Lymphocyte antigen 6G6D-mediated modulation through p38α MAPK and DNA methylation in colorectal cancer

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Abstract

In addition to being novel biomarkers for poor cancer prognosis, members of Lymphocyte antigen-6 (Ly6) gene family also play a crucial role in avoiding immune responses to tumors. However, it has not been possible to identify the underlying mechanism of how Ly6 gene regulation operates in human cancers. Transcriptome, epigenome and proteomic data from independent cancer databases were analyzed in silico and validated independently in 334 colorectal cancer tissues (CRC). RNA mediated gene silencing of regulatory genes, and treatment with MEK and p38 MAPK inhibitors were also tested in vitro. We report here that the Lymphocyte antigen 6G6D is universally downregulated in mucinous CRC, while its activation progresses through the classical adenoma-carcinoma sequence. The DNA methylation changes in ly6G6D promoter are intimately related to its transcript regulation, epigenomic and histological subtypes. Depletion of DNA methyltransferase 1 (DNMT1), which maintains DNA methylation, results in the derepression of ly6G6D expression. RNA-mediated gene silencing of p38α MAPK or its selective chemical inhibition, however, reduces ly6G6D expression, reducing trametinib’s anti-inflammatory effects. Patients treated with FOLFOX-based first-line therapy experienced decreased survival due to hypermethylation of the ly6G6D promoter and decreased p38α MAPK signaling. We found that cancer-specific immunodominant epitopes are controlled by p38α MAPKs signaling and suppressed by DNA methylation in histological variants with Mucinous differentiation. This work provides a promising prospective for clinical application in diagnosis and personalized therapeutic strategies of colorectal cancer.

Keywords: Lymphocyte antigen 6G6D, DNA methylation, Colorectal cancer, Anti-tumor immunity

Background

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide [1]. Although inherited genetic susceptibility plays a key role in a subset of cancers, the vast majority of CRCs are sporadic and not inherited [1, 2]. A Western diet, smoking, obesity, diabetes, alcohol consumption, and exposure to carcinogens are important risk factors for the development of sporadic CRC [2]. Early detection of CRC is critical for effective treatment. In recent years, genomic and high-throughput profiling of mRNA expression data from patient tissues have greatly improved our understanding of tumor biology and environmental factors leading...
can be distinguished by a large number of histopathological changes, with multiple subpopulations within a tumor showing differences in inflammatory infiltrates, mutations, and morphology [3, 4]. These studies have shown that CRC is not a uniform disease, but rather a heterogeneous collection of neoplastic diseases. A crucial contribution to the pathogenesis of CRC also depends on the anatomical location of the tumor and differs between right- and left-sided CRC [5, 6]. In routine clinical practice, CRC tissues can be distinguished by a large number of histopathological variants, but no reliable tissue-specific antigens are available to unambiguously identify these different variants [7]. In this context, we have recently identified lymphocyte antigen 6, member of the G6D family (LY6G6D), as a tissue-specific antigen that is unique to colorectal cancer and has minimal or relatively low expression in normal colorectal mucosa [8]. We know that LY6G6D is induced de novo in CRC, but the mechanism of its diffuse activation remains unclear. The LY6G gene cluster on human chromosomes 6 belongs to the major compatibility complex (MHC) class III. Recently, several genes encoding the III class region have been described and appear to be involved in both global and specific inflammatory responses [9]. It has been suggested that genes of the LY-6 family can be regulated in original ways, for example, by forming chimeric transcripts with their neighbors or undergoing an intron retention event, a mechanism that would prevent expression in most cell lines and tissues [10]. Thus, it might be possible that blockade of LY6G6D expression may be triggered only at a specific developmental stage or in specific pathological processes, but no conclusive data are available in this study, we show that the LY6G6D gene is characterized by relatively high basal DNA methylation in both the promoter and gene body regions, and its expression is activated by DNA hypomethylation through the classical adenoma-carcinoma sequence. Conversely, LY6G6D expression is downregulated and its promoter hypermethylated in mucinous CRC, regardless of anatomical location. In vitro studies and bioinformatic predictions revealed that p38α MAPK and DNA methyltransferase 1 (DNMT1) play important roles in controlling LY6G6D expression. Finally, we show that hypermethylation of the LY6G6D promoter may be a predictor of resistance to FOLFOX therapy, which is used as a basic scaffold for the treatment of patients with metastatic CRC.

