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Molecular patterns in deficient mismatch repair colorectal tumours: results from a French prospective multicentric biological and genetic study

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Background: To test the prognostic value of tumour protein and genetic markers in colorectal cancer (CRC) and examine whether deficient mismatch repair (dMMR) tumours had a distinct profile relative to proficient mismatch repair (pMMR) tumours.

Methods: This prospective multicentric study involved 251 stage I–III CRC patients. Analysed biomarkers were EGFR (binding assay), VEGFA, thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) expressions, MMR status, mutations of KRAS (codons 12–13), BRAF (V600E), PIK3CA (exons 9 and 20), APC (exon 15) and P53 (exons 4–9), CpG island methylation phenotype status, ploidy, S-phase, LOH.

Results: The only significant predictor of relapse-free survival (RFS) was tumour staging. Analyses restricted to stage III showed a trend towards a shorter RFS in KRAS-mutated ($P=0.005$), BRAF wt ($P=0.009$) and pMMR tumours ($P=0.036$). Deficient mismatch repair tumours significantly demonstrated higher TS (median 3.1 vs 1.4) and TP (median 5.8 vs 3.5) expression relative to pMMR ($P<0.001$) and show higher DPD expression (median 14.9 vs 7.9, $P=0.027$) and EGFR content (median 69 vs 38, $P=0.037$) relative to pMMR.

Conclusions: Present data suggesting that both TS and DPD are overexpressed in dMMR tumours as compared with pMMR tumours provide a strong rationale that may explain the resistance of dMMR tumours to 5FU-based therapy.

Colorectal cancer (CRC) is heterogeneous with regard to genetic alterations (Grady and Carethers, 2008; Cancer Genome Atlas Network, 2012), suggesting distinct natural histories emerging from different genetic instabilities. Burrell *et al* (2013) have recently suggested that replication stress and chromosome segregation errors may promote intratumour genetic heterogeneity.

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The majority of sporadic CRC display chromosomal instability, whereas ~15% of sporadic cases exhibit microsatellite instability resulting from deficiencies in mismatch repair (MMR) enzymes. This deficient MMR (dMMR) status is associated with a favourable prognosis irrespective of tumour staging (Popat *et al*, 2005; Bertagnolli *et al*, 2011; Hutchins *et al*, 2011; Roth *et al*, 2012; Sinicrope and Sargent, 2012). So far, among potential biomarkers that could guide the decision to initiate adjuvant CRC treatment, none has been validated with sufficient level of evidence for routine use, and pathologic tumour staging is still considered to be the main prognostic factor in current practice (Ueno *et al*, 2012). Adjuvant treatment is currently a standard in stage III CRC. For stage II CRC (tumours without apparent lymph node metastasis), the majority of patients will be cured by surgery alone, whereas a subset of patients will develop recurrence. The QUASAR randomized study demonstrated that adjuvant fluorouracil (5FU)-folinic acid treatment significantly improved survival of stage II CRC patients; however, absolute improvement was small (Quasar Collaborative Group *et al*, 2007). Thus, there is still a need to identify prognostic and predictive biomarkers for optimal personalised medicine in CRC, particularly in stage II patients.

This was the challenge we faced when launching this prospective study, such studies being considered the gold standard (Van Schaeybroeck *et al*, 2011). This French multicentric study included 256 stage I–III CRC patients who received, or not, adjuvant chemotherapy. The primary objective was to test prognostic/predictive markers covering relevant protein expression and genetic abnormalities. Since MMR status not only influences prognosis (Popat *et al*, 2005; Bertagnolli *et al*, 2011; Hutchins *et al*, 2011; Roth *et al*, 2012; Sinicrope and Sargent, 2012) but also sensitivity to fluoropyrimidine-based adjuvant treatment (Ribic *et al*, 2003; Popat *et al*, 2005; Sargent *et al*, 2010), our intention was also to examine whether dMMR tumours exhibit a distinct protein profile as compared with proficient MMR (pMMR) tumours. Such a broad prospective multiparametric approach has seldom been reported.

MATERIALS AND METHODS

Patients. This French prospective multicentric study (nine hospitals) was conducted in 251 out of 256 CCR patients included between May 1995 and November 2002 (last follow-up November 2008). All presented non-metastatic histologically documented adenocarcinoma (stages I–III) of the colon (or non-irradiated rectum) with complete surgical resection. Non-inclusion criteria included known hereditary non-polyposis colorectal cancer (HNPCC). The choice of adjuvant chemotherapy was guided according to institutional practices. Patients must have received no previous chemotherapy and present no other malignant tumour. The study was conducted in accordance with the Good Clinical Practice guidelines (ethics committee approval, written informed consent). For all patients, primary tumour was collected at the time of surgery. Normal colorectal mucosa was collected in 137 patients. Histological control, performed on 87 tumour samples, showed that 94% of samples fully fitted histological quality criteria (>40% tumour cells and good quality). The five tumours that did not meet histological quality prerequisites were excluded from the analysis; analyses were thus performed on 251 patients.

Tumoral analyses. Tumours (100–200 mg) were immediately frozen and stored in liquid nitrogen. Tumour handling is described in the Supplementary Material, as well as flow cytometry analysis (ploidy and S-phase fraction). EGFR concentrations in crude membranes were analysed by a ligand-binding assay based on competition between ¹²⁵I-EGF and unlabelled EGF (Santini *et al*,

1991). The sensitivity limit was 1 fmol mg⁻¹ protein. Thymidylate synthase (TS) activity was measured in the cytosol according to the tritium-release assay (Etienne *et al*, 2002). The sensitivity limit was 10 fmol min⁻¹ per mg protein. Tumour VEGFA expression was measured in the cytosol using the Human VEGF Quantikine ELISA kit from R&D Systems Inc. (Minneapolis, MN, USA) (Onesto *et al*, 2006). The quantification limit was 15 pg ml⁻¹.

