Wild-type APC predicts poor prognosis in microsatellite-stable proximal colon cancer

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Background: APC mutations (APC-mt) occur in ~70% of colorectal cancers (CRCs), but their relationship to prognosis is unclear.

Methods: APC prognostic value was evaluated in 746 stage I–IV CRC patients, stratifying for tumour location and microsatellite instability (MSI). Microarrays were used to identify a gene signature that could classify APC mutation status, and classifier ability to predict prognosis was examined in an independent cohort.

Results: Wild-type APC microsatellite stable (APC-wt/MSS) tumours from the proximal colon showed poorer overall and recurrence-free survival (OS, RFS) than APC-mt/MSS proximal, APC-wt/MSS distal and APC-mt/MSS distal tumours (OS HR > 1.79, P < 0.015; RFS HR > 1.88, P < 0.026). APC was a stronger prognostic indicator than BRAF, KRAS, PIK3CA, TP53, CpG island methylator phenotype or chromosomal instability status (P < 0.036). Microarray analysis similarly revealed poorer survival in MSS proximal cancers with an APC-wt-like signature (P = 0.019). APC status did not affect outcomes in MSI tumours. In a validation on 206 patients with proximal colon cancer, APC-wt-like signature MSS cases showed poorer survival than APC-mt-like signature MSS or MSI cases (OS HR > 2.50, P < 0.010; RFS HR > 2.14, P < 0.025). Poor prognosis APC-wt/MSS proximal tumours exhibited features of the sessile serrated neoplasia pathway (P < 0.016).

Conclusions: APC-wt status is a marker of poor prognosis in MSS proximal colon cancer.
Colorectal cancer (CRC) is a leading cause of cancer-related death in the Western world (Stewart and Wild, 2014). Despite advances in surgery and oncology, the average 5-year survival rate remains below 60%. Treatment decisions are primarily based on disease staging performed by imaging and tumour histopathological assessment. However, there exists substantial heterogeneity in prognosis within tumours of identical stage.

Approximately 70% of sporadic CRCs are initiated by biallelic inactivation of the APC tumour-suppressor gene, resulting in aberrant activation of WNT/β-catenin signalling (Christie et al., 2013). The majority of APC-mutated (APC-mt) cancers are thought to develop via the classic adenoma-carcinoma pathway, characterised by premalignant adenomatous polyps with tubular and/or villous architecture, and carcinomas associated with TP53 mutation and chromosomal instability (CIN) (Fearon and Vogelstein, 1990). Another major pathway to CRC is the sessile serrated pathway, accounting for 15–20% of cases (Leggett and Whitehall, 2010; Snover, 2011; Bettington et al., 2013). This pathway is typified by precursor sessile serrated adenomas, wild-type APC (APC-wt), BRAF mutation, a CpG island methylator phenotype (CIMP-high), poor differentiation and mucinous histology, and approximately half of sessile serrated pathway cancers show late development of microsatellite instability (MSI). A further alternate pathway has been proposed, perhaps comprising 5–10% of CRC, which may arise from traditional serrated or tubulovillous adenomas and exhibits CIMP-low, KRAS and PIK3CA mutation and a microsatellite stable (MSS) genotype (Leggett and Whitehall, 2010; Day et al., 2013). Notably, these pathways show differential distributions throughout the large intestine, with the sessile serrated pathway more frequent in the proximal colon, and the classic pathway more common in the distal colon and rectum.

Individual molecular characteristics associated with the neoplasia pathways including CIN, MSI, CIMP and mutations in KRAS, BRAF, PIK3CA and TP53 have all been evaluated as indicators of patient prognosis. Multiple studies have demonstrated good outcomes for early-stage cancers with MSI, and combined with data indicating that MSI cancers may not benefit from 5-FU-based chemotherapy, it has been proposed that moderate- and high-risk stage II patients exhibiting an MSI phenotype may forego adjuvant chemotherapy (Popat et al., 2005; Guastadisegni et al., 2010; Ng and Schrag, 2010). Conversely, there are emerging data that presence and extent of CIN are associated with inferior outcomes (Walther et al., 2008; Mouradov et al., 2013). Mutation in BRAF has been reported as an indicator of poor prognosis, in particular for patients with metastatic disease, but this relationship is complex, because of the strong positive association between BRAF mutation and MSI (Samowitz et al., 2005; French et al., 2008; Tye et al., 2011; Lochhead et al., 2013). Accordingly, recent data suggest that BRAF or KRAS mutation are associated with poor prognosis specifically in patients with MSS cancers (Phipps et al., 2015; Sinicropi et al., 2015). Evidence for the prognostic values of CIMP, PIK3CA and TP53 mutation is inconsistent (Murro et al., 2005; Russo et al., 2005; Prenen et al., 2010; Day et al., 2013).