### Methods

**In silico analysis of non-pathological and colorectal cancer datasets**

Gene expression profiles, RNA-sequencing and clinical data were downloaded using the TCGA biolinks R/Bioconductor package [11]. To better correlate the expression of LY6G6D with distinct histological types, we analyzed an impendent dataset (affymetrix transcriptomic profiles, GSE4045) corresponding to serrated primary CRCs, which are morphologically different from conventional adenocarcinomas [12]. In total, 519 transcriptomic profiles of COAD, including 478 tumor and 41 normal samples, and 176 transcriptomic profiles of READ, including 166 tumor and 10 normal samples were collected from TCGA. To adjust gene-level effects and distributional difference within and between samples we applied the GC content correction and upper-quantile normalization to raw data using the EDASeq R/Bioconductor package [13]. All the differential expression analyses between two considered conditions were performed using the edgeR R/Bioconductor package [14]. Genes with an adjusted P-value (Benjamini & Hochberg correction) ≤ 0.01 and log2 Fold Change ≥ 1.5 were considered significantly differentially expressed. To test if the candidate transcriptomic datasets show statistically significant differences between two biological states (i.e., tumor and normal) the CRC expression profiles were analyzed by GSEA, using the Cluster Profiler R/Bioconductor package [14]. Enrichment map was used for visualization of the GSEA results. Further, Gene Ontology-Biological Process terms show statistically significant differences between two biological states (i.e., tumor and normal) the CRC expression profiles were analyzed by GSEA, using the Cluster Profiler R/Bioconductor package [14]. Enrichment map was used for visualization of the GSEA results. Further, Gene Ontology-Biological Process terms overrepresentation analysis of the commonly up-regulated genes in tumor versus normal samples, both in COAD and READ, was computed using the Cluster Profiler R/Bioconductor package [15]. The Genotype-Tissue Expression (GTEx) biobank [https://www.gtexportal.org/home/] was used to analyze normalized RNA-seq data indicated as Transcripts Per Million (TPM) from 54 nondisease tissue sites [16]. We collected data from human proteome atlas platform to integrate Transcriptomic data and the relative protein expression of the candidate genes. Subcellular and tissue distributions of proteins encoded by genes of interest were visualized by immunohistochemical (IHC) staining. High resolution IHC images and protein distribution across different cancer tissues were downloaded from [https://www.proteinatlas.org/][18]. The graphical representation of gene expression data and the derived survival curves were imported from GEPIA, a bioinformatics web tool for analyzing RNA sequencing expression data across TCGA [18]. Another interactive web resource named UALCAN, for analyzing cancer OMICS including Proteomic data from Clinical Proteomic Tumor Analysis Consortium (CPTAC), was used to identify or validate...
the correlation between gene expression data and pathological parameters [19]. The cBioPortal database (https://www.cbioportal.org/) was used to integrate mRNA expression, genomic data, somatic cell mutation, DNA methylation and DNA copy number changes in cancer samples [20]. The significance of genes in determining the overall survival was analyzed using the Kaplan–Meier curve. We considered as statistically relevant a p-value less than 0.05. To further establish the role of pathways involved in drug resistance, we analyze gene expression profiles and proteome data from oxaliplatin treated CRC patients and in vitro cellular models treated with and without oxaliplatin (GSE83129 and GSE83131) [21].