Tumoral DNA analyses, namely KRAS mutations (codons 12–13), BRAF V600E mutation, P53 mutations (exon 4 to exon 9), PIK3CA mutations (exons 9 and 20), MMR status, allelic loss (LOH) on chromosomes 8p, 17p, 18q and CpG island methylation phenotype (CIMP), are detailed in the Supplementary Material. LOHs were only analysed on the subgroup of patients with available normal mucosa.

Tumour expression levels of TS, thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) were measured using quantitative real-time RT-PCR, using the *GAPDH* gene as reference (see details in Supplementary Materials). The sensitivity limit was 0.05 arbitrary units for TS and TP, and 0.5 arbitrary units for DPD.

Normal mucosa analyses. Normal mucosae (100–200 mg) were handled like tumour samples and resulting DNAs were analysed for candidate-gene polymorphisms.

Gene polymorphism analyses. The following gene polymorphisms were analysed on tumour DNA (251 patients): *TYMS* (5'-UTR 28 bp, rs34743033; G>C mutation in the 3R allele, rs11540151; 3'-UTR 6 bp deletion, rs11280056), methylene tetrahydrofolate reductase (*MTHFR* 677C>T, rs1801133 and 1298A>C, rs1801131), *EGFR* (intron 1 CA repeats, rs11568315; -216G>T, rs712829; -191C>A, rs712830 and 497Arg>Lys, rs2227983) and the *EGF* gene (61A>G, rs4444903). For *TYMS* 5'-UTR genotype, patients were classified as a function of the number of theoretical E-box-binding sites likely to bind USF proteins: class 2 (2R2R or 2R3RC or 3RC3RC), class 3 (2R3RG or 3RC3RG) and class 4 (3RG3RG). For *EGFR* intron 1 polymorphism, patients were split into three groups: both alleles <17 vs both alleles ≥17 vs others. In addition, analyses were performed on paired-normal mucosa DNA for 137 patients.

Statistics. χ^2 -tests for Hardy–Weinberg equilibrium (bi-allelic genotypes) were performed on <http://www.oege.org/software/hwe-mr-calc.shtml>. The distribution of quantitative tumour markers – that is, EGFR, VEGFA, TS activity, TP and DPD expressions – did not fit a Gaussian distribution and were analysed as continuous variables using non-parametric tests. Relationships between continuous variables were analysed by means of Spearman rank correlations. The impact of categorical variables on continuous variables was tested by means of Mann–Whitney test or Kruskal–Wallis test (exact *P*-values computed according to the Monte Carlo method). Links between categorical variables were assessed by Fisher's Exact tests. For statistics, genotypes were considered as ternary categorical variables, with the exception of *EGFR* -216G>T and -191C>A genotypes, both considered as binary variables (rare homozygous cases merged with heterozygous cases).

The primary efficacy variable was relapse-free survival (RFS), computed from the date of surgery until the date of first relapse defined as local recurrence or metastasis (deaths were not considered with the exception of one patient lost to follow-up who died from his cancer). Median follow-up was computed according to the inverse Kaplan–Meier method. Comparison of RFS between groups was tested by the log-rank test adjusted for tumour staging. Univariate and multivariate Cox proportional hazard regression models were also applied for testing categorical or Gaussian continuous variables (that is, log₁₀-transformed TS,

TP and DPD expression, and EGFR) and for estimating relative risks (RRs) along with 95% confidence intervals (CIs 95%). For survival analyses, quantitative tumour markers were also analysed as binary variables according to their median values (\leq vs $>$ median). For stepwise multivariate analyses, both forward and backward analyses were performed ($P = 0.05$ for entry, $P = 0.10$ for removal). All tests were two-sided. All P -values < 0.050 were reported. At the time of study initiation and sample size calculation (300 patients), the number of prognostic biomarker to be tested was limited (TS, P53 mutation, S-phase). Owing to the enlargement of tested variables subsequent to knowledge evolution, we have thus considered a P -value as significant when ≤ 0.001 (Bonferroni correction). We considered P -values comprised between 0.001 and 0.05 as indicating tendencies. Statistics was drawn up on the SPSS software v 15.0 (Paris, France).

RESULTS

Patient and tumour characteristics. Patient description is given in Table 1. There were 30 stage I, 116 stage II and 105 stage III patients. The mean age was 69.1 (extremes 29–90). The S-phase fraction, assessable on 204 tumours, ranged from 1.46 to 50% (mean 17.6, median 17). Tumour localisation was significantly linked to tumour staging (right/transverse colon being preferentially stage III, $P < 0.001$). A trend towards an association was observed between the ploidy status and tumour localisation (aneuploid or multiploid status in 48% of right/transverse colon vs 69% in left/sigmoid/RS junction vs 76% in rectum, $P = 0.004$) or histology (mucinous adenocarcinoma more frequently diploid (68.8%) than non-mucinous adenocarcinoma (36%, $P = 0.048$)).

