The non-coding variant rs1800734 enhances DCLK3 expression through long-range interaction and promotes colorectal cancer progression

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Genome-wide association studies have identified a great number of non-coding risk variants for colorectal cancer (CRC). To date, the majority of these variants have not been functionally studied. Identification of allele-specific transcription factor (TF) binding is of great importance to understand regulatory consequences of such variants. A recently developed proteome-wide analysis of disease-associated SNPs (PWAS) enables identification of TF-DNA interactions in an unbiased manner. Here we perform a large-scale PWAS study to comprehensively characterize TF-binding landscape that is associated with CRC, which identifies 731 allele-specific TF binding at 116 CRC risk loci. This screen identifies the A-allele of rs1800734 within the promoter region of MLH1 as perturbing the binding of TFAP4 and consequently increasing DCLK3 expression through a long-range interaction, which promotes cancer malignancy through enhancing expression of the genes related to epithelial-to-mesenchymal transition.
A n individual’s risk to develop colorectal cancer (CRC) is affected by a broad spectrum of genetic variants that abolish the functions and/or alter the expression of target genes. In CRC, two types of genetic variants have been extensively discussed to contribute to disease onset and progression: (1) protein-coding mutations and (2) non-coding variants, in particular in DNA regulatory elements. To date, the majority of studies have focused on protein-coding mutations. Key coding mutations such as APC/CTNNBI, KRAS/BRAF, PIK3CA, TP53 and SMAD4 have been intensively characterized1. However, even though a great number of non-coding risk variants for CRC have been identified in genome-wide association studies (GWAS)2–9, their molecular functions have rarely been determined.

Functional genetic variants in distal DNA regulatory elements may alter transcription networks in several ways such as by affecting transcription factor (TF) binding. Single-nucleotide polymorphisms (SNPs) within TF-binding sites may affect the local chromatin accessibility10–12 and/or alter the expression of gene targets through mediating different chromatin interactions13–15. Therefore, identification of variant-specific TF interactors is of great importance to understand regulatory consequences of the variants. However, DNA oriented methods such as DNase I sequencing (DNase I-seq)16, systematic evolution of ligands by exponential enrichment sequencing17 and chromatin immunoprecipitation with massively parallel DNA sequencing (ChIP-seq)18 are biased by DNA motif knowledge or limited by the availability of antibodies, resulting in a biased identification of TF-binding dynamics at disease-associated loci. A recently developed proteome-wide analysis of disease-associated SNPs (PWAS) enables to identify DNA–TF interactions in an unbiased manner19. A similar approach has been used to characterize a protein–DNA interaction map for ultra-conserved elements20. Therefore, we performed a large-scale PWAS study to comprehensively understand TF-binding landscape related to CRC.

As the outcome from our PWAS screen, we further investigated the functions of a SNP located in the promoter region of MLH1 gene (MLH1-93G>A or rs1800734), which has been associated with the risk of several cancer types including CRC21–22, endometrial cancer23, glioblastoma24 and lung cancer25. Here we identified a molecular function of this SNP in promoting cancer malignancy through a novel gene target named DCLK3.

Results

PWAS identified TF occupancy switching at the 116 loci. We selected 116 SNPs associated with CRC risk2–9 for PWAS analysis including the following: (1) typed and imputed GWAS significant SNPs (for imputed SNPs, linkage disequilibrium (LD) $r^2 \geq 0.2$) from 8q24.21 (MYC-335), 15q13.3 (GREM1) and 18q21.1 (SMAD7); (2) SNPs with functional evidence rs16969681/15q13.3 (ref. 26), rs58920878/18q21.1 (ref. 27), rs16888589/8q23.3 (ref. 28) and rs4444235/14q22.2 (ref. 29); (3) 3 SNPs from 3p22.2 (MLH1 region); and (4) GWAS significant SNPs in DNase I-seq peaks in minimal one of 15 fetal large intestine tissues and 12 CRC cell lines (Supplementary Data 1). The PWAS analysis identified 731 TF-binding alterations between reference (Ref) and alternate (Alt) alleles ($P < 0.01$, A/B significance test) (Supplementary Data 2). Compared with proteome data, TF–DNA interactome data showed a clear enrichment for TFs (Supplementary Fig. 1a) and the altered binding events mediated by known TFs (Supplementary Data 3) showed stronger allele preference than other interactions ($P = 4.4 \times 10^{-6}$, Mann–Whitney U-test) (Supplementary Fig. 1b). Overlay of the pulldowns showed a consistent allele preference between two replicate experiments (Supplementary Fig. 1c).