**Differential DNA methylation analysis**

Comparison of the averaged methylation values was made between clinical groups at the CpG site level using Wilcoxon’s test for paired samples. DNA methylation analysis was performed using The Cancer Genome Atlas (TCGA) data and SMART App (http://www.bioinfo-fo-zs.com/smart app), a web application for comprehensively analyzing DNA methylation data across TCGA project [22]. UALCAN mentioned above, was used as an independent platform to validate gene methylation in relation to cancer subtypes. To discriminate DNA methylation profiling the following criteria were used: β-difference > 0.2 and a FDR-corrected p value < 0.05. The Beta value indicates levels of DNA methylation ranging from 0 (unmethylated) to 1 (fully methylated). We considered Hyper-methylation and hypo-methylation levels based on that detected in normal non-neoplastic colonic mucosa. These same criteria were used to calculate the methylation difference among the CpG site level variants identified. The average of the β-values of differential CpG sites in the encoding regions and transcription start site (TSS) were used to establish the relationship between gene transcription and methylation profile. To ensure consistency of data processing, we compared samples with publically accessible samples with raw idat files. The human LY6G6D gene promoter and encoding regions were retrieved from http://genome.ucsc.edu/cgi-bin/hgGateway a genome, a genome browser to search and analyze genome sequence and annotation data. MethPrimer program at http://www.urogene.org was used to identify the distribution of CpG island in defined regions of LY6G6D structure. We also collected independent series of genome-wide DNA methylation datasets which included normal colonic mucosa, low-grade adenoma and high-grade adenoma (GSE139404) [23]. Genome-scale DNA methylation data (GSE148766) derived from in house primary-resistant or drug-sensitive mCRCs treated with 1st-line FOLFOX or FOLFIRI backbone chemotherapy were analyzed [24]. To establish whether the loss of three DNMTs, 1, 3A, and 3B, affect DNA methylation profiles and transcription at LY6G6D locus, we collected genome-wide DNA methylation and gene expression data from (GSE93136) [25]. Finally, 5-FU resistant cell line from its parental wild type CRC HCT-8 cell line treated both with 5-FU for 0 and 72 h and evaluated three replicates were assessed to verify the methylation pattern of LY6G6D using the Illumina 450 k Methylation Beadchip (GSE81006) [26].

**CRCs dataset of tissue microarrays analysis**

Colorectal cancer tissues including a subset of adjacent normal tissues were obtained from the San Filippo Neri Hospital, Rome, Italy as previously reported [8]. The recruitment of samples was performed following the ethical guidelines, protocol number: 1703/2016 of September 2016 from the San Filippo Neri Hospital, Rome, Italy as already reported [8]. The procedure for tissue microarrays (TMAs) preparation and analysis was performed as previously described [8]. Briefly, the corresponding area on the matching formalin-fixed, paraffin-embedded tissue (donor block) was then identified and marked. Tissue cylinders with a 2 mm diameter were punched from representive tissue areas of each donor tissue block and placed into one recipient paraffin blocker. Each TMA spot included at least 50% tumor cells.

**Immunohistochemistry**

Immunohistochemistry (IHC) analysis was performed as previously reported [8] using anti-LY6G6D (ab139649 Abcam, Cambridge, UK) on 4-μm-thick histological TMA sections. Mismatch repair (MMR) was investigated using the following antibodies: human mutS homolog 2 (anti-MSH2, ab92372), MutL homolog 1 (anti-MLH1, ab92312), human mutS homolog 6 (anti-MSH6, ab92471) from (Abcam Cambridge, UK). LY6G6D positive intratumoral cells were counted automatically with ImageJ-based software. All the cell counts were expressed as cells mm⁻² as already reported [8]. The proportion of cancer cells stained was scored regardless of intensity as follows: Negative, as the complete absence of staining in more than 95% of tumor cells; Weak/moderate, characterized by a limited number of tumor cells scattered in a background of either negative or positive tumor cells; High or intense as a homogeneous staining in virtually all tumor cells.

**Cell lines, treatment and western blot analysis**

The human colorectal carcinoma HCT116 cell line was obtained from ATCC (CCL-247) and authenticated by microsatellite markers analysis. According to reports [27], p38α shRNA was inserted into the pSuper.retro. puro vector to produce HCT116 cells that have p38α
permanently knocked down. These cells were then selected with 2 g/ml puromycin. Cells transfected with the empty vector were also produced as a control. The SB203580 (Calbiochem; 559,389) was used to selectively inhibit p38α at concentration of 5 μM. The Trametinib, a selective MEK1/2 tyrosine kinase inhibitor, was used as described in [8]. The inhibitors were dissolved in sterile dimethylsulfoxide (DMSO) and a 10 mM working solution and stored in aliquots at −20 °C. Working concentrations were diluted in culture medium just before each experiment. For western-blot analysis, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 5 mM EGTA, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na3VO4 and 20 mM NaF and centrifuged (at 13,000 rpm 10 min, 4 °C). Supernatants (total cell extracts) were stored at −80 °C. Protein concentration was determined by the Bradford method. Western-blot analysis was carried out as previously described [8] using total cell extracts. Proteins were separated by electrophoresis using SDS-page gels and transferred to nitrocellulose membranes that were probed with the following antibodies against: P-p38MAPK (9211); P-ERKs (9101) Cell Signaling Technology and anti-LY6G6D (ab139649 Abcam, Cambridge, UK). β-actin, dilution 1:10,000, Sigma Aldrich) was used as loading control.

