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Original Research

Impact of polymorphisms within genes involved in regulating DNA methylation in patients with metastatic colorectal cancer enrolled in three independent, randomised, open-label clinical trials: a meta-analysis from TRIBE, MAVERICC and FIRE-3

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KEYWORDS
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Abstract ***Background:*** CpG island DNA hypermethylation and global DNA hypomethylation are hallmark characteristics of colorectal cancer (CRC). Therefore, we aim to explore the effect of genetic variations within the genes that regulate the DNA methylation and demethylation pathways on outcomes in patients with metastatic CRC (mCRC) treated with first-line therapy and enrolled in three independent, randomised, open-label clinical trials.
Methods: A total of 884 patients with mCRC enrolled in TRIBE, MAVERICC and FIRE-3 trials were included. Single-nucleotide polymorphisms (SNPs) within genes involved in DNA methylation and demethylation pathways were analysed. The prognostic value of each SNP across all treatment arms was quantified using the inverse-variance–weighted effect size, a meta-analysis approach implemented in the METASOFT software.
Results: In the meta-analysis, DNMT3A rs11681717 was significantly associated with overall survival (hazard ratio = 1.26; 95% confidence interval [CI] 1.08–1.46; $P = 0.002$; false discovery rate [FDR] = 0.016), accounting for seven tests in the DNA methylation pathway. In addition, there was suggestive evidence of association for ten-eleven translocation (TET) genes variance with tumour response (TET1 rs3814177, odds ratio [OR] = 0.76, 95% CI 0.59–0.97, $P = 0.025$, FDR = 0.087; TET3 rs7560668, OR = 1.44; 95% CI 1.10–1.89; $P = 0.009$; FDR = 0.062).
Conclusions: We showed that polymorphisms within the genes responsible for the DNA methylation and demethylation machineries are correlated with outcomes in patients with mCRC who were enrolled in three independent, randomised, open-label, phase II/III clinical trials. In addition, we demonstrated the feasibility of a meta-analysis approach to identify stronger and more convincing association between gene polymorphisms and outcome, potentially leading the way to a new method of analysis for similar data set.

1. Introduction

The body of knowledge describing epigenetic abnormalities in human cancers has been largely developed over the last few decades. It is now believed that alterations in the epigenetic landscape are a hallmark of cancer. Unlike genetic mutations, the potential reversibility of epigenetic modifications encouraged the advancement of epigenetic therapies, especially, in haematologic malignancies but also in solid tumours [1].

The epigenetic biomarker research in colorectal cancer (CRC) has led to the identification of methylation markers for early detection, prediction of prognosis and treatment response. To date, some methylation markers for early detection of CRC (such as SEPT9, NDRG4 and BMP3) have been incorporated in the US Food and Drug Administration–approved commercial tests, Epi proColon® and Cologuard®, respectively [2]. In advanced disease, the availability of blood-based epigenetic biomarkers would increase tumour detection at the earlier and more treatable stage, as well as a better selection of patients who would benefit from specific treatments. However, to date, neither predictive nor prognostic biomarkers have made the translation to clinical practice.

Among epigenetic alterations, DNA methylation has been the most widely studied in CRC and considered as one of the main molecular pathways that lead to CRC development. Promoter CpG island DNA hypermethylation has been shown to promote CRC by silencing tumour suppressor gene expression. Moreover, global DNA hypomethylation is also considered a common characteristic of CRC, promoting genomic instability and proto-oncogene activation [3].

The CpG island methylator phenotype (CIMP) represents about 15% of CRCs, characterised by a unique epigenome with a high frequency of specific CpG island DNA methylation [4]. CIMP tumours have been shown to be associated with both clinical (right-sided colon cancer, female gender and older age) and molecular ($BRAF^{600E}$ mutation and microsatellite instability high) features [5]. However, the lack of a global consensus for the definition of the CIMP status and the controversial results reported in several prior studies regarding to its predictive and prognostic values [6] have impeded the application of the CIMP status in clinical practice so far.

DNA methyltransferases (DNMTs) are responsible for the transfer of a methyl group from *S*-adenosyl-L-methionine to the C-5 position of cytosine residues in DNA. Previous reports have highlighted a significant association between *DNMT3A* polymorphisms and increased risk of CRC [7], as well as the association between *DNMT3B* overexpression and CIMP-high CRC [8].

