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Colorectal cancer patient outcome is influenced by tumour-adherent bacteria through epigenetic regulation of cardinal genes including PHLPP1

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SUMMARY

Objective How tumour-adherent microbiome can influence outcomes in patients with colorectal cancer (CRC).

Methods and analysis CRC tissues (n=97) were analysed using RNAseq, whole exome sequencing (WES) and 16 r sRNA sequencing. Inflammatory/immune cells were quantified by immunohistochemistry in mice and human tissues. Patient outcomes were analysed according to virulent bacteria and gene RNA levels in tumour tissues. We performed faecal microbiota transfer (FMT) in germ-free mice using presurgery stool samples from nine patients with sporadic CRC. Changes in gene mutations and DNA methylation in the colonic mucosa were measured and compared with those in mice receiving FMT from nine healthy individuals. Immune cells in the tumour tissues of donor and mouse recipients were compared.

Results FMT from CRC donors led to preferential DNA demethylation, but specific host genes, including pleckstrin homology domain and leucine-rich repeat protein phosphatase 1 (PHLPP1), were hypermethylated compared with FMT from healthy donors. CRC faecal samples showed elevated levels of virulent bacteria, reduced levels of commensals and triggered colon inflammation in mice. Notably, PHLPP1 was downregulated in CRC tumours, and its expression varied in accordance with other CRC cardinal genes (n=137, as identified in the scientific literature to be involved in colon carcinogenesis), and was most pronounced in inflammatory bowel disease associated CRC. Tumours with reduced PHLPP1 expression are more inflammatory and correspond to patients with a higher relative abundance of adherent virulent bacteria and poorer outcomes.

Conclusion The composition of CRC tumour-adherent bacteria predicts epigenetic modifications and altered expression of cardinal CRC genes such as PHLPP1. PHLPP1 downregulation characterises patients with CRC with inflammatory tumours and poor survival outcomes.

INTRODUCTION

Colorectal cancer (CRC) is among the three most common cancers, causing over 600 000 deaths annually worldwide.¹ The majority of

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Colorectal cancer (CRC) is one of the most prevalent cancers in humans. The majority of them are sporadic, suggesting a major causative role for environmental factors.
- ⇒ Gut microbiota analysis is becoming a pivotal source for identifying these factors and the mechanism they involve.
- ⇒ However, studies including all CRC types to decipher putative role of environment versus host gene parameters are lacking.

WHAT THIS STUDY ADDS

- ⇒ By analysing microbiome and tumour tissues of 97 patients with CRC, including sporadic, inflammatory bowel disease (IBD), and Lynch syndrome associated CRCs representing both hereditary and presumed environment-related CRCs, we identified bacteria as well as host genes associated with tumour growth and clinical outcomes of patients.
- ⇒ The pleckstrin homology domain and leucine-rich repeat protein phosphatase 1 (PHLPP1), a tumour suppressor gene that regulates signalling pathways associated with cell growth, survival and inflammation, is epigenetically regulated and transcriptionally downregulated in patients with CRC infected with tumour-adhering virulent bacteria. Reduced PHLPP1 expression is indicative of poor overall patient survival and is common in IBD-associated CRC, as well as a subset of sporadic CRCs. Faecal microbiota transfer from patients with CRC to germ-free mice causes hypermethylation of the PHLPP1 gene and its transcriptional downregulation, corresponding to an increased incidence of pre cancerous lesions.
- ⇒ These findings support the notion that virulent bacteria influence the outcome of patients with CRC by epigenetically inhibiting tumour suppressor genes, as exemplified by PHLPP1.



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CRCs are sporadic, and only less than 5% of cases involve germline mutations (eg, familial adenomatous polyposis coli or Lynch

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Our results suggest combined testing including bacteria and host gene markers should be validated through mass screening or a diagnostic approach in high-risk patients with IBD.
- ⇒ A panel of methylated genes identified in circulatory DNA in patients with CRC may be used during the surveillance period after therapy.
- ⇒ The modulation of microbiota in individuals with high risk of cancer should be evaluated as a tool to prevent cancer.

syndrome - LS- in the mismatch repair gene system), suggesting a major causative role for environmental factors.

The genetic events that cause sporadic CRCs are used to classify CRCs and determine preferred therapeutic strategies. However, a subset of patients with CRC experiences accelerated disease progression, possibly linked to the expression of immune checkpoint molecules by regulatory T cells (Tregs) and resistance to medical therapies including immune checkpoint inhibitors.² In addition to genetic mutations, epigenetic events contribute to cancer susceptibility. The high frequency of aberrant DNA methylation in specific Cytosine phosphate Guanine (CpG) islands defines a subtype of CRCs named the CpG island methylator phenotype that evolves high tumorous mutation levels and response to therapy.^{2,3}

The epigenome connects the microbiome to host gene expression, helping organisms sense and respond to environmental changes with significant pathophysiological roles in the aetiology of CRC⁴ as they may silence the expression of tumour suppressor genes and directly impact cancer initiation and progression. Microbial metabolites like short-chain fatty acids (SCFAs) and virulence proteins act as epigenetic regulators, influencing host gene expression and epigenetic programming. SCFAs modulate the host epigenome via DNA methylation and histone modifications, impacting the host.⁵ We found that several gene promoters are hypermethylated in the tumour tissues and blood of patients with CRC in a microbial-dependent manner and tested their use as candidate biomarkers for CRC screening.⁶ Gut microbiota dysbiosis is characterised by a panel of 20 bacterial species that are strongly associated with CRC development⁷ through an epigenetic pathway^{6,8} and could influence the response to therapies.

In inflammatory bowel disease (IBD), another emerging GI burden that enhances the risk of developing CRC, the gut microbiota has been shown to enhance inflammatory cell infiltrates and hypermethylation of tumour suppressor genes.^{9–11} These findings prompted our investigation into bacterial-mediated DNA changes that lead to the downregulation of tumour suppressor genes in CRC. We investigated this in tumour tissues in patients with CRC and in the colonic mucosa of germ-free mice after faecal microbiota transfer (FMT). Notably, we focused on a well-studied tumour suppressor gene, pleckstrin homology domain and leucine-rich repeat protein

phosphatase 1 (PHLPP1). PHLPP1 is a Ser/Thr phosphatase that negatively regulates signalling by protein kinases such as PKC, Akt/mTOR, ERK1/2 and STAT1, affecting cell survival, growth, senescence, autophagy and inflammation, and is considered a tumour suppressor gene in colon carcinogenesis.^{12–16} Downregulation of PHLPP1 has been reported in a wide range of gastrointestinal carcinomas,¹⁷ particularly CRC, where its loss of function contributes to cancer metastasis and therapy resistance.^{14, 18–20} Genetic ablation of PHLPP1 exacerbates tumour growth and invasion.¹⁴ The molecular mechanism responsible for the downregulation of PHLPP1 in CRC is suggested to be DNA methylation of the promoter.²¹

Here, we report differential DNA methylation of PHLPP1 in the colonic mucosa of germ-free mice after FMT from patients with CRC compared with FMT from healthy donors. We investigated tumour tissues of 97 patients with CRC, including sporadic CRC, IBD-associated CRC and LS-associated CRCs, and found increased ratios of virulent relative to commensal tumour adherent bacteria correlated with intratumour downexpression of the PHLPP1 gene, increased deoxythymidine monophosphate (dTMP), a precursor of DNA synthesis, chronic inflammation and poor patient outcomes. We conclude that enrichment of virulent microbiota in the CRC gut causes downregulation of PHLPP1 gene expression, contributing to worse disease outcomes.