Statistical analysis
The statistical analyses were carried out using Prism version 4.02 (GraphPad Software, Inc), GeneSpring R/bioconductor v.12.5 and R based package. All p values represent two-sided tests of statistical significance with p value < 0.05.

Results
LY6G6D is selectively and strongly expressed in colon and rectal adenocarcinomas
To comprehensively evaluate the gene expression profile of LY6G6D, we analyzed GTEx data with gene expression profiles from 54 nondiseased tissue sites. LY6G6D and its neighboring gene lymphocyte antigen 6 family member G6F (LY6G6F), as well as two other gut specific transcription factors encoding the Caudal Type Homeobox 1 and 2 genes, CDX1 and CDX2, were included in the analysis [28]. As expected, CDX1 and CDX2 were mainly expressed in the small and large intestine. LY6G6D was most strongly expressed in testicular and prostate tissues, and moderate expression was found in the normal colon tissue “sigmoid colon”. In contrast, LY6G6F was most strongly expressed in blood, followed by testis, but not in colonic tissue (Additional file 1: Fig. S1A, B). These results suggest that LY6G6D/6F gene expression is restricted to specific human tissues, and that normal colon tissues are characterized by weak to moderate expression of LY6G6D. We next analyzed the expression profiles of LY6G6D and LY6G6F in tumor tissues from 31 different types in TCGA, using GTEx data for comparison. In tumor tissues, the highest expression of LY6G6D was in colon carcinoma, and LY6G6D was significantly overexpressed only in colon tumor tissues. The expression of LY6G6D was five times higher in colorectal carcinomas than in the corresponding normal mucosa (Fig. 1A and Additional file 1: Fig. S1C). Of note, the expression of LY6G6F was mainly found in CRC tissue, but on average, the expression in tumors was less than one-fold higher that that in normal tissue (Additional file 1: Fig. S1C). Overall, the in silico data showed consistent upregulation of LY6G6D found only at CRC.

Downregulation of LY6G6D characterizes mucinous colon adenocarcinoma
To decipher the variations of LY6G6D expression in different histological subtypes of colorectal carcinoma, we examined the gene expression profiles in the TCGA database, which includes samples of colon adenocarcinoma (COAD, N = 478) and rectal adenocarcinoma (READ, N = 166), respectively. We found that the expression levels of LY6G6D were significantly lower in mucinous adenocarcinomas (MAD) than in classical colon adenocarcinomas (CAD), regardless of anatomic location (Fig. 1B). The low expression pattern of LY6G6D in mucinous cancer resembled that in normal colonic mucosa (Additional file 1: Fig. S1D). Interestingly, a clear overlap was observed between classical adenocarcinoma and tubular/villous adenoma, suggesting that upregulation of LY6G6D occurred early in adenoma and persisted after malignant transformation following the well-defined sequence from adenoma to adenocarcinoma (Fig. 1B).

By examining gene expression profiles in serrated colorectal carcinomas, we recapitulated the results obtained in TCGA and found that mucinous subtypes had lower expression of LY6G6D compared with non-mucinous colorectal carcinomas, even within specific histological variants (Additional file 1: Fig. S1D). Mucinous CRC is a specific histological subtype commonly associated with the proximal colon and characterized by dedifferentiation and mucin production [29]. Because LY6G6D has not previously been associated with differences in histotype, we analyzed the global gene expression profiles of mucinous and classical adenocarcinomas in the COAD and READ TCGA databases. Using a stringent FDR of 0.01, 205 up-regulated and 112 down-regulated genes were identified in COAD. On the other hand, 231 and 41 genes were up- and down-regulated in READ, respectively (Fig. 1C, D, Additional file 1: Fig. S2A and Additional file 2: Table S1, Additional file 3: Table S3). Overlapping
the above results, a total of 70 genes were upregulated and 16 downregulated in mucinous CRC compared with classical adenocarcinoma. In particular, we found that the common downregulated cluster included the gene LY6G6D (Fig. 1D). In addition to the well-known MUC2 gene involved in mucin processing and secretion, analysis of the upregulated genes revealed pathways not previously known to be overexpressed in mucin cancer, including inflammatory chemokines such as CXC motif chemokine ligand (CXCL) 8, CXCRL1 and CXCRL2 receptor, and IL-1β-inflammosome [30] (Fig. 2A). Our analysis of the downregulated genes revealed biological changes in immunity (LY6G6D), protein digestion and absorption (XPNPEP2), lysine degradation (PIPOX), and vitamin digestion and absorption (SLC19A3) (Additional file 1: Fig. S2B). Mucinous dedifferentiation was associated with the CIMP-H/MSI phenotype, a high frequency of BRAF and KRAS mutations, and fewer changes in the p53 pathway, consistent with the literature [29] (Additional file 1: Fig. S2C, D, S3A). Thus, these data clearly indicate that LY6G6D is one of the genes that are generally downregulated in mucinous CRC.