Although *DNMT3A* and *DNMT3B* are expressed at low levels in somatic tissues, both are overexpressed in human cancers, including CRC, and are thought to be involved in generating cancer-specific DNA methylation profiles [9]. DNMTs can also act as corepressors to

silence gene expression, in part through their association with histone deacetylases that help maintain chromatin in a compact and silent state [10].

On the other hand, the DNA demethylation machinery and the major players involved in this pathway have been poorly understood [11]. The ten-eleven translocation (TET) family of enzymes was discovered as the main regulators of DNA demethylation in 2009 [12,13]. The three mammalian TET proteins, namely TET1, TET2 and TET3, are Fe^{2+}- and 2-oxoglutarate–dependent dioxygenases that successively oxidise 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) and then can further oxidise 5hmC to 5-formylcytosine and 5-carboxylcytosine [14]. In addition, TET activities are increased by vitamin C, which induces DNA demethylation [15]. *TET1* downregulation and 5hmC reduction are common features of CRC [16]. *In vitro* and *in vivo* experiments showed that *TET1* downregulation is not only linked to tumour progression and malignancy but is also necessary for tumour initiation and growth in CRC, leading to a downregulation of the inhibitors of the Wnt pathway, that eventually is constitutively activated [17]. In addition, 5hmC, the main product of TET enzymes, was shown to regulate gene expression during colon cell differentiation and controls gene expression in human colon cancers [18].

The impact of the genes involved in the DNA methylation and demethylation machineries on prognosis in metastatic CRC (mCRC) has not been well established. Therefore, the significant role of epigenetic modifications in CRC development and progression, together with these promising preclinical data, led us to explore the effect on outcomes of genetic variations within these genes in mCRC patients treated with first-line therapy and enrolled in three independent, randomised, open-label clinical trials.

2. Materials and methods

2.1. Patient population and study design

A total of 884 patients with mCRC enrolled in randomised, open-label TRIBE (NCT00719797) [19], MAVERICC (NCT01374425) [20] and FIRE-3 (NCT00433927) [21] trials were included in this study. Patients were randomly assigned to two different treatment arms in each trial. In the TRIBE trial, patients received either FOLFIRI plus bevacizumab or FOLFOXIRI plus bevacizumab; in MAVERICC trial, they received either FOLFIRI plus bevacizumab or mFOLFOX6 plus bevacizumab and in FIRE-3 trial, they received either FOLFIRI plus bevacizumab or FOLFIRI plus cetuximab.

All patients provided informed consent for the molecular analysis, and local ethics committees for each participating site approved this study.

2.2. Selected polymorphisms and genotyping

Single-nucleotide polymorphisms (SNPs) within genes involved in DNA methylation and demethylation pathways were selected according to two major criteria: (1) minor allele frequency in Caucasians ≥10% (www.ensembl.org) and (2) potential role in changing gene function based on public databases (https://snpinfo.niehs.nih.gov; https://www.ncbi.nlm.nih.gov). Linkage disequilibrium (LD) among selected SNPs was identified through SNAP search service (http://archive.broadinstitute.org/mpg/snap/).

Genomic DNA from blood samples was extracted using the QIAmp DNA easy kit (Qiagen, Valencia) and then genotyped through the OncoArray, which is a custom array manufactured by Illumina (San Diego, CA, USA), including 530K SNP markers [22]. Fourteen functional SNPs within ten genes involved in DNA methylation (*DNMT1/3A/3B* and *MDM2*) and demethylation pathways (*TET1/2/3, IDH1/2* and *MBD4*) were analysed. The characteristics of these SNPs are depicted in Supplementary Table S1.

2.3. Statistical analysis

SNP variation within DNA methylation and demethylation pathways was evaluated for association with mCRC patient outcomes, overall survival (OS), progression-free survival (PFS) and tumour response (TR). OS was defined as time from randomisation until death from any cause. Patients still alive were censored at the last date of follow-up. PFS was defined as time from randomisation until disease progression, death or until the last follow-up in patients who were alive and remained free of disease progression. TR was defined as the percentage of patients who achieved either a complete or a partial remission according to the Response Evaluation Criteria in Solid Tumours classification. Associations between SNPs and clinical outcomes were estimated separately for each treatment arm. Survival outcomes were modelled using Cox proportional hazards regression, and TR was modelled using logistic regression. Regression models included the following study-specific adjustment covariates; the TRIBE trial included gender, age, Eastern Cooperative Oncology Group (ECOG) performance status, primary tumour site, liver-limited disease, adjuvant chemotherapy, BRAF status and RAS status; the MAVERICC trial included age, ECOG performance status, the number of metastases and primary tumour resected and the FIRE-3 trial included gender, ECOG performance status, liver-limited disease and BRAF status. Models also included the first three principle components of the European ancestry SNPs, computed separately for each cohort. SNPs were coded using an additive genetic model for the number of variant alleles, i.e., the

common homozygote is represented by 0, the heterozygote by 1 and the variant homozygote by 2.