Although mutations in APC have a principal role in CRC initiation, their relation to outcome remains unclear. Most previous studies have not found an association between the presence of APC mutation and prognosis, but these analysed small patient cohorts, only screened limited regions of the APC gene, and did not account for prognostically important features such MSI, CIN, BRAF mutation and tumour location (Dix et al., 1994; Lovig et al., 2002; Conlin et al., 2005; Hsieh et al., 2005; Meguid et al., 2008; Chen et al., 2009; Wong, 2010; Birnbaum et al., 2012).

Evaluation of APC prognostic value is further complicated by the observation that proximal and distal tumours differ substantially in their APC mutation spectra. In general, somatic APC mutations tend to cluster in codons 1282–1581, the so-called mutation cluster region, producing truncated proteins retaining 1–3 intact 20-amino-acid repeats (20AARs), functional domains that are critical for β-catenin regulation (Miyoshi et al., 1992; Christie et al., 2013). However, when assessed by tumour location, proximal cancers show a marked enrichment for mutations leaving 2–3 20AARs, while distal cancers show predominance of mutations leaving 0–1 20AARs, indicating distinct WNT/β-catenin signalling thresholds for tumourigenesis in these embryologically distinct regions (Rowan et al., 2000; Albuquerque et al., 2002; Christie et al., 2013). A single prognostic study considered location of APC mutation and suggested that patients who have lost all β-catenin binding sites (15- and 20-AARs) of APC may have shorter cancer-related survival than patients with mutations that have retained one or more binding sites (Lovig et al., 2002); however, this study did not consider tumour location.

Here, we examined whether APC mutation presence or genotype are indicators of patient prognosis when accounting for tumour location, MSI, CIN, CIMP, KRAS, BRAF, PIK3CA and TP53 status, analysing the largest CRC cohort (n = 746) to date in which the entire coding region of APC has been sequenced. We then identified the gene expression signature of APC mutation status using microarray analysis that could predict disease outcome. We validated the prognostic value of the APC classifier using an independent cohort of 206 patients from a publicly available microarray dataset (GSE39582) (Marisa et al., 2013) (Supplementary Figure 1).

**MATERIALS AND METHODS**

**Patients.** We analysed 746 patients with stages I-IV CRC who had undergone treatment at the Royal Melbourne Hospital (Parkville, VIC, Australia), Western Hospital Footscray (Footscray, VIC, Australia), Royal Adelaide Hospital (Adelaide, SA, Australia) and St Vincent’s Hospital Sydney (Darlington, NSW, Australia). Patients with familial polyposis syndromes, ulcerative colitis or Crohn’s disease-associated CRC were excluded. All patients gave informed consent, and this study was human research ethics committee-approved (WEHI HREC 12/19). Clinicopathological characteristics and pre- and post-operative treatment details were collected using a multi-site database. Tumours from caecum to transverse colon were defined as proximal, and those from splenic flexure to rectum as distal. For patients with early stage disease (I-III), patient follow-up data were collected prospectively according to national guidelines, with 3-monthly clinic visits and testing for carcinoembryonic antigen levels, 12-monthly computed tomography scans of the chest, abdomen and pelvis for 2 years after diagnosis, and then 6-monthly clinic visits and carcinoembryonic antigen testing until 5 years from diagnosis. For patients with stage IV cancer, standard follow-up was with imaging every 8–9 weeks while patients remained on active therapy. Clinical follow-up while on active therapy was on a 4-weekly basis.

**Mutation detection.** Mutations in APC (entire coding region), KRAS (codons 12, 13 and 61), BRAF (V600E), PIK3CA (exons 9 and 20) and TP53 (exons 4 to 9) have been determined previously (Christie et al., 2013; Day et al., 2013). Briefly, DNA was extracted from macrodissected tumour and matched normal tissues, and Sanger sequencing performed in both orientations on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Any detected mutations were confirmed by resequencing of tumour and matched normal DNA from new PCR product.

**Microsatellite instability, CIN and loss of heterozygosity assessment.** Microsatellite instability status was defined using the Bethesda five-marker microsatellite panel (Boland et al., 1998). Microsatellite instability was considered present if instability was seen at 2 or more markers. Tumour CIN status and loss of heterozygosity
at APC have been determined previously using single nucleotide polymorphism microarray analysis (Human 610-Quad BeadChip, Illumina, San Diego, CA, USA) on tumour and matched normal DNA samples and OncoSNP software (Isis Innovations, Oxford, UK) (Yau et al., 2010; Christie et al., 2013; Mouradov et al., 2013).

CpG island methylator phenotype analysis. Tumour CIMP data have been reported based on MethyLight real-time PCR for the IGF2, SOCS1, RUNX3, CACNA1G and NGN1 marker panel, and the reference ALU (Day et al., 2013). Tumours with a percentage of methylated reference value of greater than 10 for ≥3 CIMP markers were classified as CIMP-high (CIMP-H), those with 1–2 methylated markers as CIMP-low (CIMP-L), and 0 methylated markers as CIMP-0.