Proteogenomic profiling reveals DNA methylation changes in Mucinous gene signature

To further explore the role of down-regulated genes, we integrated mRNA and protein expression data by immunohistochemistry (IHC) from the Human Protein Atlas. We found that approximately 50% of the genes encode secreted proteins (Fig. 2B). A subcluster
of genes encoding proteases (PRSS33), protease inhibitors (R3HDML), and cytoskeletal factors (TNNC2) was overexpressed only in colon tumor tissues and resembled the expression pattern of LY6G6D (Additional file 1: Fig. S3B, C). For example, the IHC data showed intense extracellular positivity of ISM2 in classical adenocarcinomas compared with mucinous tumors, reflecting the differences in gene expression profiles (Fig. 2C, D). To validate the differential expression of LY6G6D in different CRC subtypes, we examined our in-house CRC tissue databases. Overall, we evaluated the immunohistochemical expression of LY6G6D in (n = 334) colorectal cancer tissues consisting of 51 (15%) mucinous and 283 (85%) non-mucinous adenocarcinomas, respectively. More than 50% of the malignant tissues showed moderate LY6G6D positivity regardless of histological type. However, negative staining was more common in mucinous carcinomas than in non-mucinous carcinomas (25% versus 10%). In contrast, we found that strong/intense membranous/cytoplasmic immunoreactivity of LY6G6D was less frequent in mucinous carcinomas than in non-mucinous carcinomas CRC, (18–35%) (p < 0.05) (Fig. 3A). All cases were also analyzed for mismatch repair protein expression by IHC. More than 50% of mucinous tumors were MMR-deficient, compared to 15% of classical adenocarcinomas, which is consistent with the literature [29]. Next, we examined the tumor microenvironment for infiltration of LY6G6D+ cells in mucinous and non-mucinous tumors. We found no relevant difference in the intratumoral expression of LY6G6D (Fig. 3A). These results suggest that changes in LY6G6D expression are primarily related to cancer cell-intrinsic properties in CRC tissues. We next inquired about the causes of downregulated expression. Of note, in classical carcinomas, there was coamplification and overexpression of the R3HDML, TNNC2, and DUSP15 locus at chromosome band
suggesting functional cooperation between these coamplified genes (Additional file 1: Fig. S3D). In contrast, we found no relevant genetic abnormalities in mucinous CRCs. In light of these data, we examined the effects of DNA methylation variations in COAD and READ samples using TCGA data. We found that the majority of genes exhibited high basal DNA methylation in normal colonic tissue and similarly in mucinous CRC. In contrast, a striking 70% of genes in classic adenocarcinomas exhibited DNA hypomethylation. Remarkably, we found that only LY6G6D was methylated differently in mucinous tissue than in adenocarcinoma, regardless of the primary tumor site (Fig. 3B, C). Therefore, to obtain a more detailed picture, we examined the DNA methylation profiles of the entire LY6G6D gene. We examined three sequences: region (A) upstream of the transcription start site (TSS); region (B) overlapping with TSS; region (C) located in the coding region, the “gene body” (Additional file 1: Fig. S4A). We found consistent changes in DNA methylation patterns at the TSS and gene body levels (Additional file 1: Fig. S4B). Specifically, the methylation changes around TSS showed a strong and negative linear relationship with the transcription levels of the gene LY6G6D ($R = -0.91, p = 2.2e-16$, Additional file 1: Fig. S4C).