Adjuvant 5FU-based chemotherapy was administered in 25.9% of stage II and 58.1% of stage III patients. The median interval between surgery and chemotherapy onset was 41 days. The median follow-up was 88 months. At the time of analysis, 60 patients had developed metastasis or recurrence and 91 patients had died.

Among conventional histoprognostic factors, only tumour staging was significantly linked to RFS in univariate (RR = 5.7 and 12.6 for stages II and III, respectively, as compared with stage I; $P = 0.001$, Supplementary Figure S1A) and multivariate analyses. A trend was observed towards a longer RFS in left/sigmoid/RS junction tumours (RR = 0.67 and 1.6 for left/sigmoid/RS junction and rectum, respectively, as compared with right/transverse; $P = 0.029$, Supplementary Figure S1B). A log-rank analysis adjusted for tumour staging and restricted to stage II–III patients showed no significant impact of chemotherapy. A multivariate Cox analysis confirmed the absence of interaction between chemotherapy and tumour staging.

Analysis of tumour DNA features. Results of MMR, CIMP, mutation and allelic loss analyses, and their relationships with tumour localisation and histology, are presented in Supplementary Table S2. None of these DNA molecular features were significantly related to tumour staging. Deficient mismatch repair tumours were observed in 14% of patients. Deficient mismatch repair status was significantly associated with the right/transverse colon ($P < 0.001$) and diploid tumours ($P < 0.001$). A tendency towards an elevated dMMR rate in mucinous adenocarcinoma was observed ($P = 0.023$). P53 mutations (48.8% in total) were less frequent in the right/transverse colon ($P < 0.001$) and diploid tumours ($P < 0.001$). The S-phase fraction was significantly higher in p53-mutated tumours as compared with p53 wt tumours (median 21% vs 11%, respectively; $P < 0.001$). We observed 32.7% of KRAS mutations at codons 12 and 13, which were mutually exclusive of BRAF V600E mutation found in 9.6% of tumours ($P < 0.001$). KRAS and BRAF mutations were more frequent in the right/transverse colon ($P < 0.001$ and 0.004, respectively). BRAF

Table 1. Description of patients and tumours (N = 251)

	No. of patients	%
Gender		
Men	150	59.8
Women	101	40.2
Primary localisation		
RC or TC	95	37.9
LC or sigmoid or recto-sigmoid junction	122	48.6
Rectum	33	13.1
Multiple	1	0.4
Stage		
Stage I (adjuvant chemotherapy)	30 (0)	12.0 (0)
Stage II (adjuvant chemotherapy)	116 (30)	46.2 (25.9%)
Stage III (adjuvant chemotherapy)	105 (61)	41.8 (58.1%)
Adjuvant chemotherapy^a		
FUFOL	47	51.6
LV5FU2	29	31.9
FOLFOX	6	6.6
FOLFIRI	4	4.4
Combinations	5	5.5
Histology		
Poorly differentiated	7	2.8
Moderately differentiated	139	55.4
Well-differentiated	88	35.1
Mucinous	17	6.7
Ploidy status		
Diploid	86	38.6
Aneuploid	130	58.3
Multiploid	7	3.1
Unknown	28	–
Patient follow-up		
No event	179	71.3
Local recurrence	9	3.6
Metastasis	47	18.7
Both	4	1.6
Lost to follow-up ^b	12	4.8
Patient death		
Alive	155	61.8
Cancer-related death	46	18.3
Treatment-related death	2	0.8
Unrelated-cancer death	37	14.7
Unknown cause of death	6	2.4
Lost to follow-up ^b	5	2.0

Abbreviations: LC = left colon; RC = right colon; RFC = relapse-free survival; TC = transverse colon.

^aThe median number of administered cycles was 6 (range 2–21) and the median interval between the start of chemotherapy and surgery was 41 days (range 7–162).

^bAmong the 12 patients lost to follow-up (that is, with unknown status for disease-free survival), survival status was obtained for seven of them: six unknown cause of death and one cancer-related death (considered as an event in RFS analysis).

mutations were more frequent in diploid tumours ($P < 0.001$) and in poorly/moderately differentiated tumours ($P = 0.002$). A tendency towards a lower rate of APC mutations (75.4% in total)

was observed in diploid tumours (57%, $P=0.034$). In contrast, a trend towards a higher rate of PIK3CA mutations (12.3% in total) was observed in diploid tumours (20.3%, $P=0.012$). CIMP phenotype was identified in 17.7% of patients and was significantly associated with right/transverse colon ($P<0.001$).

Associations between tumour DNA features are detailed in Supplementary Table S3. Deficient mismatch repair tumours were significantly prone to be p53 wt, BRAF-mutated and CIMP-positive ($P<0.001$). All dMMR tumours were APC wt ($P=0.011$). Deficient mismatch repair tumours tended to be KRAS wt (82.4% in dMMR vs 64.6% in pMMR tumours, $P=0.049$). BRAF mutation status was significantly linked to p53 wt status ($P<0.001$). All BRAF-mutated tumours were CIMP-positive ($P<0.001$). P53-mutated tumours were significantly prone to present allelic loss on 8p, 17p and 18q ($P<0.001$).

None of the above DNA features had a significant impact on RFS in univariate analyses adjusted for tumour staging. Analyses restricted to stage II patients did not reveal significant impact of MMR status, and KRAS or BRAF mutation status (Supplementary Figure S2, Figure 1A). In contrast, analyses restricted to stage III patients showed a tendency towards a longer RFS in dMMR tumours (log-rank, $P=0.036$, Figure 2A), KRAS wt tumours (log-rank, $P=0.005$, Figure 2B) and, unexpectedly, in BRAF-mutated tumours (log-rank, $P=0.009$, Figure 1B). An analysis restricted to stage III patients with KRAS wt tumours confirmed the trend towards a better RFS in BRAF-mutated tumours ($P=0.02$).