METHODS**Subjects and materials**

Patients referred to university hospitals for colonoscopy were enrolled in several prospective cohorts as described.⁶ Cohort studies were financed by the French government (INCA, PHRC, ANR announcements). All regulatory processes were performed prior to the first subject being enrolled, colonoscopy results were registered and participants signed an informed consent form. Endoscopy and/or surgical tissue samples (tumour and normal neighbours) were obtained and stored in either formalin or azote liquid until preparation for analysis.

DNA and RNA isolation and quantitative PCR

Tumour tissues and normal neighbours were homogenised with QIA shredders prior to DNA and RNA isolation using the AllPrep DNA/RNA mini kit (Qiagen). RNA was reverse transcribed using a cDNA reverse transcription kit (Applied Biosystems) and quantified by the SYBR Green PCR mix (Applied Biosystems) system with GAPDH expression for normalisation. The primers and methods are described elsewhere.⁵

Microbiota analysis

The 16S rRNA gene sequencing was performed on tissue DNA samples (extraction using GNOME DNA Isolation Kit-MP Biomedicals) as previously described,²² and after amplification by PCR of the V3 to V4 region submitted to a 250bp paired-end sequencing protocol on the

Illumina MiSeq platform. Raw FASTQ files were demultiplexed, quality-filtered and assigned using Qiime2 with the SILVA-123 database. The intergroup high similarity and intragroup low similarity of the microbiota were assessed by β -diversity and PCoA (generated by Qiime using unweighted UniFrac metrics). We subjected the study populations to the Qlucore program analysis for differences between different CRC patient subgroups. All microbiome statistical tests were performed using packages or the Shaman Webserver, with sex, age and BMI adjusted for multiple testing.

DNA methylation using methylation chips

The GoldenGate Methylation Cancer Panel I (Illumina) was used to probe 500 ng of each bisulfite-converted DNA sample of humans (n=18, 9 patients with CRC and 9 controls), including tissue (n=18), stool (n=18) and blood (n=18). Stool samples were used for FMT in germ-free mice and methylation levels ranging from 0% to 100.0% were as described.⁶ A bisulfite-converted universal human methylated DNA standard (Zymo Research) served as a calibrator, and positive control and DNA-free distilled water as a negative control.

Metabolomic analysis on tumour tissue sections

To analyse metabolites in the tumour tissues, they were immediately flash-frozen following collection and then stored at -80°C until processed for metabolomics. This analysis was performed in 34 sporadic CRC patient samples. Tissues were visualised to obtain the tumour area, and bacteria were stained by fluorescent in situ hybridisation (FISH) and imaging of the tumours was performed using a 7T-MALDI-FTICR (SolariX, Bruker) in full scan (75–1000) and negative ion mode, yielding 2.122 peaks. FISH analysis and MALDI-imaging were conducted for in situ metabolite analysis based on the presence of bacteria in 12 patients with sporadic CRCs according to low (gene expression <3.49) and high (gene expression >3.49) PHLPP1 expression levels, with a value of $p < 0.05$, fold change >1 or <-1 , and a volcano plot showing the differentially expressed metabolites using the EnhancedVolcano R package. Exact m/z values with free database analyses after comparisons were performed using MultimagingTM software (ImaBiotech) and Data-Analysis (Bruker Daltonics), metabolite annotations were performed according to Lipid maps (<http://www.lipidmaps.org/>) and Metlin as detailed elsewhere.²³

Inflammatory and immune cell characterisation in human tumour tissue environment

RNAScope in situ hybridisation was performed on formalin-fixed paraffin-embedded (FFPE) tumour tissue sections (5 μm) using the Multiplex Fluorescent Reagent 2.5 HD kit (Advanced Cell Diagnostics). Tumour (n=69 patients) and adjacent normal areas on tissues (n=22 patients) were analysed. Colocalisation analyses of PHLPP1, CD8 and IFN γ stained cells was conducted in two groups (low vs high PHLPP1) and in two (sporadic,

IBD) CRC types according to the methods described.²³ To validate RNAScope results, we performed a quantification study on serial sections of tumour and normal neighbouring tissues (n=95 CRC cases; 2 excluded due to missing material) using immunohistochemistry (IHC) and morphometric analysis. Inflammatory and immune cells were quantified by using specific antibodies targeting CD3, CD4, CD8, CD68, CD163, mastocytes, FoxP3, ROR γ T cells, Ki67 and Granzyme which were purchased from Sigma (France) and used according to the manufacturer's instructions. Reactions were revealed using DAB. Digital quantification of stained cells was performed using QuPath software V.2.1 (<https://qupath.github.io/>).

Statistical analyses

The characteristics of the study population were described using numbers for qualitative variables and mean \pm SD for quantitative variables. All patients underwent a survey programme for 3 years or more. They have considered failure or success regarding CRC in cases of death, disease progression or recurrence after surgical or endoscopic R0 removal of the cancer. Patients were screened at every 6-monthly visit and body CT scan was performed until recurrence was observed and were followed up until death without additional scheduled exams. Only patients with stages III and IV (metastatic dissemination) received chemotherapy and/or palliative care in neoadjuvant and adjuvant therapy when appropriate, according to international consensus protocols. No patient with stage I or II disease received adjuvant chemotherapy until recurrence was observed.

The statistical analysis for patient survival was performed using Qlucore V.3.2. software. Principal component analysis (PCA) was used to visualise the data set in a three-dimensional space, after filtering out variables with low overall variance to reduce the impact of noise and centring and scaling the remaining variables to zero mean and unit variance. Significantly differential variables between the low and high PHLPP1 gene RNA subgroups were identified after fitting a linear model for each variable with gene-level class as a predictor and the sample factor as a nuisance covariate. Values of p were adjusted for multiple testing using the Benjamini-Hochberg method.^{24 25} The probability of survival (disease-free or no progression was considered with death censored) was calculated according to the class of PHLPP1 gene expression through a Kaplan-Meier construction.

Multivariate Cox models were fitted to compare inflammatory and immune cell infiltrates on IHC slides in different types of CRCs (IBD, Lynch and sporadic) while considering potential confounders. For comparisons between classes of PHLPP1 gene expression, the χ^2 test or Fisher's exact test and Mann-Whitney test were used when appropriate.

Whole exome sequencing

A total of 59 paired samples (normal/tumour) were used and fastq files were quality checked using fastQC

(V.0.11.9) and trimmed. For variant calling, we used Varscan software (V.2.2.3, default parameter values except for the purity, which was set to 0.4) and SNPs and indels were filtered based on their value of p ($p < 0.05$) and status ('Somatic'/'LOH'); snpEff software (V.5.0) to annotate variants and an inhouse Python script to compile statistics were used.