Many of the 731 TFs showed $>8$-fold affinity to one of the alleles at these loci (Fig. 1a), for example, TFAP4 at rs1800734 and RUNXI/RUNX2/CFBP at rs1714640. As expected, top pathways associated with the 731 TFs included key CRC drivers such as WNT and transforming growth factor-β pathways (Fig. 1b).

It is well-known that DNase I-hypersensitive sites coincide with regulatory elements and are the hotspots for TF binding10. To better predict in vivo TF binding, it is necessary to consider the hypersensitivity of tested regions. Therefore, we ranked all the SNP-TF interactions based on PWAS fold change (Ref/Alt allele) and DNase I hypersensitivity (DHS) of the SNP loci. A total of 27 significant allele-specific SNP–TF binding events were considered to be important (Fig. 1c and Supplementary Table 1). Many selected TF–SNP interactions (Fig. 1d and Supplementary Fig. 1d) were validated using ChIP-seq data (Supplementary Fig. 1e). Based on this selection, the top candidate SNP is rs1800734 (MLH1-93G>A) in the 5’-untranslated region (UTR) region of MLH1 gene. Therefore, we decided to focus on this SNP.

PWAS identified specific interactors of rs1800734. Our PWAS screen identified TFAP4 as an allele-specific interactor with an almost 16-fold higher affinity for G-allele, whereas ELF1 showed higher affinity for the A-allele of rs1800734 in LoVo cells (Fig. 1d). The A-allele abolishes TFAP4 binding due to a point mutation at the last position of the E-box (Fig. 2a), which simultaneously creates an E26 transformation-specific (ETS) family binding motif (Supplementary Fig. 2a). We corroborated and extended this finding by label-free-based DNA pulldown with SNU175 and COLO320 extracts (Fig. 2b and supplementary Fig. 2b). Notably, other basic helix-loop-helix (bHLH) and ETS family members also displayed allele-specific binding, including MYC and ELF2 (Supplementary Fig. 2b,c), indicating that these TFs can compete with TFAP4 and ELF1 at this locus. Using ChIP-seq as an orthogonal technique, we validated TFAP4 and ELF1 binding at the rs1800734 in the SNU175 and COLO320, cell lines heterozygous for this locus. Consistent with PWAS, TFAP4 ChIP-seq showed a dominant preference for G-allele binding in the two cell lines (Fig. 2c). ELF1 did not show significant allele-binding preference (Supplementary Fig. 2d), which may be due to the competitive binding interference by other ETS proteins.