Analysis of tumour expression features. Table 2 illustrates the relationships between quantitative tumour expression (EGFR, VEGFA, TS, TP and DPD) and tumour characteristics. Both TS and DPD expression levels showed a trend towards an association with tumour staging (lower in stage I) and with tumour localisation (greater in the right/transverse colon, reaching significance for DPD with $P<0.001$). EGFR expression also showed a tendency towards greater levels in the right/transverse colon (vs the left colon and rectum), and for lower levels in mucinous tumours (vs others). A two-fold higher DPD expression was observed in mucinous tumours (vs others, $P=0.005$). TS, TP and DPD expression levels were higher in diploid tumours (vs aneuploid/multiploid tumours, significant for TS expression, $P<0.001$). In addition, trends towards correlations between the S-phase fraction and cytosolic VEGFA concentrations ($r=+0.17$, $P=0.015$) or DPD expression ($r=-0.19$, $P=0.009$) were observed.

The strongest correlations between tumour expression features were observed between DPD expression and TP expression ($r=0.38$, $P<0.001$) as well as between DPD expression and TS activity ($r=-0.23$, $P<0.001$) (Supplementary Table S4).

Table 3 illustrates the relationships between expression markers and tumour DNA features. Deficient mismatch repair tumours were significantly associated with elevated TS and TP expression ($P<0.001$) and showed a trend to express high DPD ($P=0.027$), high TS activity ($P=0.004$) and high EGFR ($P=0.037$). P53 wt tumours were significantly associated with elevated TS and DPD expression ($P<0.001$). CIMP-positive tumours were significantly associated with elevated TS activity ($P=0.001$). LOH on 17p was significantly associated with elevated VEGFA concentration and low DPD expression ($P=0.001$).

None of these expression markers had a significant impact on RFS (taken as continuous variables, or binary variables, with adjustment for tumour staging). Analyses restricted to stage II–III patients, adjusted for tumour staging and adjuvant therapy, did not reveal any significant impact of TS activity or TS expression on RFS.

Analysis of gene polymorphisms. Gene polymorphism data are detailed in Supplementary Material (Supplementary Table S1). No significant relationship was observed between any of the genotypes

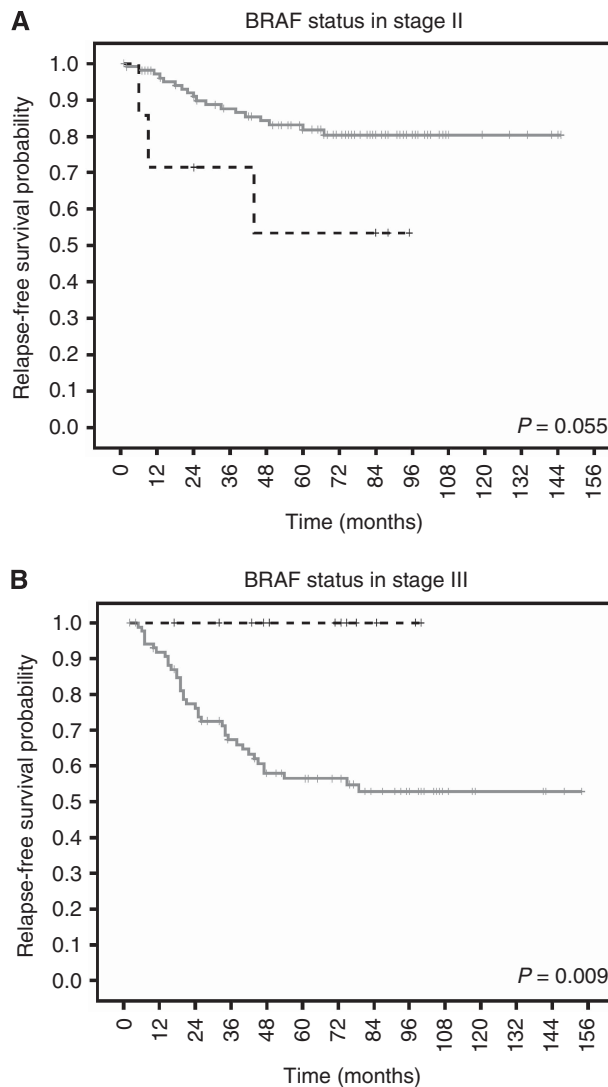


Figure 1. Relapse-free survival probability according to BRAF mutation status in stage II (A) and stage III (B) patients. Overall log-rank test adjusted for tumour staging (stages II–III), $P=0.11$. (A) Stage II patients with BRAF wt tumour (full green line, 104 patients, 18 events) or BRAF-mutated tumour (dotted black line, seven patients, three events): log-rank: $P=0.055$. Analysis restricted to the 75 stage II patients with KRAS wt tumour (68 BRAF wt, 14 events; 7 BRAF-mutated, 3 events): log-rank, $P=0.14$. (B) Stage III patients with BRAF wt tumour (full green line, 87 patients, 37 events) or BRAF-mutated tumour (dotted black line, 13 patients, 0 event): log-rank, $P=0.009$. Analysis restricted to the 63 stage III patients with KRAS wt tumour (50 BRAF wt, 18 events; 13 BRAF-mutated, 0 events) showed a similar pattern: log-rank, $P=0.022$. The full colour version of this figure is available at *British Journal of Cancer* online.

and expression markers or other tumour characteristics. None of the above genotypes had a significant impact on RFS in univariate analyses adjusted for tumour staging.