RNAseq

Paired-end reads were quality checked using fastQC (V.0.11.9) and trimmed for 8 Lynch, 19 IBD and 69 sporadic cases. Two sets of analyses were performed. One analysis included 137 cardinal genes involved in CRC (list in online supplemental file 1) with an FDR cut-off of 0.1 and considered according to 'failure (death- or progression disease) vs Success (remission or stable disease)' through an unsupervised procedure. This consisted of filtering by fold change 1.5, t test ($<$), and genes that were significantly associated with the failure (all with $p = 0.002$ and $q = 0.186$ and without any co-factor eliminated factors). According to the median value of PHLPP1 RNA, our series was divided into two subgroups (high vs low). The second analysis included all genes and consisted of classifying gene RNAs according to CRC types and PHLPP1 subgroups.

CMS classification

Three algorithms were used for consensus molecular subtype (CMS) evaluation using R packages: CMSCaller^{26 27} and CRC Assigner using default parameters. (see 'itmo-RNASEQ-CMS-ALL4packages_rev-09-03-2021-Validated-11-03-2021. ods' in online supplemental file 1).

Methods for gene networks around the PHLPP1 gene

For the gene network of PHLPP1, we selected 13 361 high expression level genes from the RNASEQ data set using a threshold of $\log_2(\text{CPM}) > 1$, then computed Pearson correlations (with absolute coefficient value > 0.3) between each gene and the PHLPP1 gene for the Lynch ($n = 8$), IBD ($n = 19$) and sporadic ($n = 69$) subgroups. The value of p was computed using 1000 permutations with $p < 0.05$ as significant and the Cytoscape (v3.8.2) program was used for figures.

Mice germ-free study experiments

Fresh stool samples were obtained from nine women and nine men in the CCR1 cohort for FMT in germ-free mice; there were nine patients with CRC and nine with normal colonoscopy giving CRC microbiota (CRC μ), and normal microbiota (N μ), respectively, as described.⁶ FMT was performed in male C3H/HeN germ-free 8-week-old mice: $n = 53$ in the 7-week study [(CRC μ transfer, $n = 30$, and N μ transfer, $n = 23$ in the 7-week study; $n = 132$ in the 14-week study (CRC μ transfer, $n = 66$, and N μ transfer, $n = 66$)]. In addition, mice were given intraperitoneal injections of either the carcinogen AOM (Sigma, 8 mg/kg body weight once a week for 3 weeks or 10 weeks), or saline. Mice were euthanised according to the recommendations of

the American Veterinary Medical Association Panel on Euthanasia.

RESULTS

Transfer of fresh stool of patients with CRC in germ-free mice induces methylation of various genes in the colonic mucosa

We observed a synergistic effect of CRC μ and AOM treatment in causing DNA changes in the mice colon epithelium. We found that a large fraction of the methylated loci in DNA extracted from the colonic mucosa belonged to cardinal genes involved in colon carcinogenesis (online supplemental table S1).²⁸ We also reported global DNA demethylation in the colonic mucosa, with hypermethylation of some antioncogenes (ie, hypermethylated > 0.8 or hypomethylated < 0.2) and significant increases in proinflammatory cytokines (IL2, IFN γ , TGF β , TNF α , IL-6 and IL1 α , IL β) in CRC μ FMT recipients compared with N μ recipients.⁶ Notably, we found DNA methylation of the PHLPP1 gene in the colonic mucosa as a consequence of CRC μ FMT compared with N μ FMT. PHLPP1 was downregulated in the CRC μ recipient group, as assessed by qPCR performed on mouse RNA recovered from the colonic mucosa of mice (online supplemental figure S1A). We also reported a decrease in butyrate producer Firmicutes in the CRC patient donor microbiota,⁶ consistent with reports by others^{29 30} suggesting that microbial dysbiosis is related to changes in the expression of the PHLPP1 gene as reported in a wide range of human diseases, including CRC.³¹

Inflammatory and immune cell infiltrates in mice colonic mucosa after human FMT

Mice receiving CRC μ had significantly higher numbers of immune cells infiltrating mucosal crypts than N μ mice recipients, as reflected by CD11b+ cells ($p < 0.05$), Treg cells ($p < 0.05$) and CD3+ cells ($p < 0.01$) (online supplemental figure S1B,C). These differences were less obvious in the lymphoid aggregates and lymphoid follicles. Innate lymphoid cells, which are known to respond to the gut microbiota, remained unchanged. Interestingly, in CRC microbiota donors' tumour tissues, we did not observe a significant difference in Granzyme+CD3+ and ROR γ t+FoxP3+ immune cells within the tumours as compared with the normal neighbouring tissues, whereas CD3 immune cells were significantly ($p < 0.05$) higher (online supplemental figure S2). The tumour tissues were distinguished by the absence of Granzyme B+CD3+ immune cells and the absence of ROR γ t+FoxP3+ immune cells compared with the neighbouring normal tissue.

Pathology and clinical description of patients

We investigated 97 patients with CRC (table 1) presenting with adenocarcinoma of stage I, II, III or IV according to the TNM international classification. RNA levels were used to characterise tumours according to the CMS classification by using a supervised analysis based on an

Table 1 Characteristics of patients

N (%)	
N	97
Age, years, mean (SD)	61.1 (11.1)
Female/male	31/66
Family history of	
Colonic neoplasia	14 (14.5)
Comorbidities	
IBD	20 (21)
Diabetes	12 (12.3)
Hypercholesterolaemia	13 (13.4)
Daily medications*	70 (72)
Immunosuppressive/anti-TNF α	19 (19.5)
Location of tumours	
Colon	78
Rectum	19
TNM staging	
I	22 (23)
II	22 (23)
III	28 (29)
IV	25 (26)
PHLPP1	
Tumour/normal methylation >1.2	60 (62)
Genes correlated with PHLPP1 genes#	136/13600
Kras/Nras	28 (29)
Braf	2 (3)

*only daily medicine introduced at least three months prior to the baseline are considered
IBD, inflammatory bowel disease; PHLPP1, pleckstrin homology domain and leucine-rich repeat protein phosphatase 1.

algorithm, as described by Guinney *et al*²⁶ and modified by Eide *et al*.²⁷

PHLPP1 downregulation in CRC

An unsupervised analysis of tumour RNAs identified five distinct clusters within the tumour tissues (figure 1A–F). Cluster 3, which exhibited mild variations in expression, included PHLPP1, along with other cardinal genes such as APC (a common initiator of CRC), FBXW7 (an F-box protein suppressor of JUN and MYC), oncogenes such as BRAF, MAP3K3, PDGFRA, FGFR1, genes related to autophagy and protein traffic (TRAPPC), immunomodulating genes (RORC, TLR4, CXCL1, SOCS1), chromatin modifiers (TCF4, TOPRRS), DNA repair genes (PMS2, MLH1, MSH2, MSH6, methylguanine methyltransferase (MGMT)), the G2/M cell cycle checkpoint gene MCPH2, and the core circadian genes CLOCK and ARMTL2 (BMAL).