Microarray analysis. Gene expression profiles were determined for 52 MSS proximal colon cancers using the Affymetrix (Santa Clara, CA, USA) GeneChip Human Exon 1.0 ST Array version 2 according to the manufacturer recommendations. The microarray data have been deposited in the Gene Expression Omnibus database (GSE63624). Data were normalised using the Robust Multi-array Average algorithm (Irizarry et al., 2001), Differences between groups were assessed using the Kruskal-Wallis test for continuous variables and Fisher’s exact test for categorical variables. Odds ratios (ORs) and 95% confidence intervals (CI) were obtained from Fisher’s exact test calculations. Outcome analyses were performed for overall survival (OS) and recurrence-free survival (RFS) from date of surgery, censored at 5 years. Patients who had received radiotherapy or were stage IV and had undergone additional surgery for metastases were excluded. Univariate survival distributions were compared using the log-rank test. Cox proportional hazards models were used to estimate survival distributions and hazard ratios for multivariate analyses, adjusting for gender, age at diagnosis, tumour stage and treatment; to facilitate comparisons between multiple APC-wt/-mt tumour groups, hazard ratios were retrieved for all pairwise combinations of reference states. Comparisons between models with and without inclusion of specific molecular variables were made using the likelihood ratio test and the Akaike Information Criterion. Complete case analysis was used for all statistical calculations. Statistical analyses were two-sided and considered significant if P < 0.05.

RESULTS

Clinicopathological and molecular features of CRC patients. In the cohort of 746 patients, the median age at presentation was 70 years and 55% were male (Table 1). Seventy cancers were stage I, 229 stage II, 347 stage III and 100 stage IV; 315 were from the proximal colon, 242 from the distal colon and 189 from the rectum. Clinical follow-up information was available for 685 patients for OS. For patients with stages I-III CRC, RFS data were available for 415 cases. The median duration of follow-up was 47 months for OS, and 37 months for RFS. Among the 685 patients with available outcome data, 296 had received standard adjuvant 5-fluorouracil-based chemotherapy (unknown in 33 cases).

The cancers had molecular features that were similar to those found in other studies (Table 1). Truncating APC mutations were detected in 68.4% (510 of 746) of tumours, and 31.1% (201 of 627 with available single nucleotide polymorphism array data) showed loss of heterozygosity at APC. Among the APC-mt cases, 76.7% (335 of 437) had two hits (2 mutations or 1 mutation and loss of heterozygosity). The frequencies of mutations in BRAF, KRAS, PIK3CA and TP53 were 9.0% (67 of 746), 35.1% (262 of 746), 12.5% (93 of 746) and 55.4% (413 of 746), respectively; 73.6% (465 of 632) of tumours were CIN, 13.4% (100 of 746) MSI, 17.3% (85 of 746) CIN and 55.4% (413 of 746), respectively; 73.6% (465 of 632) of tumours were CIN, 13.4% (100 of 746) MSI, 17.3% (85 of 746) CIN and 55.4% (413 of 746), respectively. Multivariate analyses, adjusting for gender, age at diagnosis, tumour stage and treatment; to facilitate comparisons between multiple APC-wt/-mt tumour groups, hazard ratios were retrieved for all pairwise combinations of reference states. Comparisons between models with and without inclusion of specific molecular variables were made using the likelihood ratio test and the Akaike Information Criterion. Complete case analysis was used for all statistical calculations. Statistical analyses were two-sided and considered significant if P < 0.05.

Validation of prognostic capability of APC gene signature in an independent dataset. We validated the prognostic capability of the APC gene signature using a publicly available independent microarray dataset on stages I to IV CRCs (GSE39582) (Marisa et al., 2013). Raw data were Robust Multi-array Average - normalised and batch-corrected using the ComBat algorithm, and restricted to proximal tumours with available outcome data (n = 206). For genes with multiple alternative probes, those with the largest variance across samples were selected. Gene probe data were mean-centred and scaled, and the SVM model used to predict APC-wt status. Statistical analyses were conducted using the statistical computing software R (The R Development Core Team, 2013). Differences between groups were assessed using the Kruskal-Wallis test for continuous variables and Fisher’s exact test for categorical variables. Odds ratios (ORs) and 95% confidence intervals (CI) were obtained from Fisher’s exact test calculations. Outcome analyses were performed for overall survival (OS) and recurrence-free survival (RFS) from date of surgery, censored at 5 years. Patients who had received radiotherapy or were stage IV and had undergone additional surgery for metastases were excluded. Univariate survival distributions were compared using the log-rank test. Cox proportional hazards models were used to estimate survival distributions and hazard ratios for multivariate analyses, adjusting for gender, age at diagnosis, tumour stage and treatment; to facilitate comparisons between multiple APC-wt/-mt tumour groups, hazard ratios were retrieved for all pairwise combinations of reference states. Comparisons between models with and without inclusion of specific molecular variables were made using the likelihood ratio test and the Akaike Information Criterion. Complete case analysis was used for all statistical calculations. Statistical analyses were two-sided and considered significant if P < 0.05.