DCLK3 is a novel target of rs1800734. Given the position of the SNP in the promoter of MLH1, we further investigated whether predisposition of rs1800734 in CRC is due to DNA methylation of MLH1 promoter as proposed9,10. We tested hypersensitivity and transcription of G- and A-allele in the two heterozygous cell lines, which showed the two alleles are equally accessible and transcribed (Fig. 2d). The neighbouring gene EPM2AIP1, also reported to be regulated by rs1800734 (ref. 31), was similarly unaffected by rs1800734 (Fig. 2d). Hence, we conclude that rs1800734 does not result in allele-specific epigenetic silencing of either MLH1 or EPM2AIP1 in these cell lines. We sought to confirm our observation in the Systems Biology of Colorectal Cancer (SYSCOL) cohort of paired healthy and tumour tissues (healthy: n = 288, tumour: = 289). A strong expression quantitative trait loci (eQTL) between rs1800734 and MLH1 was observed in the healthy tissues ($P = 0.001$, linear regression model) but the A-allele was associated with increased MLH1 expression. This eQTL was lost in the tumours and remained only a weak association in microsatellite stable (MSS) tumours ($P = 0.025$, n = 236, linear regression model) (Supplementary Fig. 3a). Intriguingly, we identified a correlation between rs1800734 and DCLK3 expression in the healthy ($P = 0.029$, linear regression model).
Figure 1 | PWAS screen systematically identified allele-specific TF binding at the selected CRC risk loci. (a) Allele-specific binding of the 731 candidate TFs at the 116 CRC risk loci. Chromatin environment of the 116 SNPs was described by DHS of these loci in the 15 fetal large intestine tissues and 12 CRC cell lines, and histone modifications (H3K4me3, H3K4me1 and H3K27ac) within ±1 kb regions around these loci in the LoVo and HCT116 cell lines. The TFs with P-value <10^{-30} and absolute Log2(fold change) > 3 was listed in the bubble plot. Bubble size represents -Log_{10}(P-values) of the interactors in the pull-down screen (n=2 pulldowns per SNP, P-values: A/B significance test). (b) Pathway annotation of the 731 TFs (HR, gonadotropin-releasing hormone receptor pathway; WNT/PDGF/CCKR/transforming growth factor (TGF)-β, Wnt/PDGF/Cholecystokinin/TGF-β signalling pathways). (c) TF-SNP interactions ranked by fold changes in the PWAS screen and DHS at the SNP loci. Bubble size indicates the -Log_{10}(P-values) of the TF-SNP interactions (n=2 pulldowns per SNP, red and blue bubbles: P-value<0.05, Z-test). (d) The top three candidate TF-SNP interactions (n=2 pulldowns per SNP, red and blue dots: P-values<0.01, A/B significance test).
and tumour ($P = 0.031$, linear regression model) samples, and this association was highly significant in the MSS patients ($P = 0.004$, linear regression model) (Fig. 2e), indicating this locus may act as a distal enhancer and regulate \textit{DCLK3}.

The \textit{A}-allele positively regulates transcription of \textit{DCLK3}. To establish the functional relation between rs1800734 and \textit{DCLK3} expression, two isogenic cell lines homozygous for \textit{G}- or \textit{A}-allele were generated from COLO320 using CRISPR-CAS9 technique (Fig. 3a). Successful targeting of rs1800734 was confirmed by Sanger sequencing (Fig. 3b). No other mutation was observed in the surrounding region. ChIP–quantitative PCR (qPCR) confirmed higher TFAP4 binding to the \textit{G}-allele (Supplementary Fig. 3b) and the level of \textit{MLH1} and \textit{EPM2AIP1} transcription was identical in the three isogenic lines (Supplementary Fig. 3c). Importantly, the eQTL association between rs1800734 and \textit{DCLK3} expression was replicated and a fivefold difference in \textit{DCLK3} expression was observed between \textit{G}- and \textit{A}-homozygotes ($P = 0.004$, linear regression model) (Fig. 2e), indicating this locus may act as a distal enhancer and regulate \textit{DCLK3}.

\textbf{rs1800734 regulates DCLK3 through long-range interactions.} A capture Hi-C study has suggested that rs3806624 in the promoter of \textit{EOMES} affects AZI2, a gene 640 kbp downstream to this SNP, through a long-range chromatin interaction\textsuperscript{14}. A similar constellation may apply to rs1800734. Therefore, Circularized Chromosome Conformation Capture with massively parallel DNA sequencing (4C-seq) was employed to search for long-range interactions between rs1800734 and other potential targets in the three isogenic cell lines. Using rs1800734 as a viewpoint, a significantly enhanced interaction was observed in A-homozygote...
with DCLK3 region (Fig. 3d). Increased interactions were found in the promoter and 3′-UTR region of DCLK3 in two independent experiments (Fig. 3e) and, in addition, the 3′-UTR interaction appeared to increase chromatin accessibility (Fig. 3f).

In conclusion, the A-allele of rs1800734 increases the DCLK3 transcription through increased chromatin interaction and enhanced chromatin accessibility. DCLK3 is a potential oncogenic and tumour progressive factor.

