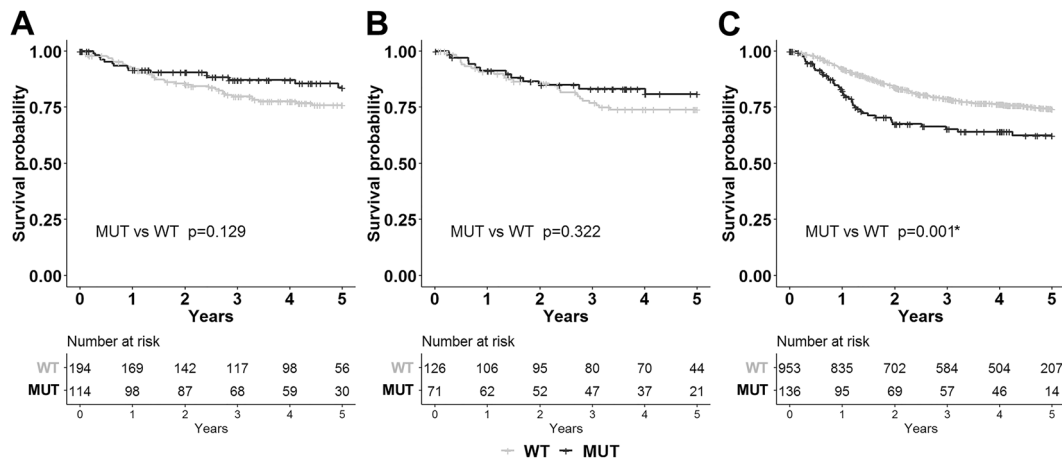


Fig. 2 Kaplan–Meier plots for disease-free survival in patients with stage II/III CRC according to BRAF mutation status. Patients are grouped into (a) individuals with mucinous tumours, (b)

individuals with tumours with a mucinous component and (c) individuals with non-mucinous tumours. WT wild-type, MUT mutated; * $p < 0.050$.

in multivariate Cox regression models using the Akaike Information Criterion (AIC) the TIL model was favoured in all cases, with AIC differences of 12.3 or greater indicating that there was essentially no support for grade, MMR and *BRAF* models being as good as the TIL model (Table 4) [43]. Corresponding results were found when using the Bayesian Information Criterion (data not shown). Accordingly, addition of TIL status significantly improved DFS models with tumour grade, MMR and *BRAF* variables ($p < 0.05$ for all comparisons, likelihood-ratio test), whereas addition of tumour grade, MMR and *BRAF* variables to models with TIL status did not significantly improve model fit (Table 5).

Discussion

Surveying a community-based cohort of 1643 patients with sporadic CRC, we found that tumours with a mucinous component exhibited similar clinico-molecular characteristics as mucinous tumours. Overall, tumour classification by mucinous differentiation did not stratify DFS in our cohort. Tumour MMR status, but not histological grade, stratified survival for mucinous tumours in univariate analysis consistent with WHO 2010 guidelines [1], and similar results were obtained for tumours with a mucinous component. However, neither tumour grade nor MMR status were independent prognostic variables for mucinous/mucinous component tumours in multivariate analyses. Our data indicate that the prognostic value of *BRAF* mutation depends upon mucinous differentiation and MMR status, with poor prognosis limited to non-mucinous pMMR tumours. Tumour classification according to TIL status was a strong independent predictor of DFS in mucinous/mucinous component tumours, superior compared with