Proximal APC-wt/MSS tumours exhibit inferior prognosis. We tested whether overall APC mutation status (wild-type vs mutated) was associated with patient prognosis when accounting for tumour MSI status and location, and adjusting for gender, age at diagnosis, tumour stage and treatment. Among patients with MSS tumours, APC-wt proximal cancers showed significantly inferior prognosis as compared with APC-mt proximal, APC-wt distal and APC-mt distal cancers for OS and RFS (OS HR ≥ 1.79, P < 0.015; RFS HR ≥ 1.88, P ≤ 0.026) (Figure 1, Table 2A), and this was retained
Table 1. Characteristics of 746 patients with colorectal cancer according to APC mutation status

|                      | APC-wt | APC-mt | P    |
|----------------------|--------|--------|------|
| Age, years           | 744    | 235    | 509  |
| Mean ± s. d.         | 69 ± 11.3 | 69 ± 11.7 | 68.9 ± 11.2 | 0.317 |
| Median               | 70     | 70     |      |
| Range                | 25–99.0 | 25–99.0 | 33.0–93.0 |
| Unknown              | 2      |        |      |
| Sex                  | 746    | 236    | 510  |
| Male                 | 413 (55.4) | 109 (26.4) | 304 (73.6) | <0.001* |
| Female               | 333 (44.6) | 127 (31.6) | 206 (26.1) |
| Stage                | 746    | 236    | 510  |
| I                    | 70 (9.4) | 15 (21.4) | 55 (78.6) | 0.080 |
| II                   | 229 (30.7) | 67 (29.3) | 162 (70.7) |
| III                  | 347 (46.5) | 124 (35.7) | 223 (64.3) |
| IV                   | 100 (13.4) | 30 (9.0) | 70 (70.0) |
| Site                 | 746    | 236    | 510  |
| Right Colon          | 315 (42.2) | 131 (41.6) | 184 (58.4) | <0.001* |
| Left colon           | 242 (32.4) | 51 (21.1) | 191 (78.9) |
| Rectum               | 189 (25.3) | 54 (28.6) | 135 (71.4) |
| Differentiation      | 718    | 221    | 497  |
| Well/Moderate        | 596 (83.0) | 162 (27.2) | 434 (72.8) | <0.001* |
| Poor                 | 122 (17.0) | 59 (48.4) | 63 (51.6) |
| Unknown              | 28     | 15     | 13   |
| Mucinous             | 737    | 231    | 506  |
| No                   | 577 (78.3) | 154 (26.7) | 423 (73.3) | <0.001* |
| Yes                  | 160 (21.7) | 77 (48.1) | 83 (51.9) |
| Unknown              | 9      | 5      | 4    |
| MSI status           | 746    | 236    | 510  |
| MSS                   | 646 (86.6) | 167 (25.9) | 479 (74.1) | <0.001* |
| MSI                  | 100 (13.4) | 69 (69.0) | 31 (31.0) |
| CIMP status          | 491    | 140    | 351  |
| CIMPD                | 299 (60.9) | 55 (18.4) | 244 (81.6) | <0.001* |
| CIMPML               | 107 (21.8) | 25 (23.4) | 82 (76.6) |
| CIMPH                | 85 (17.3) | 60 (70.6) | 25 (29.4) |
| Unknown              | 255    | 96     | 159  |
| BRAF                 | 746    | 236    | 510  |
| No                   | 679 (91.0) | 183 (27.0) | 496 (73.0) | <0.001* |
| Yes                  | 67 (9.0) | 53 (79.1) | 14 (20.9) |
| KRAS                 | 746    | 236    | 510  |
| No                   | 484 (64.9) | 185 (38.2) | 299 (61.8) | <0.001* |
| Yes                  | 262 (35.1) | 51 (19.5) | 211 (80.5) |
| PIK3CA               | 746    | 236    | 510  |
| No                   | 653 (87.5) | 214 (32.8) | 439 (67.2) | 0.095 |
| Yes                  | 93 (12.5) | 22 (23.7) | 71 (76.3) |
| TP53                 | 746    | 236    | 510  |
| No                   | 333 (44.6) | 143 (42.9) | 190 (57.1) | <0.001* |
| Yes                  | 413 (55.4) | 93 (22.5) | 320 (77.5) |
| CIN status           | 632    | 191    | 441  |
| CIN –                | 167 (26.4) | 86 (51.5) | 81 (48.5) | <0.001* |
| CIN +                | 465 (73.6) | 105 (22.5) | 360 (77.4) |
| Unknown              | 114    | 45     | 69   |