We further examined the expression of PHLPP1 in relation to the genes within cluster 3, categorising them according to CMS classification. The mean PHLPP1 RNA

expression values in subtypes CMS1, CMS2, CMS3 and CMS4 were 3.3, 3.8, 3.5 and 3.4, respectively. Notably, the lowest PHLPP1 expression levels of the PHLPP1 gene were observed in subtypes CMS2 and CMS4. CMS2 is particularly enriched in inflammatory pathways when CMS4 is specifically characterised by epithelial-mesenchymal transition activation, extracellular matrix organisation and TGF β responses,²⁷ many of them being associated with PHLPP1 expression and disease-free survival (online supplemental figure S3A).

We analysed the survival data along with the following covariates: age, tumour location (left vs right), TNM staging (I to IV), follow-up period (in months), and the distinction between ‘failure’ (progressive disease, such as recurrence, metastases or death) and ‘success’ (remission or stable disease from the baseline in stage IV) during the follow-up period. RNA-seq data revealed three genes PHLPP1, TRAnsport Protein Particle (TRAPP) and IL17R that predicted failure. These genes demonstrated similar expression patterns through an unsupervised PCA model and were correlated (online supplemental figure S3B). To identify significant differential variables between the various subgroups, we considered low and high PHLPP1 based on the median RNA value within the data set and fitted a linear model for each variable using gene-level class as a predictor and incorporated the sample factor as a nuisance covariate. Surprisingly, most patients with IBD-CRC, who showed the highest levels of inflammatory cell infiltrates in tumour tissues, were in the low PHLPP1 subgroup. Thus, we investigated IBD (omics-view) and COAD (TCGA), two cohort gene data sets, to ascertain whether the PHLPP1 gene is downregulated in tumour versus healthy surrounding tissues, and whether inflammation within the tissue influences this expression. Comparison between 475 tumorous and 41 normal tissues revealed a broad spread expression of PHLPP1 in tumorous tissues, with overall significant downregulation in tumours as compared with normal tissues (figure 1B). To verify the interdependence between the expression of genes, the rank of PHLPP1 gene RNA was determined for all genes (n=13 360) or in a limited panel of 137 cardinal gene RNAs; again, the lowest expression of PHLPP1 was observed in IBD-CRC (figures 1C, 1D). Analysis of PHLPP1 gene expression in the IBD cohort data set showed a significant decrease at the onset of the disease as compared with normal tissue, with a return to the normal value only in responders to anti-TNF α treatment (figure 1E).

Estimation of PHLPP1-related gene network functions

We conducted functional gene analysis associated with PHLPP1 using gene set enrichment analysis.³² Genes were ranked based on t-statistics from gene-wise linear models, with condition Y as the predictor and city factor as a nuisance covariate. These genes were primarily associated with the phospholipid pathway, a pivotal factor in colon carcinogenesis. This pathway has also been linked to molecular subtypes and patient outcome.²⁸ Among