DISCUSSION

Various prognostic and/or predictive signatures based on targeted molecular markers or gene expression profiles have been tested in CRC (Van Schaeuybroeck *et al*, 2011). With the exception of dMMR status, which is reproducibly associated with a good prognosis (Popat *et al*, 2005; Bertagnolli *et al*, 2011; Hutchins *et al*, 2011;

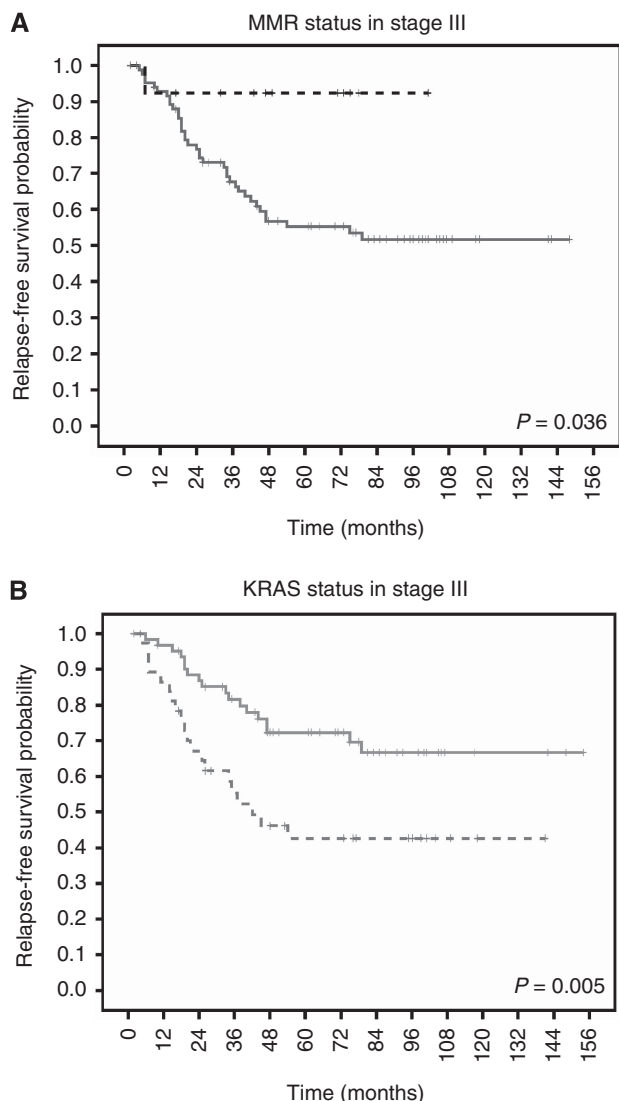


Figure 2. Relapse-free survival probability in stage III patients according to MMR status (**A**) and KRAS status (**B**). (**A**) Stage III patients with pMMR (full blue line, 85 patients, 37 events) or dMMR tumour (dotted black line, 13 patients, 1 event): log-rank, $P=0.036$. Overall log-rank test performed on stages II–III patients and adjusted for tumour staging: $P=0.18$. (**B**) Stage III patients with KRAS wt tumour (full green line, 63 patients, 18 events) or KRAS-mutated tumour (dotted red line, 38 patients, 20 events): log-rank, $P=0.005$. Relative risk = 2.40 (95% CI 1.27–4.55) for KRAS mut as compared with KRAS wt (Cox, $P=0.007$). Overall log-rank test performed on stages II–III patients and adjusted for tumour staging: $P=0.21$. Multivariate Cox analysis conducted on stages II–III patients revealed an interaction between KRAS status and tumour staging ($P=0.046$). The full colour version of this figure is available at *British Journal of Cancer* online.

Sinicrope *et al*, 2011, 2013; Roth *et al*, 2012; Sinicrope and Sargent, 2012), the clinical management of CRC is still based on clinicopathological staging (Ueno *et al*, 2012). We analysed 251 frozen tumour samples prospectively collected from stage I–III sporadic CRC patients who received or not 5FU-based chemotherapy according to physician's practice. The relevance of this study lies in the decision to administer adjuvant therapy reflecting daily therapeutic management and, more globally, in the truly prospective nature of this biological marker study. We investigated not only tumour DNA characteristics but also, less frequently, proteins that are targeted by current therapies approved in CRC,

namely, VEGFA (bevacizumab target) and EGFR (cetuximab and panitumumab target), as well as markers involved in fluoropyrimidine pharmacology, namely TS (main 5FU target), DPD (key enzyme of 5FU catabolism) and TP (involved in pyrimidine anabolism). EGFR was measured by a ligand-binding assay that quantifies high-affinity binding sites, as we previously reported that EGFR binding sites correlated with anti-EGFR efficacy *in vitro* (Magné *et al*, 2002) and that EGFR IHC analysis only partially reflects the presence of functional EGFR quantified with a binding assay (Etienne-Grimaldi *et al*, 2006). To our knowledge, this is the first such multifaceted biological prospective study conducted to date in CRC.