To better understand whether epigenetic changes in LY6G6D play a role in CRC carcinogenesis, we analyzed DNA methylation changes at TSS and in the gene body in an independent data set that included normal colonic mucosa and low- and high-grade adenomas. We found...
that DNA methylation reduced considerably from normal mucosa to the transition from low-grade to high-grade adenomas, showing that LY6G6D hypomethylation is an early step in the progression of classical adenoma-carcinoma (Fig. 3D). This is confirmed by the fact that CIMP-negative CRCs (clusters 3 and 4) have higher expression of LY6G6D than CIMP-positive (CIMP-L and CIMP-H) tumors. A similar trend was also observed in a subset of 17 CRC cell lines (Additional file 1: Fig. S4D). These data suggest that changes in CpG island methylation have critical effects on LY6G6D transcriptional regulation and mark distinct CRC subtypes.

**The expression of LY6G6D is positively regulated by p38α MAPK (MAPK14) and repressed by DNA methyltransferase 1 (DNMT1)**

We have previously shown that LY6G6D is positively regulated in a context-specific manner by the JAK/STAT5 pathway [8], but the molecular network involved in controlling its expression remains unknown. Using a regulatory network analysis strategy, we demonstrated a possible role of p38 MAPK in controlling LY6G6D expression (Fig. 4A). By integrating transcriptome and proteome data from TCGA, we found that the expression of p38α MAPK (MAPK14) tended to be lower in mucinous than in nonmucinous CRC at the protein and mRNA levels (Fig. 4A, B). Transcriptomic data showed a close association between MAPK14 (p38α MAPK) and LY6G6D gene expression in samples from CRC and derived cell lines, suggesting a possible co-regulation (Fig. 4B and Additional file 1: Fig. S5A). We therefore analyzed LY6G6D protein expression in HCT116 cells with permanent p38α knockdown (p38α shRNA), using cells transfected with the empty vector as a control [27]. LY6G6D expression decreased by approximately 50% in HCTC116 cells with p38α silencing compared to control cells (Fig. 4C). Since our previous results have shown that the STAT5/LY6G6D axis mediates resistance to the MEK inhibitor trametinib [8], we monitored LY6G6D expression levels after trametinib treatment in p38α-silenced HCT116 and control cells. As shown in (Fig. 4C, D) the level of the p38α protein was reduced by around 60–70% in p38α KD compared to control cells, and the levels of p38α were considerably lower after trametinib treatment. Remarkably, the data for LY6G6D levels showed similar trends. After trametinib treatment, we found increased LY6G6D levels in HCT116 control cells. Trametinib treatment on the other hand reduced the expression of LY6G6D in p38α knockdown cells (Fig. 4D). To further investigate the role of p38α in the regulation of LY6G6D, we treated HCT116 wild-type cells with SB203580, a known p38α inhibitor, in the presence or absence of trametinib. Treatment with SB203580 alone or in combination with trametinib resulted in downregulation of LY6G6D protein, suggestive of p38 MAP kinase-dependent regulation of LY6G6D expression levels (Fig. 5A). We next examined publicly available DNA methylation profiles and transcriptomic data in which the three DNMTs 1, 3A, and 3B were inactivated by the combination of genetic and shRNA silencing strategies. In HCT116 cells, we found that time-dependent shRNA-mediated silencing of DNMT1 reduced DNA methylation in the LY6G6D promoter four-fold compared with control. Loss of DNA methylation in DNMT1 knockdown cells was associated with significant reexpression of the LY6G6D transcript from 0.28 to 0.034 compared with control (Fig. 5B). Analysis of additional colorectal cancer cell lines, DLD1, HT29, and RKO, after DNMT1 knockdown with higher and lower shRNA efficiencies confirmed DNMT1-dependent repression of the LY6G6D transcript (Fig. 5C). In contrast, genetic deletion of DNMT3A or 3B did not significantly affect LY6G6D promoter methylation, but double knockout (with DNMT1 and DNMT3B knockout) again drastically reduced DNA methylation (Additional file 1: Fig. S5B). Therefore, maintenance of DNA methylation by DNMT1 and p38α MAPK signaling play opposite roles in transcriptional regulation and control of LY6G6D expression.