Abbreviations: CIN = chromosomal instability; CIMP = CpG island methylator phenotype; MSI = microsatellite instability; MSS = microsatellite stable. Row percentages are given in parentheses and column percentages are given in square brackets. *P<0.05.

when restricted to stage II and III cancers (OS HR ≥ 1.79, P ≤ 0.029; RFS HR ≥ 1.81, P ≤ 0.038) (Supplementary Table 1A). Accordingly, a multivariate model with an APC-tumour location interaction term provided a significantly better fit of the survival data as compared with a model with APC status and tumour location but without the interaction term (OS, P = 0.043; RFS, P = 0.011, likelihood ratio test). We found no evidence for differential outcomes between APC-mt/MSS proximal, APC-wt/MSS distal and APC-mt/MSS distal tumours (Figure 1, Table 2A, Supplementary Table 1A for stage II and III cancers).

No prognostic value of APC mutation in MSI tumours. Among patients with MSI cancers, testing for association between APC mutation status and prognosis was restricted to proximal cases owing to the low prevalence of MSI in distal tumours (n = 16). In contrast to MSS proximal cancers, the adverse prognostic influence of APC-wt status was lost in MSI proximal cancers (n = 75) with similar OS and RFS for wild-type and mutated tumours (multivariate OS, P = 0.859; RFS, P = 0.779; Figure 1, Table 2B, Supplementary Table 1B for stage II and III cancers).

Compared with MSI proximal cancers, APC-wt/MSI proximal tumours showed significantly poorer survival (OS, HR ≥ 2.05, P = 0.022; RFS, HR ≥ 3.34, P = 0.005). Microsatellite instability proximal tumours further exhibited a trend to improved outcomes as compared with APC-mt/MSI proximal cancers, although this was not statistically significant (OS, HR ≥ 0.88, P = 0.656; RFS, HR ≥ 0.59, P = 0.185) (Figure 2A, Table 3A). These trends were retained when restricting the analysis to stage II and III cancers (Supplementary Table 2A).

APC mutation genotype does not provide additional prognostic information. Given the different APC mutation spectra in proximal and distal tumours (Albuquerque et al., 2010; Christie et al., 2013), we examined whether classification of tumours by APC mutation genotype had additional prognostic value. Among patients with APC-mt/MSS cancers, outcomes were similar across tumour locations irrespective of whether truncating APC mutations left 0 or ≥ 1 15AARs required for β-catenin binding, or whether the mutations left 0-1 or 2-3 20AARs associated with residual β-catenin binding/regulatory activity (P ≥ 0.169 for all comparisons, Supplementary Table 3A and B). Further, no outcome differences were apparent when considering the number of hits in APC with one exception: proximal tumours with 1 hit in APC showed a tendency to better OS as compared with distal tumours with 1 hit in APC (HR = 0.37, P = 0.049), however, this trend was not observed for RFS (P = 0.246; Supplementary Table 3C). We did not explore APC genotypes in MSI CRCs owing to low sample numbers per group.

APC prognostic value in the context of other molecular changes. The prognostic value of APC status in proximal MSS tumours was evaluated against other molecular changes. Single-variable models with BRAF, CIN, CIMP-H, KRAS, PIK3CA or TP53 status were compared with models with addition of APC status and vice versa using the likelihood ratio test. In all comparisons, APC mutation was found to be the strongest indicator of outcome: addition of APC status significantly improved all OS and RFS models with CIMP, BRAF, KRAS, PIK3CA or TP53 or CIN status (P ≤ 0.036 for all comparisons), whereas addition of the latter molecular variables to models with APC status did not significantly improve model fit (Table 4). Accordingly, direct comparison of models with either APC mutation or the relevant molecular feature of interest using the Akaike Information Criterion favoured the model with the APC mutation in all cases (Table 4).

APC gene signature in MSS proximal tumours. For 52 patients with MSS proximal colon cancer, including 35 APC-mt patients, we were able to obtain sufficient tumour RNA of high quality for
Validation of prognostic capability of \textit{APC}-wt gene signature in an independent cohort of proximal colon cancers. We applied the \textit{APC}-wt gene signature to an independent microarray dataset on 206 stage I-IV proximal colon cancers by Marisa \textit{et al} (2013) (GSE39582). Consistent with findings in our cohort, patients with \textit{APC}-wt-like gene signature MSS tumours showed significantly poorer OS and RFS than those with \textit{APC}-mt-like gene signature MSS tumours and MSI tumours (OS HR ≥ 2.50, \textit{P} ≤ 0.010; RFS HR ≥ 2.14, \textit{P} ≤ 0.025) (Figure 2B, Table 3B, Supplementary Table 2B when restricting to stage II and III cancers). There was no significant difference in outcome by \textit{APC} class for MSI cancers (\textit{P} ≥ 0.375 for OS and RFS).