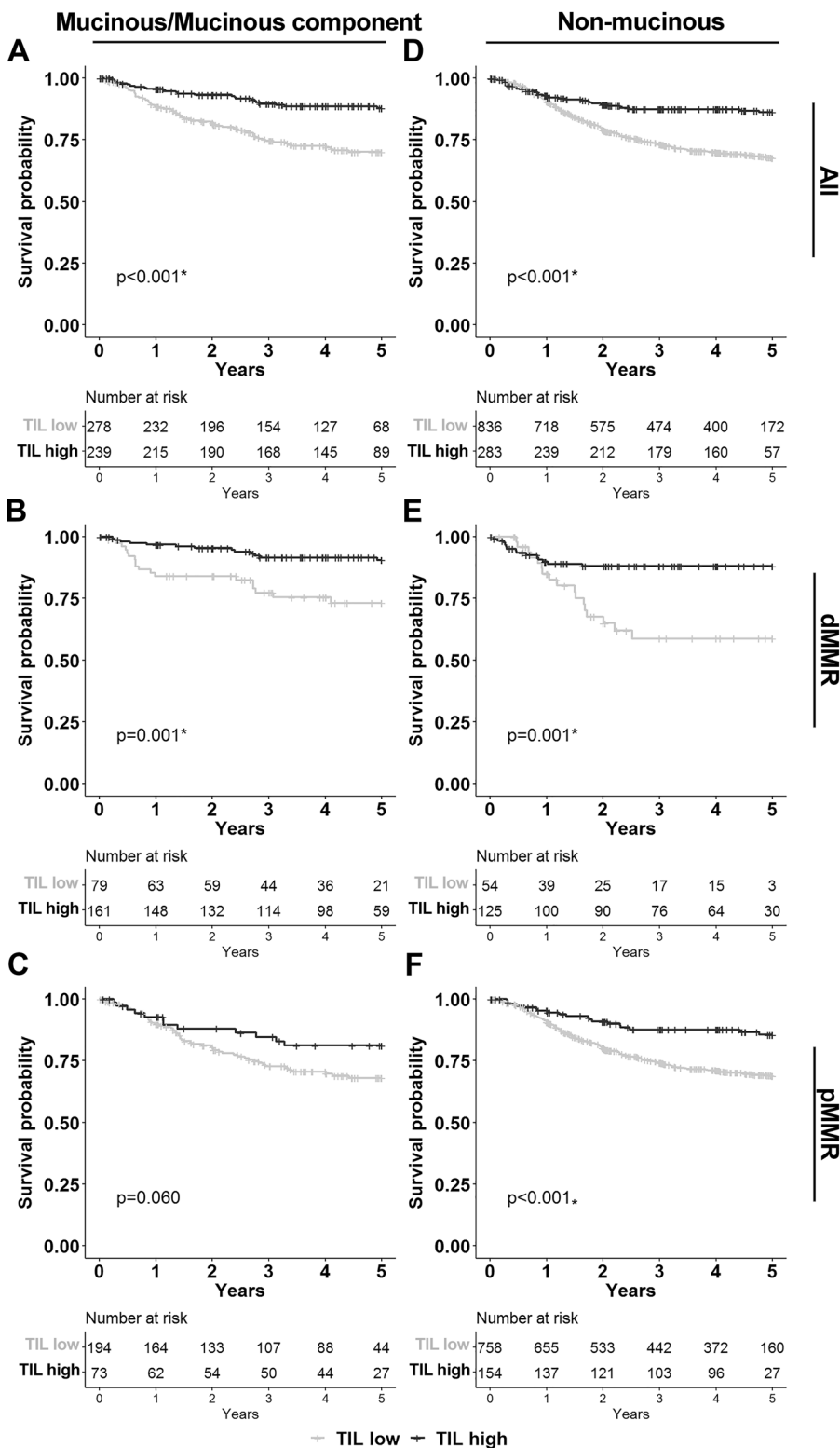
histological grade, MMR and *BRAF* mutation, highlighting the potential clinical utility of this categorisation.

The prevalence of mucinous tumours of ~19% in our cohort was in the upper range for studies from Western countries [5–7], likely related to the relatively high proportion of proximal colon tumours (~45%) in our patients. Compared with non-mucinous CRC, mucinous tumours were associated with female gender, proximal location, high grade, TIL-high, dMMR and *BRAF* mutation. Mucinous tumour associations with proximal location [3, 4, 6, 8, 11, 13, 15], dMMR [8–16] and *BRAF* mutation [10, 13–16] are well-established, indicating that our cases are representative of mucinous CRCs.

The prevalence of tumours with a mucinous component is less well defined, with previous surveys reporting rates of 4–7% for Asian and 20–28% for Western cohorts [8, 11, 13, 18–21]. In our cohort, 12% of patients had tumours with a mucinous component. We found that tumours with a mucinous component exhibited similar clinico-molecular characteristics as mucinous tumours, suggesting that the presence of any degree of extracellular mucin identifies a CRC subtype that is biologically distinct from non-mucinous carcinoma. In a previous large cohort analysis, Ogino et al. also concluded that in general mucinous and mucinous component tumours appeared similar, although they noted some differences for frequencies of MSI and MGMT loss [17]. Likewise, Yoon et al. reported that patients with mucinous and mucinous component tumours had overall similar clinicopathological characteristics, which differed from those with non-mucinous tumours [11]. Overall concordant observations were made in other studies [8, 18–21]. For our patient cohort, the only differentiating characteristic between mucinous and mucinous component CRCs was tumour stage, with the former associated with stage II disease. Interestingly, a similar

Fig. 3 Kaplan–Meier plots for disease-free survival in patients with stage II/III CRC according to TIL status.

Patients are grouped into (a–c) individuals with mucinous/mucinous component tumours, (d–f) individuals with non-mucinous tumours for (a, d) the entire cohort, (b, e) dMMR tumour, and (c, f) pMMR tumours. TIL, tumour infiltrating lymphocyte; dMMR DNA mismatch repair deficient, pMMR DNA mismatch repair proficient; * $p < 0.050$.



association was evident in the cohort investigated by Langner et al. [8].