DCLK3 is one of three doublecortin-like kinases (DCLK1, DCLK2 and DCLK3). In this family, DCLK1 has been shown to be a cancer stem cell marker in intestinal tumours. The molecular function of the DCLK3 has not been characterized in depth. We therefore performed gene set enrichment analysis (GSEA) to identify DCLK3-associated gene sets in the SYSCOL RNA sequencing (RNA-seq) cohort. Interestingly, we found that epithelial-to-mesenchymal transition (EMT)-related genes were highly correlated to the expression of DCLK3 in the healthy tissues (normalized enrichment score (NES) = 2.50), which was enhanced in the tumour samples (NES = 3.10) (Fig. 4a and Supplementary Table 2). Using a cutoff of NES at 2.00, we also identified gene sets that are preferentially enriched in the healthy versus tumour tissues (Supplementary Table 2), for example ‘MYC targets’ and ‘G2/M checkpoint’ gene sets were enriched in the healthy tissue, whereas ‘Angiogenensis’ and ‘tumor necrosis factor alpha (TNFA) signalling via nuclear factor–κB’ gene sets were enriched in the tumour tissues (Fig. 4a). As expected, common EMT markers such as CALD1, FN1, SNAI1, SNAI2, TWIST1, VIM, ZEB1 and ZEB2 were highly significantly co-expressed with DCLK3 in the tumours (Fig. 4a), indicating that DCLK3 is an EMT regulator. Furthermore, we observed elevated DCLK3 expression in the tumour compared with healthy tissues (P = 2.2 × 10⁻¹⁰, Mann–Whitney U-test) (Fig. 4b), but no difference between the microsatellite instable (MSI) and microsatellite stable (MSS) tumours (Fig. 4b). Interestingly, this elevation appeared to be correlated with the CRC progression: DCLK3 expression was at a comparable level in the healthy and adenoma tissues, and increased 2-fold in the stage I tumours and remained at this level during tumour malignancy (P = 2.2 × 10⁻¹⁰, Kruskal–Wallis test) (Fig. 4b). Therefore, DCLK3 may promote EMT events and consequently drive tumour malignancy.
Discussion

Our study generated the first TF–SNP interaction map at presumed disease-relevant loci of CRC and determined TF-binding occupancy at the 116 upmost relevant CRC risk loci. Together with GWAS and epigenetic profiling data, our PWAS screens provide a comprehensive TF-binding landscape of these loci and yielded candidate interactions for further functional investigations (Fig. 1a,c,d and Supplementary Fig. 1d,e). As an
alternative approach to investigate allele-specific TF-binding events, the PWAS approach shows several advantages over the DNA-centric methods. In contrast to ChIP-seq technique, PWAS-based identification is a hypothesis-free approach, which does not require any knowledge on the possible binding TFs at risk loci. In addition, application of PWAS approach is not limited by the availability of high-quality ChIP-grade antibodies. Even though large ChIP-seq data sets have been generated, these data nevertheless cover only a small proportion of the entire repertoire of TFs. Other DNA-centric methods, such as DNase-seq or assay for transposable-accessible chromatin with high throughput sequencing (ATAC-seq), are only capable of identifying DNA motifs present at risk loci, which makes it difficult to predict actual binding TF(s) from a family sharing similar motifs in a specific cell type. The outcome of PWAS is a reflection of TF abundance, TF ability to bind to the sequence (affinity), as well as synergistic and antagonist effects due to binding of other TFs to adjacent or overlapping sequences. Therefore, actual binding TFs in a given cell type can be predicted using PWAS method. In addition, PWAS approach also helps to identify binding TFs at the loci only partially matching consensus motif sequences and therefore cannot be predicted by motif prediction-based methods.