Proximal \textit{APC}-wt/MSS tumours exhibit features of the sessile serrated pathway. Given the differences in prognosis among MSS CRCs by \textit{APC} status and tumour location, we tested whether these were reflected at the pathological and molecular level. In pairwise comparisons between tumour groups (Figure 3, Supplementary Table 5), the poor prognosis \textit{APC}-wt/MSS cancers of the proximal colon (\textit{n} = 70) consistently showed associations with features of the sessile serrated pathway including poor differentiation, CIMP-H and \textit{BRAF} mutation (\textit{P} ≤ 0.016 for all comparisons), and to a lesser extent mucinous histology (\textit{P} ≤ 0.058) and female gender (\textit{P} ≤ 0.085, Figure 3A). \textit{APC}-mt/MSS distal cancers (\textit{n} = 318) displayed the expected classic adenoma-carcinoma pathway features such as \textit{TP53} mutation and CIN (\textit{P} ≤ 0.020 for all comparisons, Figure 3D), while \textit{APC}-mt/MSS proximal cancers (\textit{n} = 161) showed association with \textit{KRAS} mutation (\textit{P} < 0.001 for all comparisons) and to a lesser extent with \textit{PIK3CA} mutation (\textit{P} ≤ 0.054 for all comparisons), hallmarks of the alternate pathway (Figure 3B). \textit{APC}-wt/MSS distal cancers (\textit{n} = 97) showed no consistently outstanding characteristics, although some tendency
towards features of the sessile serrated pathway was noted when compared with *APC-*mt/MSS distal cancers (mucinous histology, CIMP-H and *BRAF* mutation; *P*<0.009 for all comparisons). However, these differences were much less pronounced than for MSS/*APC*-wt proximal cancers (Figure 3C).

In contrast, among good prognosis MSI proximal cancers (*n* = 82), for which the *APC* prognostic value was attenuated, no significant differences were apparent by *APC* status (Supplementary Table 6). When MSI proximal cancers were compared with the four groups of MSS cancers, these most closely resembled the poor prognosis *APC*-wt/MSS proximal group, exhibiting an overrepresentation of sessile serrated pathway features such as female gender, CIMP-H, *BRAF* mutation, poor differentiation and mucinous histology (Figure 3E, Supplementary Table 5). However, MSI proximal cancers differed from all four groups in showing little CIN and a tendency to present at earlier tumour stages. Associations for MSI distal cancers were not investigated owing to the small sample size (*n* = 16).

**DISCUSSION**

Prognostic, pathological and molecular features of CRC, including *APC* mutation spectra, are well-established to differ between proximal and distal tumours and between MSS and MSI cases (Meguid *et al.*, 2008; Albuquerque *et al.*, 2010; Wong, 2010; Sinicrope and Sargent, 2012; Christie *et al.*, 2013). In this study, the largest survey of the prognostic value of *APC* mutation to date, we have found evidence that *APC* status shows differential outcome associations across these tumour subgroups: *APC*-wt/MSS proximal tumours had inferior OS and RFS as compared with *APC*-mt/MSS proximal, *APC*-wt/MSS distal and *APC*-mt/MSS distal tumours, which showed similar outcomes. The prognostic value of *APC* mutation was lost in the MSI tumours of the proximal colon, which had the most favourable prognosis overall. Notably, attenuation of marker prognostic value in MSI cancers has previously been reported for *BRAF* mutation and CIMP (Ward *et al.*, 2003; Samowitz *et al.*, 2005; Ogino *et al.*, 2012). Poor prognosis of *APC*-wt/MSS proximal tumours was validated in an independent patient cohort (Marisa *et al.*, 2013) using a predictor of *APC*-wt status from microarray expression data. Most previous studies evaluating *APC* mutation status have not identified a relationship with prognosis (Dix *et al.*, 1994; Conlin *et al.*, 2005; Hsieh *et al.*, 2005; Chen *et al.*, 2009; Birnbaum *et al.*, 2012). However, these studies did not consider CRC groups by tumour location and MSI status, likely precluded by their smaller sample sizes (*n* = 100–218), and only performed mutation screening for limited regions of the *APC* gene. These differences in study design and size would have reduced the power to detect tumour-subgroup-specific outcome associations.

Differences in tumour prognostic behaviours by *APC* mutation, location and MSI status were strongly supported by differences at the pathological and molecular level (Supplementary Figure 5). The poor prognosis *APC*-wt/MSS proximal cancers were specifically associated with characteristics of the sessile serrated neoplasia pathway including female gender, poor differentiation, mucinous histology, CIMP-H and *BRAF* mutation (Leggett and Whitehall, 2010; Snover, 2011; Bettington *et al.*, 2013, 2014). In contrast, *APC*-mt/MSS proximal cancers showed features of the alternate pathway (KRAS and PIK3CA mutation) (Leggett and Whitehall, 2010; Day *et al.*, 2013). *APC*-mt/MSS distal cancers showed features of the classic adenoma–carcinoma pathway (TP53 mutation and CIN) (Fearon and Vogelstein, 1990), whereas *APC*-wt/MSS distal cancers had no consistently outstanding features. CpG island methylation phenotype-L, which has been associated with the alternate pathway in some studies (Leggett and Whitehall, 2010), was not overrepresented in *APC*-mt/MSS proximal cancers, although a positive association between CIMP-L and KRAS mutation was apparent for the entire cohort.