The prognostic value of tumour mucinous differentiation is controversial [2, 4–7, 11–13, 19, 23, 24]. A meta-analysis of 34 studies indicates only a slightly worse survival rate

among mucinous CRC as compared with other cases (HR 1.06, 95% CI 1.03–1.09) [23]. Similarly, most previous studies examining tumours with a mucinous component found no evidence for a clinically relevant prognostic difference as compared with mucinous or non-mucinous

tumours [8, 11, 13, 20, 22]. In agreement with these studies we observed similar outcomes by mucinous differentiation in our patients.

Our data provide some evidence to support the WHO 2010 premise that tumour MMR status, rather than histological grade, is a prognostic factor in mucinous tumours. Consistent with these guidelines, we found that MMR status stratified mucinous cancers for DFS with better outcomes in dMMR tumours in univariate analysis, while classic histological tumour grade did not stratify survival. In line with our data for MMR status, several previous studies have also reported a survival benefit for dMMR in mucinous tumours [11, 13, 14, 30, 45], although others found no survival difference [19, 46]. As observed for clinico-molecular characteristics, tumours with a mucinous component showed similar prognostic relationships as mucinous tumours, with 5-year DFS rates of 84.2% for dMMR and 69.2% for pMMR tumours, but similar outcomes by histological grade. In a comparison of mucinous and mucinous component tumours against non-mucinous tumours, the interaction between tumour grade and mucinous differentiation was significant. Nonetheless, in multivariate analysis combining mucinous and mucinous component tumours, dMMR status did not reach statistical significance as an independent prognostic predictor. This latter finding is consistent with the updated recommendation in the WHO 2019 guidelines [32], but our data do not support the

contention that conventional histological grading should be applied to mucinous tumours.

About 10% of CRCs harbour *BRAF* (V600E) mutations [47], and a recent meta-analysis of seven phase III clinical trials reported poorer survival for *BRAF* mutated stage II/III tumours compared with *BRAF* wild-type tumours [48]. Moreover, there is evidence that *BRAF* mutated pMMR tumours constitute a particularly poor prognostic group [27–29]. Our findings indicate that *BRAF* mutation prognostic value in stage II/III CRCs depends on mucinous differentiation and MMR status, with poor prognosis limited to non-mucinous pMMR tumours. Consistent with our findings, Andrici et al. previously reported similar 5-year survival rates for *BRAF* wild-type and mutated pMMR mucinous CRCs [14].

There are strong data to support host immune response against tumour as a prognostic indicator in CRC patients [36, 39–41]. Our study indicates that the prognostic value of TIL classification is retained in stage II/III tumours with mucinous differentiation, with TIL-high a strong independent predictor of good prognosis. In our cohort, the most favourable outcomes were observed for TIL-high dMMR tumours, whilst TIL-low tumours had poor outcomes irrespective of MMR status. Notably, TIL status was a superior predictor of outcome compared with histological grade, MMR and *BRAF* mutation. Addition of histological grade, MMR and *BRAF* mutation did not improve models with TIL status. These findings highlight that a standardised method of assessment of TILs should be considered for inclusion in routine reporting of CRCs irrespective of tumour mucinous component. We have previously shown that the TIL method applied in this study has good intraobserver and interobserver reproducibility (κ statistic 0.772 and 0.666, respectively) [36]. Immunohistochemical detection of TILs or the application of digital image analysis may improve scoring reproducibility, although a revised cut-point would need to be determined for these methods.

Strengths of our study include a large cohort size with detailed treatment and long-term follow-up data, focusing on stage II/III patients. Recruitment of patients occurred at multiple hospital sites capturing a broad population. DFS is

Table 4 Differences in Aikake Information Criteria (AIC) for the comparison of Cox proportional hazards models for disease-free survival in patients with stage II/III colorectal cancer classified by TIL status against models classified by tumour grade, MMR and *BRAF* mutation status.

Mucinous/mucinous component	Cases/ events	AIC (X)–AIC (TIL)
Grade	455/92	15.7
MMR status	448/92	12.3
<i>BRAF</i> mutation	444/92	15.2

Positive values of the AIC difference indicate a better fit for the models with TIL status. Cox proportional hazards models are adjusted by clinico-molecular variables and treatment.

Table 5 Comparison of Cox proportional hazards models for disease-free survival in patients with stage II/III colorectal cancer when adding TIL status to models with tumour grade, MMR or *BRAF* status (labelled X) or vice versa.