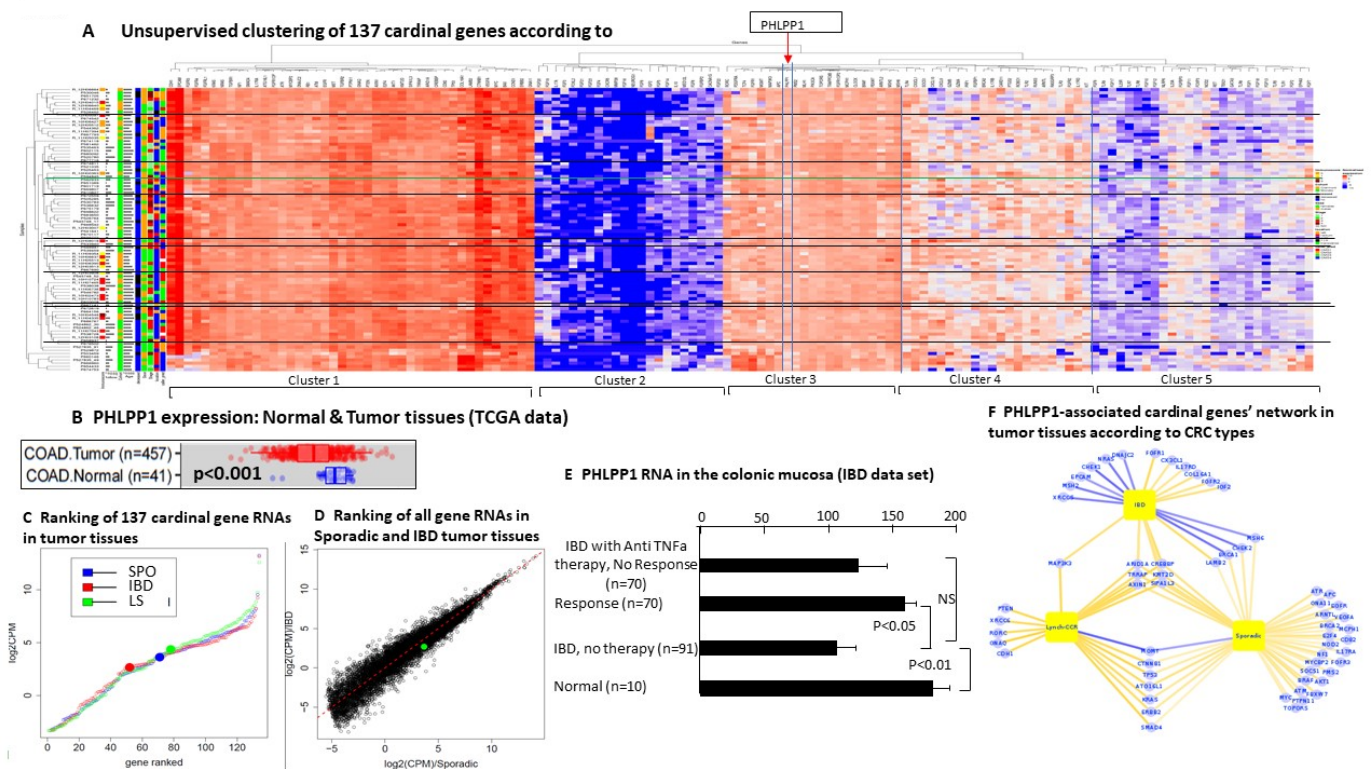


Figure 1 Unsupervised clustering of RNA expression in CRC tissues highlights the PHLPP1 gene. (A) Clustering of tumour-associated genes by patient survival outcomes: A total of 137 tumour-associated genes (listed in online supplemental table S1) were analysed for their association with survival outcomes over a follow-up period of ≥ 3 years in a cohort of 96 patients with CRC. Using RNA data from tumour tissues and an FDR cut-off of 0.1, clustering was performed to compare outcomes classified as ‘failure’ (death or disease progression) versus ‘success’ (remission or stable disease). One sample was excluded because of low read counts, leaving 96 samples for analysis. Data normalisation (mean=0, variance=1) was applied to construct a heatmap in five clusters, with cluster 3 influencing the patients’ outcome with PHLPP1 RNA levels highlighted; three genes, PHLPP1, TRAPP and IL17R, were significantly associated with failure outcomes after filtering for RNA fold change ≥ 1.5 (t-test, $p=0.002$, $q=0.186$). None of the cofactors were excluded from the analysis. (B) Comparison of PHLPP1 RNA levels in tumour and normal tissues: Data from the COAD data set (adenocarcinoma, $n=457$; normal colonic tissues, $n=41$) revealed significantly lower PHLPP1 RNA levels in tumour tissues than in normal tissues ($p<0.01$). Notably, the tumour samples showed considerable diversity and heterogeneity in PHLPP1 expression. (C) PHLPP1 RNA levels in patients with IBD: Colonic tissue RNA levels of PHLPP1 in patients with IBD, including ulcerative colitis and Crohn’s disease, were analysed using the data sets GSE23597, GSE12251, GSE52746 and GSE16879 (accessible at omicsview.org). Inflammation within colonic tissues influences PHLPP1 expression. Patients with acute-onset IBD exhibited reduced PHLPP1 RNA levels compared with normal tissues. PHLPP1 expression normalised in patients who responded positively to anti-TNF therapy. (D) Ranking PHLPP1 RNA levels in tumour tissues: In the present CRC cohort, RNA levels of PHLPP1 were ranked within all genes across sporadic, IBD-associated and Lynch syndrome CRC tumours. PHLPP1 RNA levels (green dots) were lower in IBD-associated tumour tissues than in other CRC tumour types. (E) PHLPP1 expression ranking limited to the 137 cardinal genes across CRC types: PHLPP1 expression was analysed across various CRC types, with colour-coded points indicating sporadic CRC (blue), Lynch syndrome (green) CRC and IBD-associated CRC (red). (F) Gene network correlated with PHLPP1: A network of genes (from the 137 cardinal genes) correlated with PHLPP1 expression in tumour tissues was constructed. Positive and negative correlations are represented by the orange and blue lines, respectively. CRC, colorectal cancer; IBD, inflammatory bowel disease; LS, Lynch syndrome; PHLPP1, pleckstrin homology domain and leucine-rich repeat protein phosphatase 1; TRAPP, Transport Protein Particle.

the 10 genes involved in autophagy, cell proliferation and metabolite traffic were significantly correlated with PHLPP1 gene expression. Two of these genes, AKT1 and TRAPP, were particularly significant, whereas few genes showed a trend of association (figure 1F and online supplemental figure S3). Close associations between AKT1 and different TLRs in tumour tissues were analysed according to both PHLPP1 and AKT1 gene transcripts ($r=0.62$ and $r=0.81$, respectively, $p=0.01$). Interestingly, TLRs were significantly associated with the expression

of AKT1 and PHLPP1. TLR1 and TLR2 overexpressions were observed in the CMS4 class, which is mostly (80% out of all) observed in those patients with low PHLPP1 expression, raising the hypothesis that dysbiosis might influence recurrence or progression through TLR gene activation.

In our data set encompassing gene RNA reads from tumour tissues, we identified 1576 genes that were significantly associated with at least 1 other gene out of the 13360 genes examined. Among these, 136 genes

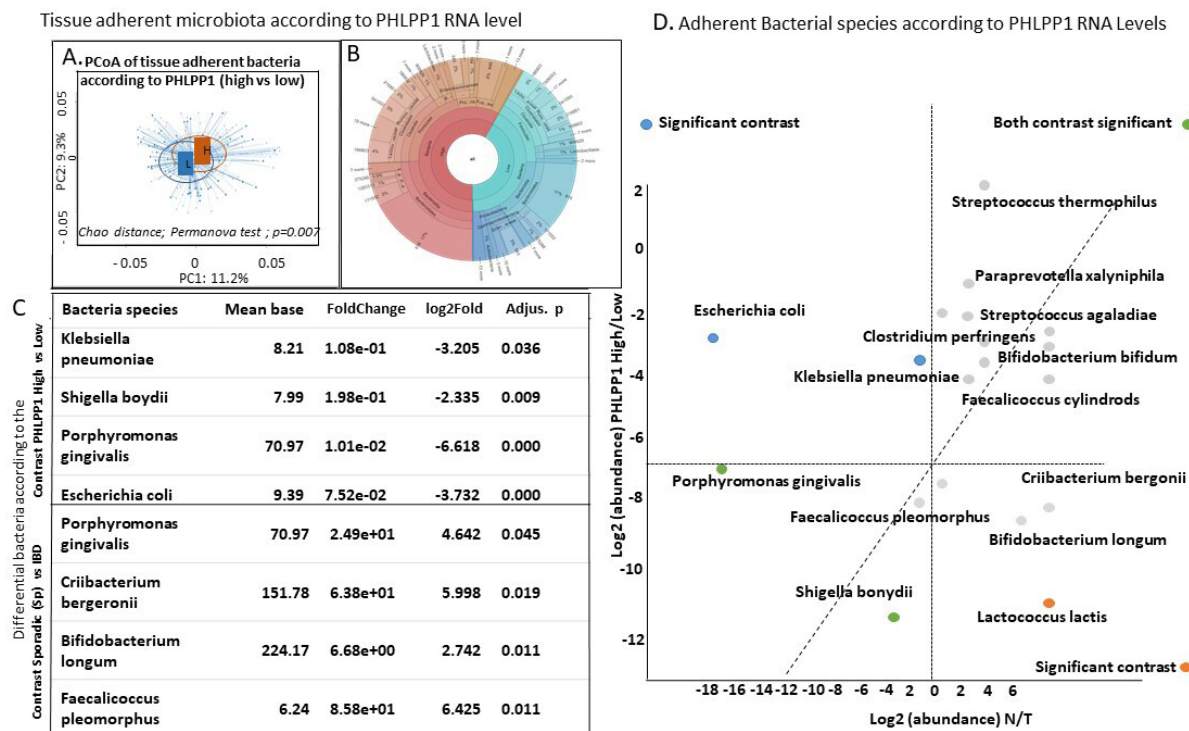


Figure 2 Differential bacterial adherence to tissues. DNA extracted from tumour (T) and adjacent normal (N) tissues was analysed using 16S rRNA sequencing (Illumina technology). The data were processed using the Shaman platform. **(A)** Tissue-adherent microbiota was analysed in tumour tissue samples revealing distinct microbiota profiles according to PHLPP1 gene expression (low vs high) based on PCoA. **(B)** A Korona plot illustrates bacterial taxa distribution relative to PHLPP1 levels (low vs high), indicating reduced taxonomic variability in low PHLPP1 (green) compared with high PHLPP1 (brown). **(C)** Several virulent bacterial species were found significantly more abundant in tumour tissues with low PHLPP1 expression and in IBD-CRC tumour tissues. **(D)** Comparisons based on both PHLPP1 expression levels (low vs high) and tissue type (tumour (T) vs normal (N)) identified elevation of virulent species (ie, *Escherichia coli*) and diminution of symbiotics (ie, *Lactococcus lactis*). CRC, colorectal cancer; IBD, inflammatory bowel disease; PHLPP1, pleckstrin homology domain and leucine-rich repeat protein phosphatase 1.

correlated with PHLPP1 (online supplemental table S2). We performed Enrichr cluster rammer analysis³³ of genes and found infectious diseases and carcinogenesis were the two main signalling pathways associated with PHLPP1 gene function.