Based on the PWAS screen, we investigated in great depths for a well-known CRC-associated SNP: MLH1+93G/4-A or rs1800734. It has been postulated that A-allele (risk allele) of MLH1 is a well-known CRC-associated SNP: MLH1. Our results suggested that TFAP4 preferentially binds to T-allele of the rs1800734 but does not affect promoter methylation and transcription of MLH1. Analysis of the SYSCOL cohort strengthened the findings in the cell lines (Supplementary Fig. 3a) and identified a new gene target DCLK3 in the MSS patients (Fig. 2e). Furthermore, CRISPR-CAS9 facilitated to generate fully comparable isogenic lines carrying G- or A-point mutation at rs1800734 locus. Therefore, mild changes in chromatin interaction, accessibility and consequently gene expression can be monitored by different genomic techniques. It has been shown that some functional SNPs in enhancer regions result in subtle changes in expression of their target genes, for example, the G-allele of rs356168 increased SNCA expression by 1.06 times in neurons and 1.18 times in neuron precursors46. Hence, accurate genome editing is required to distinguish these subtle changes. Using this model system, an increased DCLK3 transcription was detected in the A/A homozygous line (Fig. 3c), which is due to increased chromatin interaction between the two locus and consequently elevated chromatin accessibility in the DCLK3 region (Fig. 3d,e,f). These data confirmed that rs1800734, even though locates in the promoter region of MLH1, serves as a distal enhancer for the DCLK3 gene. Notably, our findings are based on the genetic data confirmed that rs1800734, even though locates in the promoter region of MLH1, serves as a distal enhancer for the DCLK3 gene. 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digested by a step buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% SDS, 100 mM EDTA pH 8.0, 1 mM Spermidine and 0.5 mM Spermine). A 9% Sucrose gradient was applied to fractionate the samples for 24 h at 25'000 r.p.m. at 16°C and the fractions with < 1 kb fragments were further purified and prepared according to the Illumina library preparation protocol.

DNase I library of other cell lines were prepared using a published protocol as described elsewhere. The cell lines were harvested under the confluence of 60% and washed with PBS. Nuclei were isolated with RSB lysis buffer (10 mM Tris-Cl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% IGEPA CA-630) at 4°C for 10 min. Then the nuclei were treated with 0.12 unit of DNase I (Roche) in the provided buffer at 37°C for 15 min before being quenched by 50 mM of EDTA. Following RNase A (Sigma) treatment at 37°C for 15 min, proteinase K (NEB) was added for an additional hour at 56°C. DNA was extracted using phenolchloroform/isoamylalcohol. Agarose Gel (2%) electrophoresis was applied to separate the released fragments (~100 bp) that were purified (Qiagen, MinElute Gel Extraction Kit), followed by Illumina TrueSeq library preparation and Sequencing (HiSeq2000).

ChIP-seq and ChIP-qPCR analysis. ChIP assays were performed following a standard protocol. Cell lines were cross-linked by a final concentration of 1% paraformaldehyde for 10 min and subsequently cross-linking reaction was quenched using 1.5 M glycine. The harvested cell lines were then lysed and sonicated to obtain ~300 bp chromatin using Bioruptor Plus sonication device (Diagonene). The sonicated chromatin was pre-cleaned by Protein A/G magnetic beads (ThermoFisher Scientific, 88802) and then incubated together with antibody conjugated beads overnight at 4°C. Antibodies against H3K27ac (Diagonene, C15410) and H3K9me3 (Abcam, ab17880) were used for ChIP assays. TFAP4 (Santa Cruz Biotechnology, sc-631X, 4 μg per ChIP assay) and ELF1 (Santa Cruz Biotechnology, sc-631X, 4 μg per ChIP assay) were used in our ChIP experiments. Posterior to the incubation, captured chromatin was washed, eluted and de-crosslinked. Resulted DNA fragments were purified and prepared according to Illumina library preparation (H3K27ac ChIP) or KAPA Hyper Prep (TFAP4 and ELF1 ChIP) protocols before sequencing, or directly quantified using SYBR Green-based qPCR assays (Supplementary Table 3).