All treatment arms differed in either population or treatment, leaving no natural division of the data into discovery vs validation sets. Therefore, we took the alternative approach of jointly evaluating cohort-specific results in a meta-analysis. This approach enabled us to evaluate (1) evidence of a common effect across cohorts, which would imply a prognostic effect, and (2) evidence of differences in an effect between cohorts, which would imply either predictive effects or population-specific effects. The prognostic value of each SNP across all treatment arms was quantified using the inverse-variance–weighted effect size estimate [23], which is most powerful when the magnitude of the effect size is the same across studies, implemented in the METASOFT software [24]. We also tested for heterogeneity of effects across arms using Cochran's Q statistic. In addition, we jointly evaluated sets of SNPs corresponding to DNA methylation/demethylation pathways to identify multiple weak effects across multiple SNPs, studies and treatment arms. Pathway tests were conducted using a statistically powerful approach called Pegasus [25] applied to the results from the meta-analyses. The Pegasus approach combines p-values for SNP-outcome association tests, modelling dependencies from independent LD estimates. Here, pathway tests were conducted using meta-analysis results, and LD among SNPs was estimated from 1000 Genomes data [26].

To investigate possible SNP effect modification by gender, primary tumour location, RAS or KRAS status, interaction terms were included in the regression models described previously and evaluated using Wald tests. For each pathway, multiple testing was accounted for using Benjamini-Hochberg false discovery rate (FDR) control at the 0.05 level. All analyses were performed using the SAS statistical package, version 9.4 (SAS Institute, Cary) and R, version 3.4.0.

3. Results

3.1. Patient characteristics

A total of 884 patients with mCRC from six treatment arms of three independent phase III trials were included in this study. Some component studies differed in treatment and other patient characteristics, such as age, performance status, primary tumour site, the number of metastases, primary tumour resected and RAS status (Table 1). Median follow-up and median survival times are summarised in Table 2.

3.2. Clinical outcomes

Nominally significant P-values ($P < 0.05$) from association tests did not appear to cluster by SNP, outcome or treatment arm (Fig. 1); however, SNPs in *DNMT* and *TET* genes (*DNMT1*, *DNMT3A* and *TET1*) did meet the significance level for association with at least one of the three outcomes after FDR adjustment.

In the TRIBE FOLFIRI/bevacizumab (bev) arm, *DNMT3A* rs11681717 and *TET1* rs3814177 had significant associations with OS ($P = 0.001$, FDR = 0.010) and TR ($P = 0.008$, FDR = 0.056), respectively. In the TRIBE FOLFOXIRI/bev arm, *DNMT3A* rs2276598 was suggestively significantly associated with TR ($P = 0.012$, FDR = 0.087). In the MAVERICC mFOLFOX6/bev arm, *DNMT1* rs2228611 was significantly associated with TR ($P = 0.001$, FDR = 0.007) (Fig. 1, Supplementary Tables S2 and S3).

The meta-analysis of effects of gene polymorphisms on outcomes across treatment cohorts yielded a single SNP within *DNMT3A*, rs11681717, that achieved the 0.05 FDR level and was therefore considered significant (Table 3). rs11681717 was associated with OS across the six treatment cohorts (meta-analysis hazard ratio = 1.26, 95% confidence interval [CI] 1.08–1.46, $P = 0.002$, FDR = 0.016), accounting for seven tests in the DNA methylation pathway. This effect appears to be driven by the results from the TRIBE study (Fig. 2A), where variant alleles were associated with shorter survival. In addition, there was suggestive evidence of association for *TET* gene variance with TR (*TET1* rs3814177, OR = 0.76, 95% CI 0.59–0.97, $P = 0.025$, FDR = 0.087 [Fig. 2B]; *TET3* rs7560668, OR = 1.44, 95% CI 1.10–1.89, $P = 0.009$, FDR = 0.062 [Fig. 2C]).

Pathway analysis suggested that genetic variation in the DNA demethylation pathway was associated with both TR and PFS (TR, $P = 0.016$; PFS, $P = 0.038$; OS, $P = 0.22$). These results are partially explained by associations involving the *TET1* SNP rs3814177, which is significant at the $P < 0.05$ level in the meta-analysis for both these outcomes. Of note, *TET1* rs3814177 has a negative coefficient for TR and positive coefficients for PFS and OS.