The KEGG and an updated EnrichR program³³ revealed that in sporadic tumour tissues, only 30 genes exhibited a negative correlation with the PHLPP1 gene; they were related to various pathways, including cytoskeleton dynamics, AMP and insulin signalling, autophagy, mitophagy and NOTCH signalling. By contrast, in IBD-CRC tumours 95 genes were negatively associated with the PHLPP1 gene that were predominantly involved in the 'RNA splicing' pathway. The number of genes showing negative correlations in IBD cases was three times higher than that observed in sporadic cases, suggesting distinct patterns of gene regulation depending on the CRC type (online supplemental figure S4A and S4B,C).

Importantly, six genes, AXIN1, TRRAP, KMT2D, ARID1A, CREBP and SIPA1, were positively correlated with PHLPP1 gene expression in the three CRC subtypes. Genes co-associated with PHLPP1 in CRC-IBD promoted inflammation (IL17RD, CX3CL1), fibrosis (COL1GA1), cell survival (FGF1, FGF2, IFG1), cell cycle and DNA

repair when there was a negative correlation with MGMT, a DNA repair gene that maintains genomic stability. CRC-IBD showed negative and sporadic CRC, showing positive associations of PHLPP1 with BRCA1, CHECK2 and MSH2, a DNA repair gene. Altogether, this analysis indicates that PHLPP1 interacts with several other genes involved in oncogenesis and inflammation.

PHLPP1 gene mutation in tumour tissues

The results of whole-exome DNA sequencing (WES) in tumour and healthy neighbouring tissues were aligned with the RNA expression in the same samples. Among 39 patients with low (n=12) or high (n=27) intratumour PHLPP1 expression, we could only find a trend for higher mutation/deleterious polymorphism frequency and low PHLPP1 gene expression that did not reach significance (online supplemental figure S5). Mutations in the coding sequence of PHLPP1 were rare. Specifically, exome mutations with potentially high or moderate impact on PHLPP1 expression included missense mutations in one IBD-CRC and in two sporadic CRC tumours, and frame-shift mutations in two sporadic CRC tumours (online supplemental table S3). In the remaining sporadic CRCs, synonymous mutations with little or no impact on gene

expression were observed. The tumour of one patient with CRC-IBD showed mutations in additional genes (eg, TBX21, TRAPP) whose RNA was associated with the PHLPP1 RNA expression.

The DNA methylation of the PHLPP1 promoter was investigated by methylation-specific qPCR in a subset (including six out of nine CRC patient donors) of tumour tissue samples because tumour tissue material was not available in all cases. DNA samples from tumour tissues and normal neighbouring specific methylation in the PHLPP1 gene promoter^{6 34} and differences between normal and tumour samples were estimated and classified as hypermethylated when 'T (tumoral)-NT (normal neighbor)' levels were >1.2 (online supplemental figure S5C).

Hypermethylated PHLPP1 promoters or trends to hypermethylation in tumour tissues as compared with neighbouring healthy tissues were detected in 13 out of 13 samples with low and in 1 out of 8 tumour samples with high RNA gene expression (total 21 tumour tissues, 13 low and 8 high RNA expression levels investigated). Methylated genes in tumour tissues, which were almost higher than those in normal neighbouring tissues, showed significantly higher levels in high than in low PHLPP1 expressed gene (online supplemental figure S5B). In one CRC tumour tissue sample, there was a discrepancy between the RNA level and methylation of the PHLPP1 gene promoter. Interestingly, in this case, the PHLPP1 gene was highly methylated in normal tissues neighbouring the tumour, suggesting that epigenetic changes in the normal colonic mucosa may have preceded tumour development. Such a mechanism has been reported in an experimental model of DNA methylation in gingival mucosa by *Porphyromonas gingivalis*, in which enhancement of methyltransferase enzymes such as DNMT3b levels was observed not only in the gingival tissue but also in the gut epithelium,³⁵ particularly in patients with IBD-CRC. Nevertheless, these results suggest that down-regulation of the PHLPP1 gene in tumours could result from epigenetic rather than somatic gene mutations, as reported.^{6 21 36 37}

Inflammatory and immune cell infiltrate associations with PHLPP1 expression in human tissues

Based on the mean number of stained cells (\pm SD), we observed distinct patterns of myeloid cells, macrophage cells, mastocytes as well as immune cells (CD68, CD163, CD3, CD4, CD8, FOXP3, ROR γ T) in tumour tissues compared with adjacent normal tissues (figure 3A). Notably, macrophages exhibited significantly higher infiltration in tumours with low PHLPP1 gene expression than in those with high expression. This observation may be attributed to IBD-associated cases. Furthermore, the patterns of co-associations such as 'CD3-CD8', and 'CD3-macrophages (eg, CD68, CD163)', 'CD8-macrophages' and 'CD8 with Granzym A or Caspase', differed between tumours with low and high PHLPP1 gene expression (figure 3B) with significant ($p < 0.05$) difference between

low and high tumour tissues regarding ratios of CD8/CD3 (figure 3C), CD163/CD3 and Granzym/CD3 immunostained cells. We achieved RNAscope in situ hybridisation to assess the expression of PHLPP1 alongside immune markers CD8 and IFN γ in tumour tissues. In IBD-CRC cases, the number of CD8, cytotoxic T cells and IFN γ -producing cells coexpressing PHLPP1 was significantly lower than in sporadic CRCs (figure 3D-F). This finding was corroborated by immunohistochemical analysis, which revealed diminished infiltration of granzyme B, CD3 T cells and altered patterns of macrophage and Treg markers in IBD-CRC compared with sporadic cases.

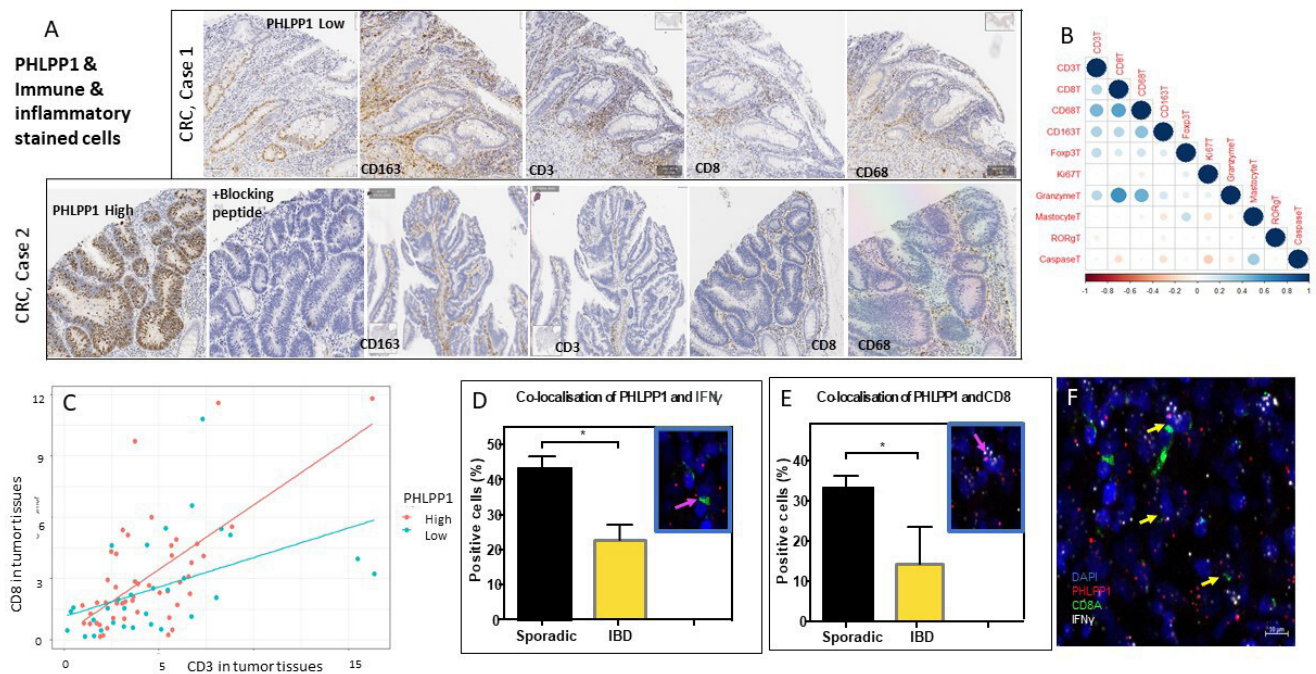
Bacteria (genera, species) characterisation in tissue samples according to PHLPP1 expression