The frequencies of analysed somatic mutations with 12% *PIK3CA* mutations at exons 9 and 20, 33% *KRAS* mutations at codons 12–13 and 9.6% *BRAF* V600E mutations, closely matched the literature data (Hutchins *et al*, 2011; Roth *et al*, 2012; Liao *et al*, 2012). Thirty-four tumours out of the 243 analysed for MMR status exhibited the dMMR status, accounting for 14%, a figure that closely matches the literature data (Hutchins *et al*, 2011; Roth *et al*, 2012; Sinicrope and Sargent, 2012; Merok *et al*, 2013; Sinicrope *et al*, 2013). None of these 34 dMMR tumours were known HNPCC, 18 were *BRAF*-mutated indicating sporadic cancer, and among the remaining 16 *BRAF* wt only five patients were below 50-years old suggesting a possible HNPCC. According to the literature data (Van Schaeybroeck *et al*, 2011), dMMR status was significantly associated with right colon localisation, *BRAF* mutation, *P53* wt, CIMP-positive and diploidy (all $P<0.001$), and significant inter-relationships between the above-cited markers were highly consistent with each other.

None of these markers had a significant impact on RFS adjusted for tumour staging. Analyses restricted to stage II patients did not reveal significant relationships. In contrast, analyses restricted to stage III patients showed a strong tendency towards a shorter RFS in patients bearing *KRAS*-mutated tumours ($P=0.005$, Figure 2B) or *BRAF* wt tumours ($P=0.009$, Figure 1B), and a weak tendency towards a longer RFS in dMMR tumours ($P=0.036$, Figure 2A). Even though the prognostic value of *KRAS* mutation in CRC patients remains controversial (Andreyev *et al*, 1998, 2001; Richman *et al*, 2009; Ogino *et al*, 2009a; Roth *et al*, 2010; Gavin *et al*, 2012; Eklöf *et al*, 2013), a study conducted on more than 1500 stage II CRC patients from the QUASAR trial (FU/FA vs observation) reported a significantly increased risk of recurrence in *KRAS*-mutated tumours, irrespective of adjuvant chemotherapy (Hutchins *et al*, 2011). More consistent is the prognostic value of *BRAF* V600E mutation, most studies having reported a poorer overall survival in *BRAF*-mutated tumours whatever the tumour staging (Richman *et al*, 2009; Souglakos *et al*, 2009; Ogino *et al*, 2009b; Roth *et al*, 2012; Gavin *et al*, 2012), whereas two of them conducted on stage II–III colon cancer receiving 5FU-based adjuvant therapy showed that RFS was not associated with *BRAF* mutations (Roth *et al*, 2012; Gavin *et al*, 2012). Our data show that among the 13 stage III *BRAF*-mutated tumours, 9 were dMMR. This limited population did not allow us to further discriminate between the impact of *BRAF* mutation itself and dMMR status. In this regard, relevant information is provided by the study by Gavin *et al* (2012) reporting that the poor prognostic values of *BRAF* mutation and pMMR status were additive. This absence of interaction between *BRAF* and MMR status on specific survival, or overall survival, was recently confirmed (Lochhead *et al*, 2013).

Tumour samples were also analysed for the S-phase fraction and TS, TP, DPD, EGFR and VEGFA expression. None of these markers, or gene polymorphisms, had a significant impact on RFS. Numerous studies have explored the predictive/prognostic value of tumour TS expression (IHC or RT-PCR) in CRC patients receiving/not receiving 5FU-based therapy, with conflicting results (Popat *et al*, 2004). As shown in the meta-analysis by Popat *et al* (2004), a majority of studies (9 out of 13) conducted in the metastatic

Table 2. Description of tumoral expression features and significant association with classical tumour characteristics^a

	All patients	Tumour stage			Tumour localisation			Tumour histology			Ploidy	
		I	II	III	RC/TC	LC/Sig/RSJ	Rectum	Poorly/moderately differentiated	Well differentiated	Mucinous	Diploid	Aneuploid or multiploid
Membranous EGFR (fmol mg⁻¹ protein)												
Median	40				58	36	37	47	37	24		
Mean	60				66	57	51	64	57	40		
Extremes	1–516											
N	245				92	120	32	142	86	17		
Statistics		NS			P=0.009			P=0.010			NS	
Cytosolic VEGF (pg mg⁻¹ protein)												
Median	289										247	342
Mean	504										440	528
Extremes	ND to 4685											
N	251										86	137
Statistics		NS			NS			NS			P=0.029	
Cytosolic TS activity (fmol min⁻¹ per mg protein)												
Median	1074											
Mean	1712											
Extremes	ND to 12118											
N	251											
Statistics		NS			NS			NS			NS	
mRNA TS expression (a.u.)												
Median	1.51	0.97	1.63	1.45	1.79	1.39	1.23				2.12	1.32
Mean	2.25	2.25	2.61	1.82	2.65	2.13	1.57				2.87	1.68
Extremes	0.05–22.50											
N	238	29	113	96	89	117	31				83	132
Statistics		P=0.030			P=0.008			NS			P<0.001	
mRNA TP expression (a.u.)												
Median	3.75										4.18	3.53
Mean	5.45										6.33	4.93
Extremes	0.07–43.40											
N	238										83	132
Statistics		NS			NS			NS			P=0.025	
mRNA DPD expression (a.u.)												
Median	9	5.1	9.9	9.5	13.8	6.7	8.7	8	9.1	17.7	11.4	7.3
Mean	14.6	10.3	15.2	15.2	18.9	11.8	12.9	12.7	15.3	27.8	16.6	10.2
Extremes	ND to 103.5											
N	238	29	113	96	89	117	31	138	85	15	83	132
Statistics		P=0.032			P<0.001			P=0.005			P=0.002	
TP expression/DPD expression^b												
Median	0.42											
Mean	1.32											
Extremes	0.01–118											
N	238											
Statistics		NS			NS			NS			NS	

Abbreviations: a.u. = arbitrary unit; DPD = dihydropyrimidine dehydrogenase; EGFR = epidermal growth factor receptor; LC = left colon; ND = not detectable; NS = nonsignificant; RC = right colon; RSJ = recto-sigmoid junction; TC = transverse colon; TP = thymidine phosphorylase; TS = thymidylate synthase; VEGF = vascular endothelial growth factor.