**Epigenetic suppression of LY6G6D predicts poor outcomes in colon cancer chemotherapy**

Mucinous colon cancer is associated with resistance to chemotherapy compared with nonmucinous colon cancer. Although the location of the primary tumor may play a role, the reason for this disparity is unclear [29]. A global analysis of TCGA clinical data revealed that CRC patients with low LY6G6D expression had shorter survival than those with high expression (Additional file 1: Fig. S5C). Surprisingly, we found that patients with low LY6G6D expression had shorter overall survival than patients with higher LY6G6D expression in rectal but not in colonic adenocarcinoma (Additional file 1: Fig. S6A). Our immunohistochemical analysis using the in-house database revealed that tumors with negative LY6G6D staining in left-sided tumors were associated with shorter survival than cases with moderate and high expression, but not in right-sided CRCs (Additional file 1: Fig. S6A). Collectively, these data suggest that epigenetic alterations at the LY6G6D locus may be associated with clinical and molecular features of CRC. Because LY6G6D has been associated with response to therapies, we analyzed our internal database of metastatic colorectal carcinomas that were primarily resistant to FOLFOX or FOLFIRI to understand whether the DNA methylation profile in LY6G6D might play a role in response to chemotherapy-based treatments. Surprisingly, the analysis revealed
that hyper-methylation of LY6G6D was the most important predictor of resistance to FOLFOX-based therapy (Fig. 5D). Remarkably, LY6G6D hypermethylation was associated with a CIMP-high phenotype, demonstrating the association between DNA methylation changes and chemotherapy resistance (Additional file 1: Fig. S6C, D).

Since 5-fluorouracil (5 FU) and oxaliplatin are the mainstay therapy of CRC, we analyzed high-throughput DNA methylation analysis in a cell line resistant to 5FU and its parental wild-type cell line CRC HCT-8. We found that DNA methylation in LY6G6D promoter was significantly higher in cells 5-FU resistant (around three times higher) than in wild type (Fig. 5E). To better understand the potential mechanism by which oxaliplatin resistance is linked to changes in LY6G6D, high-throughput transcriptomic and proteomic data were obtained from Patients and CRC cell lines treated with oxaliplatin. We found that LY6G6D expression was downregulated in non-responders compared to those who responded to oxaliplatin treatment, and notably correlated with reduced expression or activity of MAPK14 (Fig. 5E). These data suggest that the DNA hyper-methylation at the LY6G6D gene

![Fig. 4](image-url)
and reduced activity of MAPK14 impair therapeutics to FOLFOX-based treatment.

**Discussion**

Over the past years, advances in cancer immunology have radically changed therapeutic strategies for the treatment of many types of solid tumors, including colorectal cancer. So far, it is well documented that immune checkpoint inhibitors are effective in a small number of mismatch repair deficient (MMRd) malignancies [3, 4, 31]. Stable mismatch repair cancers account for the majority of CRCs and exhibit primary immune checkpoint resistance. Unfortunately, targeted therapies and immunotherapy have only shown their benefits in a limited subset of patients. Thus, it is necessary to define the molecular, immunological and biological landscape of
each patient with CRC [31, 32]. LY6G constitute a gene cluster belonging to the MHC class III. The functions of many LY6G genes are still unknown, their molecular alterations in human diseases remain largely unexplored [9]. Recently, our group discovered LY6G6D as a tumor-specific antigen of stable mismatch repair CRCs [8]. Here, we show that expression of LY6G6D is activated following the classical adenoma-carcinoma sequence but downregulated in mucinous CRC, regardless of primary tumor site. Consistently, IHC profiling in our CRC database has clearly demonstrated that low expression of LY6G6D is typical of Mucinous CRC. In an attempt to explain the biological basis of our observations, we found that DNA methylation changes in the LY6G6D promoter are intimately linked to its transcriptional variations. It is well known that Mucinous tumors arise and progress through different molecular pathways [29]. They often exhibit mutations in KRAS and BRAF, microsatellite instability and a CpG island methylator phenotype. In addition to JAK/STAT5 signaling, our studies support a p38α-dependent regulation of LY6G6D, expanding the spectrum of inflammatory pathways that control its expression. In particular, we define a novel role for DNMT1 in maintaining DNA methylation and transcriptional repression of LY6G6D. In fact, downregulation of DNMT1 determines the loss of DNA methylation in LY6G6D promoter and its reexpression, in keeping with the model in (Fig. 5F). However, our findings raise an intriguing question about the impact of such changes in tumor-associated epigenetic mechanisms on malignant progression. Although the interaction between mucinous CRC and the host immune system is unknown, one might hypothesize that the pathways regulating LY6G6D may differentially compromise anti-tumor immunity at the site of the primary tumor [6]. DNA hypomethylation at the LY6G6D locus occurs in vast majority of CRCs and could be an “early warning system” to stimulate anti-tumor immune response, as for other immune antigens [33]. In the clinical setting, we found that LY6G6D hypermethylation predicted resistance to FOLFOX. Furthermore, acquired resistance to 5-FU induces hypermethylation of the LY6G6D promoter in CRC cells, confirming recent observations that DNMT inhibitors given in combination with standard with 5-FU or oxaliplatin improve therapeutic responses in patients with CRC [34]. Moreover, it has been reported that DNMT inhibitors can reshape the tumor microenvironment by increasing intratumoral T cells in vivo, and by prolonging survival of patients with stable mismatch repair CRCs [35]. Our data suggest that epigenetic control of LY6G6D may influence the response to chemotherapy via DNMT1 and p38α MAPK signaling via miR-625-3p in CRC [21]. In conclusion, LY6G6D is a tissue-specific CRC antigen regulated by epigenetic mechanisms. DNMT1 and p38α MAPK signaling play important roles in controlling LY6G6D expression. These results could be relevant for the design of personalized therapeutic strategies in patients with CRC.