Pathway tests using the Pegasus approach for effects across multiple SNPs were not significant for the DNA methylation pathway (TR, $P = 0.90$; PFS, $P = 0.16$; OS, $P = 0.13$). None of the SNPs analysed here exhibited evidence of heterogeneity of effects across the six treatment arms after accounting for multiple tests (Table 3).

4. Discussion

Here, we demonstrate that SNPs in DNA methylation and demethylation pathways are significantly correlated with clinical outcomes in patients with mCRC.

Genome-wide association studies (GWASs) have spread our knowledge in terms of novel pathways with specific roles in carcinogenesis, and the application of GWASs has provided opportunities for drug

Table 1
Baseline characteristics of patients enrolled in the six treatment arms within the three clinical trials analysed.

Characteristics	Total N = 884	TRIBE N = 324		MAVERICC N = 324		FIRE3 N = 236		*P* value[a]
		FOLFIRI BEV N = 215	FOLFOXIRI BEV N = 109	FOLFIRI BEV N = 163	mFOLFOX6 BEV N = 161	FOLFIRI BEV N = 107	FOLFIRI CET N = 129	
Sex								0.063
Male	571	132(61%)	66(61%)	103(63%)	101(63%)	70(65%)	99(77%)	
Female	313	83(39%)	43(39%)	60(37%)	60(37%)	37(35%)	30(23%)	
Age								0.005
≤65	589	156(73%)	78(72%)	101(62%)	117(73%)	62(58%)	75(58%)	
>65	295	59(27%)	31(28%)	62(38%)	44(27%)	45(42%)	54(42%)	
Performance status								<0.001
ECOG 0	586	177(82%)	95(87%)	97(60%)	81(50%)	56(52%)	80(62%)	
ECOG 1	296	37(17%)	14(13%)	66(40%)	79(49%)	51(48%)	49(38%)	
Unknown	2	1(1%)	0(0%)	0(0%)	1(1%)	0(0%)	0(0%)	
Primary tumour site								<0.001
Right-sided	261	53(25%)	30(28%)	67(41%)	64(40%)	25(23%)	22(17%)	
Left-sided	599	147(68%)	73(67%)	96(59%)	97(60%)	81(76%)	105(81%)	
Unknown	24	15(7%)	6(6%)	0(0%)	0(0%)	1(1%)	2(2%)	
Number of metastases								<0.001
≤2	657	178(83%)	89(82%)	106(65%)	101(63%)	83(78%)	100(78%)	
>2	219	37(17%)	20(18%)	57(35%)	60(37%)	20(19%)	25(19%)	
Unknown	8	0(0%)	0(0%)	0(0%)	0(0%)	4(4%)	4(3%)	
Liver-limited disease								0.64
No	379	150(70%)	70(64%)	NA	NA	75(70%)	84(65%)	
Yes	181	65(30%)	39(36%)	NA	NA	32(30%)	45(35%)	
Primary tumour resected								<0.001
No	447	80(37%)	31(28%)	153(94%)	148(92%)	12(11%)	23(18%)	
Yes	437	135(63%)	78(72%)	10(6%)	13(8%)	95(89%)	106(82%)	
Adjuvant chemotherapy								0.081
No	760	188(87%)	94(86%)	143(88%)	146(91%)	86(80%)	103(80%)	
Yes	123	27(13%)	15(14%)	20(12%)	15(9%)	21(20%)	25(19%)	
Unknown	1	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	1(1%)	
RAS status								<0.001
Wildtype	233	50(23%)	34(31%)	NA	NA	66(62%)	83(64%)	
Mutant	203	110(51%)	57(52%)	NA	NA	17(16%)	19(15%)	
Unknown	124	55(26%)	18(17%)	NA	NA	24(22%)	27(21%)	
BRAF status								0.59
Wildtype	432	168(78%)	88(81%)	NA	NA	81(76%)	95(74%)	
Mutant	30	10(5%)	9(8%)	NA	NA	4(4%)	7(5%)	
Unknown	98	37(17%)	12(11%)	NA	NA	22(21%)	27(21%)	

BEV, bevacizumab; CET, cetuximab; ECOG, Eastern Cooperative Oncology Group.
Unknown group is not included in the analysis.
[a] *P*-value is based on the Chi-square test.

Table 2
Follow-up and survival time summary.