ATAC-seq analysis. ATAC libraries of the SNU175 and COLO320 cell lines were prepared by a documented protocol with some modifications. In brief, nuclei were isolated using a lysis buffer consisting of 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂ and 0.1% IGEPA CA-630 detergent and then tagmented with 2 μl of Tn5 transposase and 12.5 μl 2× TD buffer (illumina, Nextera DNA Library Preparation Kit). The resulted DNA fragments underwent two sequential nine-cycle PCR amplification, and in between two PCR reactions the libraries were cross-linked by a final concentration of 1% paraformaldehyde for 10 min and subsequently cross-linking reaction was quenched by 50 mM of EDTA. The cross-linked DNA was then sonicated to obtain ~300 bp chromatin using Bioruptor Plus sonication device (Diagonene). Subsequently, two sgRNAs (400 ng each) and a 199 bp single-stranded DNA were transfected into the DH5α competent (2) calculate absolute Log2(fold change) of every TF–SNP interaction between Ref and Alt alleles, and then z-score transform the absolute Log2(fold change) of all the interactions; (3) average the z-scores of DHS and absolute Log2(fold change), and convert the average z-scores into P-values based on normal statistical distribution.

eQTL analysis. The eQTL analysis was performed as described in a previous publication. Germline genotypes of these patients were genotyped on the Illumina 25k Exome v1.0 and imputed to 1000 genomes phase 3 release using IMPUTE2. For cis-eQTL analysis, we normalized gene quantification separately for healthy and tumor samples. Technical covariates were determined using the PEER program and 20 PEER factors were used in normalization. The cis region was defined as ±1 Mb from the transcription start site for each gene. The associations between genotypes and gene quantification were obtained using the FastQTL software. A reduced genome was generated by extracting the flanking the NAFTA restriction sites (30 bp on each strand from the NAIII restriction sites to downstream) using the hg19 reference human genome in order to improve the mappability of our 4C-seq data. Subsequently, the mappability of the reduced sequences from each strand was evaluated and the uniquely mappable NAIII restriction sites were kept for downstream analysis. The reads from each library were parsed based on the bait-specific primer sequence and mapped to the reduced hg19 genome using BWA-ALN with the default parameters. A Bioconductor package HTSeq was used to extract 4C fragments from the 4C sliding window used to detect significant interactions and calculate interaction difference.
Pathway annotation and GSEA analysis. TFs binding differentially to the 116 loci were annotated to PANTHER pathways using PANTHER Classification System. GSEA analysis was applied to identify the gene sets correlated to DCLK3 expression. The search was performed against hallmark gene sets in Molecular Signatures Database (MsigDB, v5.1) [86].

Data availability. Raw and processed LC-MS/MS data and sequencing data are available at ProteomeXchange (http://www.ebi.ac.uk/pride/) and Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession numbers PXD004435 and GSE83968, respectively. The publicly available data used in this study were downloaded from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) using the following accession numbers: (1) H3K4me1 and H3K4me3 ChIP-seq: GSM1240111, GSM45304, GSM1208810, GSM1208881; (2) DNase I-seq: GSM736493, GSM736600, GSM736587, GSM665815, GSM665818, GSM665826, GSM701490, GSM701495, GSM701513, GSM774213, GSM774214, GSM774214, GSM774220, GSM774233, GSM774233, GSM817162, GSM817188; (3) TF ChIP-seq: GSM1010902, GSM1208883, GSM1208842, GSM1208830, GSM803354, GSM1010847, GSM1208763, GSM1010765, GSM1010790, GSM10120852, GSM1208958, GSM791411, GSM791412, GSM782123, GSM772208, GSM7112302, GSM1192303. The detailed information of in-house generated and public data sets used in this study are listed in Supplementary Table 4. All other remaining data are available within the article and Supplementary Information files, or available from the authors upon request.

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Author contributions
N.Q.L., N.C.H. and H.G.S. conceived and designed this study. N.Q.L., M.t.H., L.N.N., T.P. and O.J. performed the proteomics, genomics and cell biology experiments. N.Q.L., S.-Y.W., J.B.S., H.O., E.T.D., R.S.H. and N.C.H. carried out statistical and bioinformatics analyses. J.B.B. and C.L.A. provided the SYSCOL RNA-seq data, and J.Y. and J.T. provided some sequencing data of the LoVo cell line for this study. H.G.S. supervised the whole study. N.Q.L. and H.G.S. drafted the manuscript, which was reviewed by all the authors.

Additional information
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