Identification and quantification of tissue-adherent bacteria showed significant differences between subgroups of patients with low and high PHLPP1 gene expression at both the genus and species taxonomic levels (figure 2A,B). PCoA projection showed significant separations between low and high PHLPP1 gene expression stratified by normal, tumorous tissue status or CRC types.

We observed differences between tumour and normal neighbouring tissues in the abundance of adherent virulent bacteria and low versus high expression levels of PHLPP1 (figure 2). Briefly, *Treponema*, *Leptotrichia* and *Fusobacterium* genera were significantly associated with tumour location (vs normal neighbouring) and low (vs high) PHLPP1 gene expression in all CRC types. Several virulent species, such as *Escherichia coli*, *Shigella bonydii*, *Klebsiella pneumoniae*, *Porphyromonas gingivalis*, were higher in tumour tissues with low PHLPP1 than in high PHLPP1 gene expression (figure 2C). By contrast, some commensal species (eg, *Lactobacillus lactis*) were diminished in IBD-CRC and sporadic CRC tissues with low (vs high) PHLPP1 expression (\log_2 fold change >4.9, adjusted $p < 0.05$) (figure 2D). These findings suggest that the virulent/symbiotic bacterial ratio is inversely related to PHLPP1 expression.

In situ metabolomic analysis on tumour tissue sections

Thus, to map the expression of metabolites, we performed FISH of FFPE tumour sections (FFPE-FISH) to select tumour areas with (n=12) and without (n=13) adherent bacteria. We then overlapped the expression of metabolites with the presence of tumour-adherent bacteria and related it to the low versus high expression of PHLPP1 (online supplemental figure S6). Untargeted metabolomic analysis of the tumour sections was performed using MALDI mass spectrometry. We applied the Metabolomics Workbench³⁸ to FISH staining and mass spectrometric measurements on tumour sections to compare low versus high PHLPP1 expression (online supplemental figure S6A,B). Several differences relating to PHLPP1 or bacteria to lipid and phospholipid metabolites were observed (online supplemental table S4). However, only the levels of dTMP were significantly co-associated with both PHLPP1 expression and the presence



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Figure 3 PHLPP1 expression and inflammatory-immune cell infiltrates in CRC tissues. (A) PHLPP1 expression in tumour cases. Two representative CRC cases were illustrated as representative of low *PHLPP1* RNA and high *PHLPP1* RNA expression, respectively. (B) Immune cell staining and quantification. The normal colon tissues were infiltrated by CD3 and CD4, Granzyme B immunoreactive cells when higher were CD3, CD8, CD68 and CD163 cells in tumour tissues. Stained markers included myeloid-macrophage markers (CD68 and CD163), cytotoxic and apoptotic markers (Caspase 1 and Granzyme A), T cell markers (CD3 and CD8). Quantification of stained cells was performed using the QuPath program, with significant correlations (R) visualised as the size of circles within the corresponding network links. (C) Patterns of immune infiltrates and PHLPP1 expression. Differences in CD8/CD3 cell patterns were observed based on *PHLPP1* RNA expression levels and CRC types with red and green points denoting tumours with high and low *PHLPP1* RNA expression, respectively. In situ co-localisation of PHLPP1 and immune markers (D–F). Representative RNAscope images showing double (D–E) colocalisation of PHLPP1 with IFN γ (D) or with CD8 (E) and triple localisation PHLPP1/IFN γ /CD8 (F); they illustrate significantly diminished cells in IBD–CRC than in sporadic CRC tissues. CRC, colorectal cancer; IBD, inflammatory bowel disease; PHLPP1, pleckstrin homology domain and leucine-rich repeat protein phosphatase 1.

of tumour-adherent bacteria (n=12 patients with CRC, with 9 high vs 3 low status), as compared with samples that lacked adherent bacteria (n=13 patients with CRC, 4 low vs 9 high status) (online supplemental figure S6B). dTMP is an intermediate product of both the de novo and salvage pathways of nucleotide synthesis and is critical for DNA replication and tumour cell proliferation.^{39 40} Therefore, dTMP was considered a marker of the zones of bacteria-related tumour growth within the tumour mass (online supplemental figure S6B). Hence, we conclude that tumour-adherent bacteria and down-regulation of PHLPP1 synergistically contribute to CRC tumour growth.

Influence of PHLPP1 gene expression on patient survival

Patient survival significantly (p=0.002, q=0.186) correlated with high PHLPP1 gene expression (figure 4); 85% of IBD–CRC cases presented with low PHLPP1 expression, in contrast with less than 15% of LS or sporadic patients with CRC. Over 3 years of follow-up, 44% of the patients

with low-level and 66% of those with high-level tumour PHLPP1 RNA expression were still alive (p<0.05). Patients with low tumour PHLPP1 expression had shorter survival (p=0.03) than those with high PHLPP1 expression, with patients with IBD–CRC having the worst outcomes.

Based on a new composite criterion ‘death, recurrences, or progressive disease–PD’ vs ‘disease-free survival–DF’ (with filtering by Fold Change and using t-test in 96 patients within 3 years follow-up), the co-expression of TRAPP and IL17R together with PHLPP1 was significantly associated with disease-free survival (online supplemental figure S3).