	TRIBE N = 324		MAVERICC N = 324		FIRE3 N = 236	
	FOLFIRI BEV N = 215	FOLFOXIRI BEV N = 109	FOLFIRI BEV N = 163	mFOLFOX6 BEV N = 215	FOLFIRI BEV N = 107	FOLFIRI CET N = 129
Follow-up						
Median (months)	48.9	54.5	23.3	26.8	26.7	29.1
PFS						
Median (months)	9.7	10.8	12.5	10.1	11.5	12.8
OS						
Median (months)	26.2	26.0	27.4	24.7	31.4	49.8

PFS, progression-free survival; OS, overall survival; BEV, bevacizumab; CET, cetuximab.

discovery and for cancer prevention [27]. However, as single GWAS has normally low sample size and low statistical power, the validation in independent cohorts or the conduction of a meta-analysis increases power and reduces false-positive findings [28]. Although this approach is a current method of choice to pinpoint the genetic variations predisposition to complex disorders [29], including CRC [30], we are the first to explore its

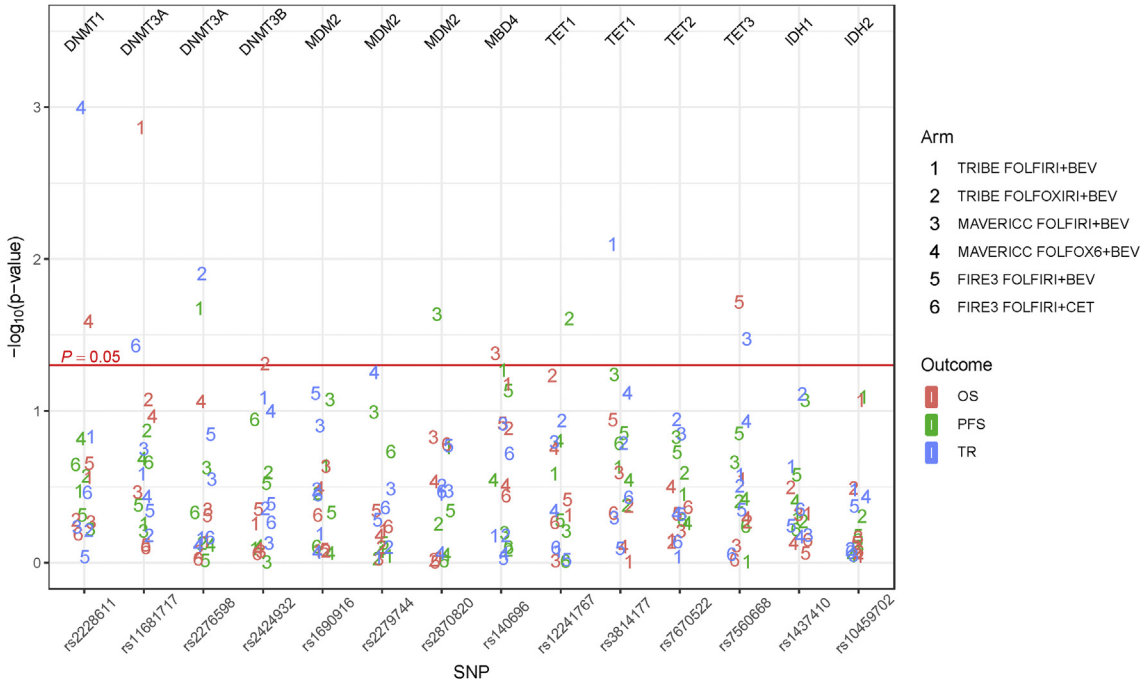

Fig. 1. P-values for associations between SNPs and outcomes (TR, PFS and OS) in six treatment arms. P-values were generated from likelihood ratio tests of association for each SNP, coded additively for the minor allele, and each outcome. OS and PFS were modelled using Cox proportional hazards regression, and TR was modelled using logistic regression. Adjustment covariates varied across studies (see Methods). TR, tumour response; PFS, progression-free survival; OS, overall survival; SNP, single-nucleotide polymorphism.

Table 3
Meta-analysis results for tumour response, PFS and OS for SNPs in methylation and demethylation pathways.