Mucinous/ mucinous component	Cases/ events	Likelihood-ratio test statistic (add TIL)	<i>P</i> value (add TIL)	Likelihood-ratio test statistic (add X)	<i>P</i> value (add X)
Grade	455/92	17.2	<0.001*	1.5	0.217
MMR status	448/92	12.5	<0.001*	0.2	0.649
<i>BRAF</i> status	444/92	15.2	<0.001*	0.0	0.983

Multivariate Cox proportional hazards models are adjusted for clinico-molecular variables and treatment. **P* < 0.05, likelihood-ratio test.

considered an appropriate sensitive endpoint for early-stage CRC, and in contrast to overall survival, is less influenced by disparities in comorbidities, treatments for recurrent disease or deaths from causes other than cancer. Tumour mucinous component was scored by a single study pathologist, with good interobserver agreement on re-examination of a subset of cases by two additional pathologists. Our large cohort size allowed for detailed comparisons of clinico-molecular features and survival between the prevalent non-mucinous and less common mucinous and mucinous component subtypes. With integrated consideration of tumour histopathologic and molecular features entering clinical care, empirical data to support clinical cut-offs for tumour classification and analyses to pinpoint confounders and prognostic interactions between variables are becoming increasingly important. Our study examined these pertinent issues for tumour mucinous component, challenging the utility of the current proportion-based definition of mucinous tumours and highlighting the differential prognostic values for tumour grade and *BRAF* mutation. To our knowledge, none of the previous studies on global TIL status in CRC has specifically assessed tumours with mucinous differentiation.

Several limitations of our study deserve comment. First, because our patient cohort was not derived from a clinical trial, chemotherapy treatment and follow-up were performed as per routine care without mandated standardisation. Second, given the low prevalence of mucinous and mucinous component differentiation, no independent large cohort was available to further validate our findings, although many of our observations were consistent with existing literature. Third our analysis of host immune response to tumour did not allow us to distinguish types of T-cell populations or consider innate immune infiltrates which may carry additional prognostic information [37, 49]. Finally, other molecular events or epidemiological factors which were not examined in this study may impact prognosis or chemosensitivity within tumour mucinous subtypes. These may include variables such as chromosome instability [50], body mass index [51] and smoking [52].

In conclusion, our study indicates that the presence of any mucinous differentiation, irrespective of the extent, demarcates CRCs with specific clinico-molecular features indicating that these tumours are distinct from non-mucinous adenocarcinomas. Mucinous tumours and tumours with a mucinous component showed marked similarities for prognostic interactions, with evidence that neither tumour grade nor *BRAF* mutation provide prognostic information in these tumours. Our results support the consideration of a standardised method to evaluate TIL status in patients with stage II/III CRC to guide patient management, irrespective of the degree of mucinous differentiation. Although further studies are required to

confirm our findings, our data provide useful information on mucinous differentiation in clinical and pathological practice of CRC.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare. This work was supported by a Cancer Australia Project Grant (APP1120882), a Cancer Council Victoria Grant-in-Aid (1060964), the Victorian Government's Operational Infrastructure Support Program, a University of Melbourne Department of Pathology Career Development Award, and an Austin Medical Research Foundation Project Grant. O.M.S. is a NHMRC Senior Research Fellow (APP1136119).

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Affiliations

David S. Williams^{1,2,3} · Dmitri Mouradov^{4,5} · Marsali R. Newman¹ · Elham Amini⁶ · David K. Nickless⁷ · Catherine G. Fang² · Michelle Palmieri^{4,5} · Anuratha Sakthianandeswaren^{4,5} · Shan Li⁴ · Robyn L. Ward^{8,9} · Nicholas J. Hawkins^{10,11} · Iain Skinner¹² · Ian Jones¹³ · Peter Gibbs^{4,5,14,15} · Oliver M. Sieber^{16,17}

¹ Department of Pathology, Austin Health, Heidelberg, VIC, Australia

² Olivia Newton-John Cancer Research Institute, Heidelberg, VIC, Australia

³ Department of Clinical Pathology, The University of Melbourne, Parkville, VIC, Australia

⁴ Personalised Oncology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

⁵ Department of Medical Biology, The University of Melbourne, Parkville, VIC, Australia

⁶ Clinipath Pathology, Sonic Healthcare, Perth, WA, Australia

⁷ Melbourne Pathology, Cabrini Health, Malvern, VIC, Australia

⁸ Prince of Wales Clinical School and Lowy Cancer Research Centre, UNSW Sydney, Sydney, NSW, Australia

⁹ Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia

¹⁰ School of Medical Sciences, UNSW Sydney, Sydney, NSW, Australia

¹¹ Faculty of Medicine, The University of Queensland, Herston, QLD, Australia

¹² Department of Surgery, Western Health, Footscray, VIC, Australia

¹³ Department of Surgery, Royal Melbourne Hospital, Parkville, VIC, Australia

¹⁴ Department of Medical Oncology, Parkville, VIC, Australia

¹⁵ Department of Medical Oncology, Western Health, Footscray, VIC, Australia

¹⁶ Department of Surgery, The University of Melbourne, Parkville, VIC, Australia

¹⁷ School of Biomedical Sciences, Monash University, Clayton, VIC, Australia