**Abbreviations**

CRC: Colorectal cancer; LY6G6D: Lymphocyte antigen 6 complex, locus G6D; BRAF: B-rapidly accelerated fibrosarcoma; ERK: Extracellular-signal regulated kinase; KRAS: Kirsten rat sarcoma viral oncogene homolog; MAPK: Mitogen-activated protein kinase; TCGA: The cancer genome atlas; IHC: Immunohistochemistry; GEO: Gene expression omnibus; GSEA: Gene set enrichment analysis; CIN: Chromosomal instability; MMR: Mismatch repair; CIMP: CpG island methylator phenotype; JAK: Janus kinase; STAT: Signal transducer and activator of transcription; ERK: Extracellular-signal regulated kinase; MSI: Microsatellite instability; FOLFOX: Folinic acid (leucovorin, FOL), fluorouracil (S-FU, F), and oxaliplatin (Eloxatin, OX); FOLFIRI: Leucovorin calcium (calcium folinate), S-fluorouracil (5-FU), and irinotecan (IRI); DNMT: DNA methyltransferase; MHC-III: Major histocompatibility complex class III; MEK: Mitogen extracellular kinase.

**Supplementary Information**

The online version contains supplementary material available at [https://doi.org/10.1186/s12935-022-02672-1](https://doi.org/10.1186/s12935-022-02672-1).

**Additional file 1:** Supplementary figures 1-6.

**Additional file 2:** Table S1. Differentially expressed genes Mucinous vs Adenocarcinoma in COAD.

**Additional file 3:** Table S2. Differentially expressed genes Mucinous vs Adenocarcinoma in READ.

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**Author contributions**

LC, FPC and MP developed bioinformatics analysis; GG, ML, MRD, VC and LC provide samples, collected and processed data; MP carried out immunostaining experiments and in vitro assays; AP provided samples, and assist in biochemical and functional studies. GG, AP and MP conceived the study; AP and MP coordinated in vitro experiments; MP supervised the study with the help of GG and AP. MP drafted the manuscript with input and final approval from all authors. All authors read and approved the final manuscript.

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**Availability of data and materials**

Our in-house DNA methylation data from (primary-resistant versus drug-sensitive tumors) have already been deposited at the NCBI Gene expression Omnibus repository (GEO) and are accessible through the accession number GSE148766. We collected independent genome-wide DNA methylation dataset from (GSE139404) and genome-wide DNA methylation and gene expression data from (GSE93136). Tissue microarrays, modified cell lines analysed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

The recruitment of samples was performed following the ethical guidelines, protocol number: 1703/2016 of September 2016 from the San Filippo Neri Hospital, Rome, Italy.
Consent for publication
The manuscript has been read and approved by all the coauthors for publication.

Competing interests
The authors declare no competing interests.

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