SNPs	Tumour response			Progression-free survival			Overall survival		
	P value for FE	Q Statistics	*P* value for Q	*P* value for FE	Q Statistics	*P* value for Q	*P* value for FE	Q Statistics	*P* value for Q
Methylation pathway									
DNMT1 rs2228611	0.460	13.841	**0.017**	0.927	6.434	0.266	0.784	8.600	0.126
DNMT3A rs11681717	0.920	8.994	0.109	0.200	4.974	0.419	**0.002 (0.016)**	7.626	0.178
DNMT3A rs2276598	0.760	9.876	0.079	0.185	5.699	0.337	0.577	3.866	0.569
DNMT3B rs2424932	0.426	6.870	0.230	0.901	5.006	0.415	0.221	3.450	0.631
MDM2 rs1690916	0.324	6.571	0.255	0.119	3.513	0.621	0.695	2.886	0.718
MDM2 rs2279744	0.741	5.626	0.344	0.271	3.380	0.642	0.815	1.357	0.929
MDM2 rs2870820	0.730	5.526	0.355	0.052	4.149	0.528	0.540	4.762	0.446
Demethylation pathway									
MBD4 rs140696	0.340	3.612	0.607	0.427	7.786	0.168	0.949	14.065	**0.015**
TET1 rs12241767	0.367	4.311	0.505	0.091	6.111	0.296	0.129	4.720	0.451
TET1 rs3814177	**0.025 (0.087)**	8.411	0.135	**0.041**	6.776	0.238	0.120	2.607	0.760
TET2 rs7670522	0.058	2.223	0.817	0.705	6.584	0.253	0.528	2.243	0.815
TET3 rs7560668	**0.009 (0.062)**	3.003	0.699	0.207	3.901	0.564	0.178	5.733	0.333
IDH1 rs1437410	0.807	5.772	0.329	**0.034**	1.550	0.907	0.432	1.669	0.893
IDH2 rs10459702	0.945	2.503	0.776	0.316	2.867	0.720	0.163	2.348	0.799

PFS, progression-free survival; OS, overall survival; SNPs, single-nucleotide polymorphisms; FDR, false discovery rate.
FE indicates fixed effects inverse-variance–weighted estimates for allelic effects that are common across studies. Q denotes Cochran's Q statistic for assessing heterogeneity, differences in effect estimates across treatment cohorts. In bold, P-values that achieved a nominal 0.05 significance level. Adjusted *P* values after FDR achieved 0.1 level are shown in the parentheses.

application to investigate the impact of genetic variants on outcomes in patients affected with mCRC. The originality of this study lies in the fact that we applied a meta-analysis approach to identify the association between functional candidate SNPs and outcomes in a large cohort of patients with mCRC. In addition, a common approach for identifying genomic elements associated with complex traits is to evaluate combinations of variants in known pathways; therefore, we applied a novel statistically powerful method (called Pegasus), which has been recently shown to outperform the other existing methods of gene scores [25], to analyse