DISCUSSION

Our study established a clear association between microbiota composition and downregulation of the PHLPP1 gene through epigenetic mechanisms. PHLPP1 is a protein phosphatase with reported tumour suppressive

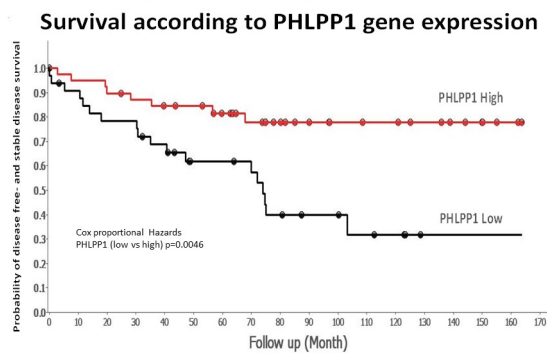


Figure 4 Patient survival based on PHLPP1 gene expression. Clinical and biological data were collected over a follow-up period of at least 3 years until death or censoring. The patients were categorised into low or high PHLPP1 RNA expression groups. Survival data were analysed using Kaplan-Meier survival curves generated using the QluCore program, with deaths censored as indicated on the curves. The curves illustrate the probability of survival (disease-free or without progression, with death censored) based on PHLPP1 expression levels, revealing a significant difference ($p=0.026$). A t-test was performed to compare PHLPP1 RNA expression levels between the recurrence/progression and disease-free survival groups (1 vs 0), yielding $p=0.002$ and $q=0.186$, with no covariates eliminated due to SD thresholds (s/smax). Active samples included 96 of 97 patients, with normalisation parameters set to mean=0 and variance=1. Missing data affected one patient, and two additional patients were excluded because of insufficient material or updated information. PHLPP1, pleckstrin homology domain and leucine-rich repeat protein phosphatase 1.

properties associated with the regulation of inflammation, cell survival and tumour growth.^{11–17} The present study provides evidence for tumour-adherent virulent microbiota-driven modulation of PHLPP1 gene expression and worse outcomes in inflammatory CRC tumours, particularly in patients with IBD-associated CRC. Furthermore, we characterised bacterial-mediated epigenetic silencing of tumour suppressor genes, exemplified by PHLPP1. As evidence, we demonstrated that dTMP—an intermediate product in nucleotide synthesis essential for DNA replication and tumour cell proliferation^{39 40}—was strongly associated with bacterial adherence to tumour tissue. Thus, we propose that tumour-adherent bacteria and the downregulation of PHLPP1 synergistically contribute to CRC tumour growth. Previous studies identified alterations in bacterial metabolites, particularly SCFAs, associated with dysbiosis in patients with CRC. SCFAs serve as key mediators of microbiome-induced epigenetic modifications, forming a dynamic interface between the microbiome and host gene expression.⁵ Although larger cohorts and longitudinal studies are needed to validate PHLPP1 downregulation and elucidate the temporal relationship between dysbiosis and tumour progression, our results underscore the significance of the microbiota, chronic inflammation and host gene expression changes in CRC pathogenesis and highlight PHLPP1 as a critical mediator linking dysbiosis to

tumour biology. Interactions between microbiota and CRC cardinal genes were strengthened by experiments involving FMT in germ-free mice. Low PHLPP1 expression in recipient mice coincided with higher levels of virulent bacteria relative to commensals and resulted in lower butyrate producer bacteria adherent to the colonic mucosa (detailed in Ref6).⁶ This imbalance was correlated with increased local inflammation noted by the infiltration of macrophages, CD3+ T cells and elevated cytokine production in both tumour tissues of CRC patient donors (online supplemental figure S1) and in the colonic mucosa of germ-free mice recipients after CRC FMT (figure 3). Altogether, these observations indicate that dysbiosis fosters a proinflammatory tumour microenvironment and influences patient outcomes through epigenetic downregulation of tumour suppressor genes, including PHLPP1, in CRC. Such a mechanism has been reported showing gut dysbiosis induces DNA methylation via SCFAs predisposing obesity-prone individuals to diabetes.⁴¹ Thus, targeting microbiota-induced epigenetic changes represents a promising avenue for personalised CRC therapies.

The PHLPP1 protein is mostly expressed in normal epithelial cells but tends to be downregulated in cancer cells, as shown by the COAD data set as well as in the data presented here, likely due to its interaction with inflammatory pathways.¹³ Our findings are consistent with inflammation-induced mutations and epigenetic DNA changes, as previously reported.^{11 21} Patients with CRC with low expression of PHLPP1 in their tumours were classified as having poor outcomes, consistent with other reports in patients with CRC^{13 17 34} and mice,¹⁴ highlighting PHLPP1 as a prognostic marker in CRC.^{17 34 42} We report, for the first time, how gut dysbiosis, exemplified by tumour-adherent virulent bacteria, is associated with patients' outcomes.^{6 8 10 43 44}

Chronic colonic inflammation is characterised by myeloid cell infiltration in the colonic mucosa, as observed in humans and reproduced after human CRC microbiota FMT in germ-free mice. Further, a significant decrease in PHLPP1 protein levels in the colonic mucosa in the acute phase of IBD and its correction after anti-TNF α therapy are consistent with low PHLPP1 expression in tumour tissues with IBD-like inflammation in the present series classified as CMS2 and CMS4 and poor prognosis.²⁷

The gut microbial community affects host homeostasis by altering the expression of genes involved in cellular differentiation and survival or inflammatory and immune responses through hypermethylation or hypomethylation of regulatory genes.^{11 45 46} Therefore, hypermethylation of specific tumour suppressor genes, including PHLPP1, can serve as a biomarker for CRC.⁴⁷ Some virulent bacteria that adhere to CRC tumours, including *E. coli*,^{48 49} *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Helicobacter pylori* and *Pseudomonas aeruginosa*, may influence their expression.^{50 51} For example, *E. coli* adherent bacteria are associated with low expression of PHLPP1 in tumour tissues.

Adherent-invasive *E. coli* has been reported to impede autophagy clearance in Crohn's disease in humans^{9,44} and with various other virulent bacteria, such as *Clostridium leptum* and *Bacteroides fragilis*, may lead to the colonic epithelial cell-specific autophagy deficiency in mice.¹⁰

Genes that are correlated with PHLPP1 in the present series are involved in splicing RNA in the context of inflammation, highlighting the importance of the epigenetic pathway.¹⁰ Restoring PHLPP1 expression or counteracting epigenetic downregulation may provide novel strategies to enhance CRC treatment. Further studies should explore whether targeting specific virulent bacteria or restoring commensal populations can reverse PHLPP1 expression and improve patient outcomes through an inhibitory kinase pathway as reported in several gastrointestinal tumours, including CRC.⁵² Notably, results from these patients suggest that a combined diagnostic approach incorporating both bacterial virulence gene markers and host genomic alterations is a promising tool for screening patients with IBD undergoing surveillance for dysplasia or CRC. We have previously demonstrated the feasibility of such a combined assay in patients with sporadic CRC, and others have shown that a limited panel of bacterial markers may serve high-risk individuals with germline mutations.⁵³

CONCLUSION

We showed that PHLPP1 expression in CRC tumours is epigenetically regulated by microbiota dysbiosis and tumour-adherent virulent bacteria and affects patient outcomes. Reduced PHLPP1 expression is indicative of poor overall patient survival and is common in IBD-associated CRC (IBD/CRC), as well as a subset of sporadic CRCs.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study involves human participants and the study protocol was approved by the Henri Mondor Hospital Ethical Committee (Comité de Protection des Personnes) Ile de France IX (no. 10-006, 15/02/2010). Mice experimental procedures were approved by the local ethics committee (Committee reference no. 45; approval no. 12/076, 2016). Participants gave informed consent to participate in the study before taking part.

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