^aComparisons of distribution of quantitative tumoral expressions (taken as continuous variables) as a function of categorical tumour characteristics were tested with the non-parametric Kruskal–Wallis test (for three-group comparison) or Mann–Whitney test (for two-group comparison). *P*-values are given.

^bFor the patient with non-detectable DPD expression (that is, <0.5 a.u.), we considered that DPD expression was 0.25 (that is half the detection limit) for the calculation of the ratio.

setting (5FU-treated patients), including ours (Etienne *et al*, 2002), have reported that elevated TS expression was associated with shorter OS (combined HR 1.74), whereas – in the adjuvant

setting – restricting the meta-analysis to patients treated with surgery plus 5FU-based adjuvant treatment did not show influence of TS expression on OS or PFS (pool HR 0.93 and 1,

Table 3. Significant relationships between quantitative tumour expression and tumour DNA features^a

	Allelic loss													
	MMR status		P53 mutation		BRAF mutation		8p LOH		17p LOH		18q LOH		CIMP phenotype	
	pMMR	dMMR	wt	mut	wt	mut	No	Yes	No	Yes	No	Yes	Neg.	Pos.
Membranous EGFR (fmol mg⁻¹ protein)														
N	205	32			221	23					52	62	128	28
Median	38	69			38	71					42	32	36	79
Statistics	P=0.037		NS		P=0.027		NS		NS		P=0.014		P=0.004	
Cytosolic VEGF (pg mg⁻¹ protein)														
N			125	119					44	75	53	65		
Median			258	360					251	433	280	393		
Statistics	NS		P=0.005		NS		NS		P=0.001		P=0.029		NS	
Cytosolic TS activity (fmol min⁻¹ per mg protein)														
N	209	34			226	24	69	46					130	28
Median	1040	2630			1045	3014	803	1460					1074	2624
Statistics	P=0.004		NS		P=0.032		P=0.011		NS		NS		P=0.001	
mRNA TS expression (a.u.)														
N	200	31	118	115	214	23					52	62	129	28
Median	1.39	3.07	1.98	1.23	1.43	2.63					1.74	1.13	1.41	2.13
Statistics	P<0.001		P<0.001		P=0.005		NS		NS		P=0.011		P=0.029	
mRNA TP expression (a.u.)														
N	200	31	118	115	214	23								
Median	3.48	5.77	4.17	3.35	3.59	4.80								
Statistics	P<0.001		P=0.007		P=0.029		NS		NS		NS		NS	
mRNA DPD expression (a.u.)														
N	200	31	118	115			67	45	42	72	52	62	129	28
Median	7.9	14.9	12.7	7.1			11.9	6.1	14.2	6.7	12.9	6.4	7.6	15.6
Statistics	P=0.027		P<0.001		NS		P=0.003		P=0.001		P=0.002		P=0.003	
TP expression/DPD expression^b														
N	200	31							42	72				
Median	0.41	0.55							0.34	0.47				
Statistics	P=0.029		NS		NS		NS		P=0.035		NS		NS	

Abbreviations: CIMP = CpG island methylation phenotype; dMMR = deficient mismatch repair; DPD = dihydropyrimidine dehydrogenase; LOH = loss of heterozygosity; MMR = mismatch repair; ND = not detectable; NS = nonsignificant; pMMR = proficient mismatch repair; TP = thymidine phosphorylase; TS = thymidylate synthase.

^aComparisons of distribution of quantitative tumoral expressions as a function of tumour DNA features were tested with the non-parametric Kruskal–Wallis test.

^bFor the patient with non-detectable DPD expression (that is, <0.5 a.u.), we considered that DPD expression was 0.25 (that is, half the detection limit) for the calculation of the ratio.

respectively). In contrast, the meta-analysis restricted to patients receiving surgery only showed a significant HR at 1.92 and 1.90 for OS and PFS, respectively, in patients with elevated TS expression relative to others (Popat *et al*, 2004). Thus, elevated tumour TS expression appears to be associated with poor prognosis in CRC.

The originality and strength of this study stem from the richness of the relationships between these protein markers and conventional tumour markers (Tables 2 and 3). None of the above-cited tumour proteins were significantly related to tumour staging. However, distribution of EGFR, TS and DPD expression differed according to tumour localisation (Table 2). These biological differences support recent data showing that left- and right-sided CRC are characterised by distinct clinicopathological and

molecular features (Missiaglia *et al*, 2013; Popovici *et al*, 2013; Sinicrope *et al*, 2013). Of note, a recent study conducted on 2580 stage III colon cancer patients receiving FOLFOX-based treatment reported that the favourable prognostic value of dMMR status was restricted to the right colon tumours (Sinicrope *et al*, 2013).