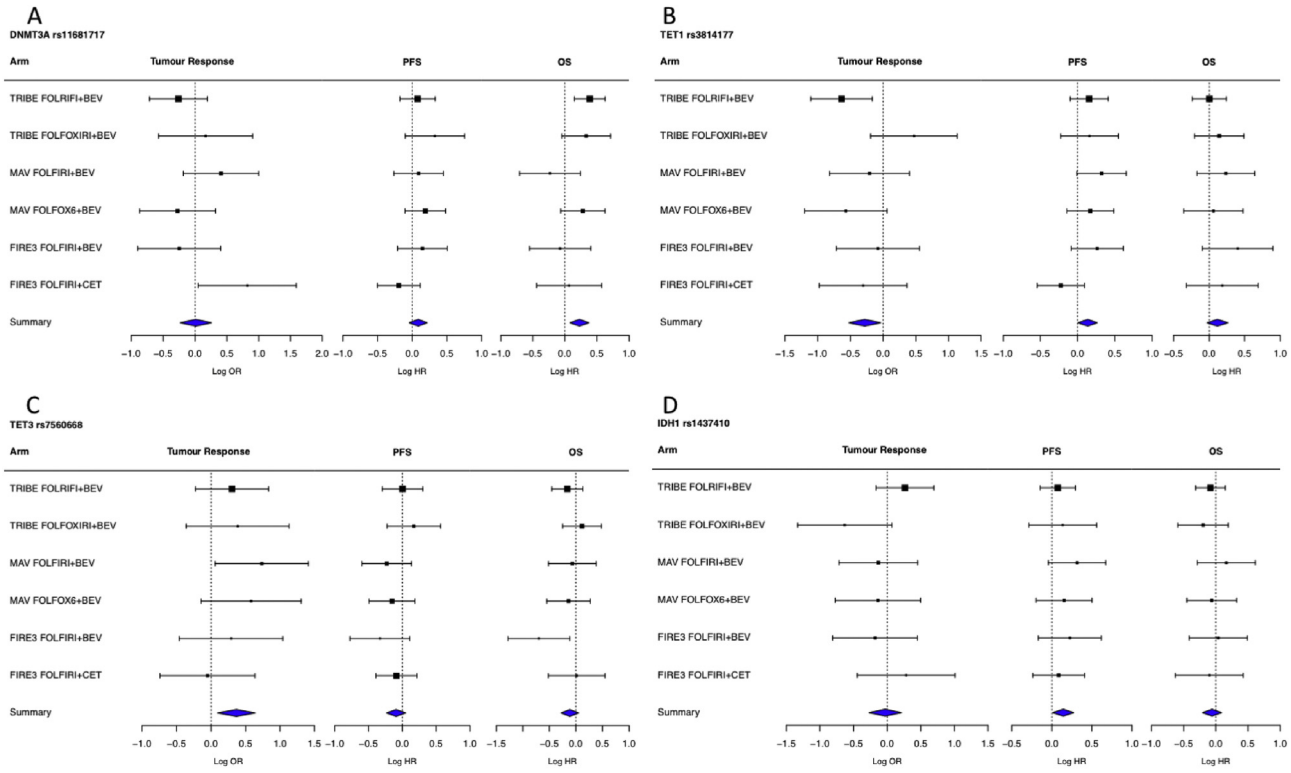

Fig. 2. Forest plots of meta-analysis results for the four significant genes. Log odds ratios (ORs) or log hazard ratios (HRs) are shown with 95% confidence intervals (CIs). The summary row shows the inverse-variance–weighted effect size with 95% CIs, combining the six estimates for the individual arms into a single summary measure. A positive log OR or HR implies a negative influence on TR or survival, respectively. SNPs with statistically significant or suggestive summary effects are plotted here. (A) *DNMT3A* rs11681717 was significantly associated with OS (HR = 1.26, 95% CI 1.08–1.46, $P = 0.002$, FDR adjusted $P = 0.016$). (B) *TET1* rs3814177 was suggestively associated with TR (OR = 0.76, 95% CI 0.59–0.96, $P = 0.025$, FDR adjusted $P = 0.087$) and PFS (HR = 1.14, 95% CI 1.01–1.30, $P = 0.041$, FDR adjusted $P = 0.14$). (C) *TET3* rs7560688 was associated with TR (OR = 1.44, 95% CI 1.10–1.89, $P = 0.009$, FDR adjusted $P = 0.062$). (D) *IDH1* rs1437410 was suggestively associated with PFS (HR = 1.15, 95% CI 1.01–1.31, $P = 0.034$, FDR adjusted $P = 0.14$). SNPs, single-nucleotide polymorphisms; OS, overall survival; FDR, false discovery rate; PFS, progression-free survival; TR, tumour response.

the effect of methylation and demethylation pathways on outcomes in this cohort of patients.

To perform our meta-analysis, we applied the additive model that assumes that there is a uniform, linear increase in risk for each copy of a specific allele. A common practice for GWASs is to examine additive models only as the additive model has reasonable power to detect both additive and dominant effects [31]. The importance and the clinical impact of these types of analysis have been recently highlighted by Nelson *et al.* [32], who estimated that drug mechanisms with genetic support would succeed twice as often as those without it, leading to lower rates of failure due to the lack of efficacy in clinical development.

Herein, we showed that *DNMT3A* rs11681717 is significantly associated with worse OS in mCRC and might serve as a prognostic factor for these patients (Table 3 and Fig. 2).

The expression of *DNMT3A* has been shown to be an independent poor prognostic indicator both in gastric [33] and in lung cancer [34]. Accordingly, *DNMT3A* mutations are highly recurrent in acute myeloid leukaemia and are independently associated with a poor outcome [35]. Recently, Lin *et al.* [36] showed a higher *DNMT3A* expression in CRC compared with normal tissue and demonstrated that *DNMT3A* mediates the function of HIF1A-AS2 (a long non-coding RNA), which affects cell proliferation, invasion and epithelial–mesenchymal transition in CRC. In addition, *DNMT3A* has been shown to be a direct target of miR-143, which is frequently downregulated in CRC, resulting in an upregulation of *DNMT3A* [37]. The restoration of the miR-143 expression in colon cell lines decreased tumour cell growth and soft agar colony formation and downregulated the *DNMT3A* expression in both mRNA and protein levels, highlighting the tumour-suppressive role of miR-143 in CRC development [37].