Among MSI cancers of the proximal colon, for which the prognostic value of *APC* mutation was attenuated, pathological and molecular features accordingly also did not differ by *APC* status. Overall, MSI proximal cancers showed the expected serrated pathway features (female gender, poor differentiation, mucinous histology, CIMP-H and *BRAF* mutation), similar to the poor prognosis proximal *APC*-wt/MSS group, but differed in showing little CIN and presentation at earlier tumour stages.
The prognostic value of APC mutation status in MSS proximal cancers was not explained by correlation with other tumour molecular features such as CIN, CIMP, BRAF, KRAS, PIK3CA or TP53 status. This is particularly notable for CIN, BRAF and KRAS mutation, which have been linked to inferior prognosis in MSS tumours (Tie et al., 2011; Pai et al., 2012; Lochhead et al., 2013; Sinicrope et al., 2015). Instead, our data suggest a more general association between tumour sessile serrated pathway features and poor prognosis, which is supported by recent gene expression-based studies. Using microarray analysis to create a gene expression classifier for BRAF-mutated CRCs, Popovici et al. (2012) identified a group of cancers including but not restricted to BRAF-mutated tumours which showed poor prognosis, proximal location and mucinous histology, consistent with serrated pathway characteristics. Similarly, De Sousa et al. (2013) used multiple microarray datasets to identify a CRC subgroup displaying a sessile serrated adenoma-like gene expression signature and inferior prognosis as compared with cancers with MSI- or CIN-associated signatures. A recent molecular profiling study has further reported poor prognosis for MSS or MSI-Low, CIMP-positive, BRAF mutated, KRAS wild-type CRCs, which likely have significant overlap with the APC-wt/MSS proximal subtype defined in our study (Phipps et al., 2015).

Methylation at the APC promoter resulting in gene silencing has been suggested as a potential alternative mechanism to APC mutation (Arnold et al., 2004). Using DNA methylation array and RNA-Seq data for 215 CRCs reported by The Cancer Genome Atlas Network, neither promoter hypermethylation nor reduced gene expression were inversely associated with APC mutation when considering all cases or the subset of MSS proximal cancers (Supplementary Figure 6) (Network TCGA, 2012). These results are consistent with our previous observation that APC promoter hypermethylation does not substitute for truncating mutations (Segditas et al., 2008).

Interestingly, AXIN2 and RNF43 were the most differentially expressed genes by APC mutation status in proximal MSS tumours, with both showing reduced expression in the APC-wt tumours. AXIN2 is a component of the central β-catenin destruction complex, while RNF43 is a transmembrane E3 ligase involved in removing WNT receptors from the cell surface (Jho et al., 2002; de Lau et al., 2014). AXIN2 and RNF43 are themselves WNT target genes and constitute negative WNT feedback loops, suggesting that their downregulation may be an alternative to APC mutation causing aberrant WNT pathway activation. AXIN2 and RNF43 are frequently mutated in MSI CRCs (Liu et al., 2000; Giannakis et al., 2014), and AXIN2 silencing by promoter hypermethylation has been observed in MSI cancers and sessile serrated adenomas (Muto et al., 2014). Furthermore, AXIN2 and RNF43 were found in the Popovici et al. (2012) signature for BRAF-mutated CRC and the De Sousa et al. (2013) gene signatures for CRC classification.

It has been suggested that APC-mutated cancers may show differential outcomes depending on mutation location within the
gene, with patients who have lost all β-catenin binding sites having shorter cancer-related survival than patients who have retained one or more binding sites (Løvig et al, 2002). We did not find evidence of outcome differences by the number of intact β-catenin binding sites or when comparing tumours with one or two detected hits. While the number of intact β-catenin binding sites appears to be important for tumour initiation, with different 'just-right' mutation spectra in the embryologically distinct proximal and distal colon (Albuquerque et al, 2010; Christie et al, 2013), APC mutation genotypes appear to have less influence on disease progression.

Although further large cohort studies will be required to validate our findings, our data suggest a potential role for joint MSI and APC testing in risk stratification for patients with CRC. In particular, more aggressive investigation and therapy may be indicated for patients with poor prognosis APC-wt/MSS proximal cancers, indicated for patients with poor prognosis APC-wt/MSS proximal cancers, according to our findings, our data suggest a potential role for joint MSI testing in risk stratification for patients with CRC.