Importantly, dMMR tumours tend to express a higher EGFR content relative to pMMR (median 69 vs 38 units, P=0.037). Even though tumour EGFR expression measured by IHC has never been shown to be predictive of anti-EGFR monoclonal antibody (mAb) efficacy, the fact that dMMR tumours tend to express elevated EGFR levels measured by a specific binding assay, and are prone to be KRAS wt, suggests that the impact of MMR status on the efficacy of anti-EGFR mAb should be explored more thoroughly.

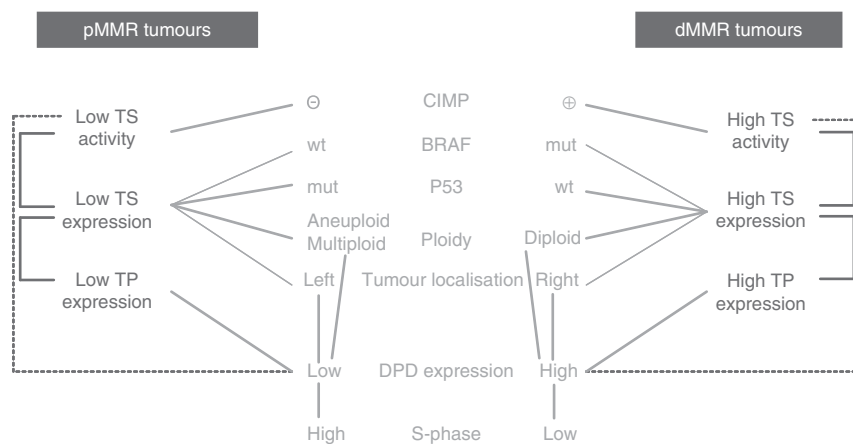


Figure 3. Exhaustive summary of tumoral protein expression (in blue) related to MMR status in the present study (TS and TP expressions, $P < 0.001$; TS activity, $P = 0.004$), and their associations with additional tumour characteristics (in orange). Connecting lines in blue indicate associations between protein expression, and connecting lines in orange indicate associations between protein expression and additional tumour characteristics. All relationships with $P \leq 0.01$ are reported. The thickness of the lines indicates the significance of the relationship (thick lines for $P \leq 0.001$; thin lines for $0.01 \leq P < 0.001$). Full lines indicate significant positive relationships. The dotted line indicates a negative correlation (inverse relationship between TS activity and DPD expressions, $r = -0.23$, $P < 0.001$). The full colour version of this figure is available at *British Journal of Cancer* online.

Given the intrinsic key role of MMR status in CRC aetiology and prognosis (Popat *et al*, 2005), we attempted to summarise tumour expression features that were related to MMR status in Figure 3. To our knowledge, present data reveal for the first time that dMMR tumours significantly exhibit elevated protein expression for TS and TP, whereas pMMR tumours express low levels of these protein markers. Of note, CIMP+ tumours, such as BRAF mut, p53 wt, diploid and right colon tumours, were prone to express high TS activity and/or expression. These observations are highly consistent with each other, considering that dMMR tumours are associated with CIMP+, BRAF mut, p53 wt, diploidy and right-side localisation. Despite a weak negative correlation between TS activity and DPD expression, dMMR tumours tend to express a two-fold higher DPD expression as compared with pMMR ($P = 0.027$, Table 3). Despite a relative low statistical power, based on a limited population of 32 dMMR tumours and 205 pMMR tumours, the present study provides original and relevant preliminary new knowledge associated with MMR status. These data would merit further confirmation on a larger set of patients.

Resistance to 5FU has been linked to TS overexpression, as reported by previous investigators (Longley *et al*, 2003) and us (Etienne *et al*, 2002). In addition, elevated intratumoural DPD expression results in an increased fluoropyrimidine catabolism at the expense of anabolism, and may cause fluoropyrimidine resistance, as recently shown by others (Gustavsson *et al*, 2009) and us at the preclinical (Beck *et al*, 1994) and clinical levels (Etienne *et al*, 1999). Two randomized trials of 5FU-based adjuvant therapy *vs* no treatment have reported that stage II–III patients bearing dMMR tumours do not benefit from 5FU-based therapy (Ribic *et al*, 2003; Sargent *et al*, 2010). In addition, a meta-analysis by Popat *et al* (2005) confirmed a significant survival advantage for 5FU-based adjuvant therapy in stage II–III pMMR patients (combined HR = 0.72, $P = 0.007$), whereas dMMR patients drew no benefit from 5FU-based treatment (combined HR = 1.24, NS). Moreover, a recent pooled retrospective study conducted on 2141 stage II–III colon cancer patients from several adjuvant trials (5FU-based treatment *vs* surgery alone) demonstrated that the subgroup of dMMR (suspected to be sporadic tumours) did not draw benefit from 5FU-based therapy (Sinicrope *et al*, 2011).

The present original data suggesting that both TS and DPD are overexpressed in dMMR tumours as compared with pMMR tumours provide a strong rationale that may explain the resistance of dMMR tumours to fluoropyrimidines, which still remain the reference therapeutic class in the chemotherapy of CRC. In conclusion, this study provides preliminary new molecular knowledge on dMMR colorectal tumours, specifically at the level of pivotal enzymes involved in 5FU pharmacodynamics. We hope the present results will be confirmed in further studies. Such confirmation may be of practical value for future optimal therapeutic management of CCR patients, as expected in the current era of personalised medicine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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