It is widely established that Wnt signalling is a hallmark of CRC, and *APC* mutations represent the main mechanism by which the Wnt pathway is constitutively activated. However, epigenetic silencing of Wnt inhibitors by DNA hypermethylation has also been observed as another potential mechanism of Wnt pathway disruption [38]. Accordingly, an elevated

DNMT3A expression coincides with repressed *SFRP5*, a Wnt antagonist, leading to an upregulation of the Wnt pathway, eventually responsible for CRC development and progression [39]. Noteworthily, the upregulation of *DNMT3A* and *DNMT3B* has been reported as a feature of the colorectal adenoma–carcinoma sequence [40], suggesting that epigenetic modifications are an early event in CRC development. Taken together, our findings add another proof of the importance and the critical role of DNMT3A, and more widely by the aberrant DNA methylation, in CRC development, progression and prognosis in patients with mCRC.

We showed that *TET1* rs3814177 and *TET3* rs7560668 were significantly associated with outcomes, highlighting their potential role in prognosis in patients with mCRC; thus, further studies are warranted to investigate whether they may be used as potential targets for drug development. As mentioned previously, α-ketoglutarate (α-KG) is required for TET protein function. α-KG is provided by isocitrate dehydrogenase (IDH) enzymes through oxidation of isocitrate. Therefore, the TET and IDH functions are strongly correlated. Although *IDH* and *TET* mutations do not seem to be common in CRC, downregulated *TET1* expression is an early event in cell transformation and has been related to colon cancer growth by leading to a constitutive activation of the Wnt pathway [17]. Interestingly, vitamin C regulates both DNA and histone demethylation, as an essential cofactor for TET dioxygenases and JMJC domain–containing histone demethylases [41,42]. Vitamin C has gained widespread attention in the last few years owing to its impact on CRC. It selectively kills KRAS- and BRAF-mutated CRC cells, leading the cell towards metabolic stress and eventual apoptosis [43]. Furthermore, vitamin C acts as an effector of 5-Aza-CdR (decitabine)-based DNA demethylation. Combining vitamin C with 5-Aza-CdR treatment of cancer cells results in a synergistic boost in DNA demethylation as both active and passive mechanisms of DNA demethylation are activated [44].

Finally, the pathway tests using the Pegasus approach for effects across multiple SNPs demonstrated the association of the DNA demethylation pathway with outcomes in terms of TR ($P = 0.016$) and PFS ($P = 0.038$), which is most likely due to the effects of TET1 variant. However, no significance was shown for the DNA methylation pathway. These results warrant some caution in the interpretation, and further studies will be needed to confirm our findings.

Immunotherapy has shown striking results in several cancer types, although only small subsets of patients benefit from these drugs. Recent studies have demonstrated the mutual relation between DNA methylation and immune system: DNA demethylating drugs, i.e. 5-azacitidine, can enhance CTLA-4 blockade-mediated T-cell responses [45] and promote a significant enrichment of immunomodulatory pathways [46]. Recently, Kato *et al.* [47] showed that *DNMT3A* alterations were associated with poorer clinical outcomes (time-to-treatment failure <2 months) in patients treated with immune checkpoint inhibitors, highlighting, once again, the strong and intertwined relationship between DNA methylation and immunotherapy.

Given the extraordinary potential of DNA methylation inhibitors as therapeutic agents, several phase I/II clinical trials in patients with mCRC are ongoing to investigate the efficacy of azacitidine, decitabine or SGI-110 in combination with standard chemotherapy, immune checkpoint inhibitors or anti–epidermal growth factor receptor antibodies, suggesting that epigenetic therapies may become an exciting new option for patients with CRC in the near future.

5. Conclusion

Epigenetic aberrations, primarily DNA methylation and demethylation, are considered to be a crucial driver of CRC development and progression. Here, we showed that polymorphisms within the genes responsible for the DNA methylation and demethylation machineries, especially *DNMT3A* rs11681717, are correlated with outcomes in patients with mCRC who were enrolled in three independent, randomised, open-label, phase II/III clinical trials. Our results highlight the strong impact that these pathways have on prognosis for patients with mCRC. In addition, we demonstrated the feasibility of a meta-analysis approach to identify stronger and more convincing association between gene polymorphisms and outcome, potentially leading the way to a new method of analysis for similar data sets.

Conflict of interest statement

Daniel J. Weisenberger is a consultant for Zymo Research Corporation. All other authors declare no conflict of interest.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.01.105.

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