### Table 3A. Cox proportional-hazards analyses of OS and RFS (A) for patients with proximal colon cancer, according to APC mutation status and microsatellite instability (discovery cohort); and (B) for patients with proximal colon cancer, according to APC gene signature and microsatellite instability (validation cohort, GSE39582 (Marisa et al, 2013)); (A) Discovery cohort

|                | Overall survival | Recurrence-free survival |
|----------------|------------------|--------------------------|
|                | HR (95% CI)      | P                        | HR (95% CI)      | P                        |
| MSI vs APC-mt/ MSS | 0.88 (0.49–1.57) | 0.656                    | 0.59 (0.27–1.29) | 0.185                    |
| APC-wt/MSS/ vs MSI | 2.05 (1.11–3.77) | 0.022*                   | 3.34 (1.45–7.68) | 0.005*                   |
| APC-wt/MSS vs APC-mt/MSS | 1.79 (1.11–2.88) | 0.016*                   | 1.97 (1.06–3.63) | 0.031*                   |
| Age (Decades) | 1.41 (1.09–1.82) | 0.009*                   | 1.08 (0.81–1.45) | 0.588                    |
| Gender (Female vs Male) | 0.92 (0.60–1.42) | 0.718                    | 0.93 (0.54–1.60) | 0.794                    |
| Stage II vs I | 1.85 (0.43–8.06) | 0.412                    | 1.19 (0.25–5.68) | 0.824                    |
| Stage III vs I | 5.04 (1.16–21.82) | 0.030*                   | 5.84 (1.26–27.04) | 0.024*                   |
| Stage IV vs I | 19.21 (4.25–86.76) | <0.001*                  |                    |                          |
| Chemotherapy (Yes vs No) | 1.03 (0.60–1.76) | 0.919                    | 0.91 (0.44–1.88) | 0.807                    |

|                | Events/N         |
|----------------|------------------|
| MSI vs APC-mt/ MSS | 91/282           |
| APC-wt/MSS/ vs MSI | 55/182           |

### Table 3B. (B) Validation cohort

|                | Overall survival | Recurrence-free survival |
|----------------|------------------|--------------------------|
|                | HR (95% CI)      | P                        | HR (95% CI)      | P                        |
| MSI vs APC-mt/ MSS | 1.21 (0.63–2.34) | 0.569                    | 0.70 (0.32–1.54) | 0.375                    |
| APC-wt/MSS/ vs MSI | 2.50 (1.25–4.98) | 0.010*                   | 3.06 (1.34–6.98) | 0.008*                   |
| APC-wt/MSS vs APC-mt/MSS | 3.02 (1.67–5.47) | <0.001*                  | 2.14 (1.10–4.18) | 0.025*                   |
| Age (Decades) | 1.45 (1.15–1.83) | 0.002*                   | 1.12 (0.87–1.44) | 0.374                    |
| Gender (Female vs Male) | 0.37 (0.22–0.63) | <0.001*                  | 0.47 (0.26–0.85) | 0.012*                   |
| Stage II vs I | 0.74 (0.22–2.51) | 0.630                    | 2.23 (0.30–16.77) | 0.437                    |
| Stage III vs I | 1.17 (0.34–4.06) | 0.809                    | 4.21 (0.55–32.46) | 0.167                    |
| Stage IV vs I | 4.86 (1.22–19.34) | 0.025*                   |                    |                          |
| Chemotherapy (Yes vs No) | 1.26 (0.68–2.32) | 0.458                    | 0.89 (0.43–1.82) | 0.742                    |

|                | Events/N         |
|----------------|------------------|
| MSI vs APC-mt/ MSS | 65/201           |
| APC-wt/MSS/ vs MSI | 49/185           |

Abbreviations: CI = confidence interval; HR = hazard ratio; MSI = microsatellite instability; MSS = microsatellite stable; OS = overall survival; RFS = recurrence-free survival. Analyses are adjusted for gender, age at diagnosis, tumour stage and treatment. To facilitate comparisons between APC wild-type/mutated tumour groups, hazard ratios are presented for all pairwise combinations of reference states.

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Figure 3. Comparison of clinicopathological and molecular characteristics between colorectal cancer groups defined by APC mutation status, microsatellite instability and tumour location. Odds ratios (circles) and 95% confidence intervals (lines) for (A) APC-wt/MSS proximal cancers, (B) APC-mt/MSS proximal cancers, (C) APC-wt/MSS distal cancers, (D) APC-mt/MSS distal cancers, relative to each other. (E) Comparison of MSI proximal cancers to the four MSS tumour groups. Abbreviations: CIN chromosomal instability; CIMP, CpG island methylator phenotype; MSS/MSI, microsatellite stable/unstable.
tumours. As APC is a large gene with mutations occurring throughout the 5' two-thirds of the coding sequence, demonstration of APC-wt status will require substantial sequencing, which would previously have been impractical for clinical purposes. However, with the increasing adoption of massively parallel sequencing in clinical molecular pathology departments, routine APC sequencing for prognostic purposes will become feasible.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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