Clinical Performance of Friend Leukemia Virus Integration 1 Methylation as a Biomarker for Colorectal Carcinoma

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Research

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Abstract

Objectives

Accumulating evidence supports the reliability of molecular biomarkers and cancer, the current situation is lacking. This present study was aimed to investigate clinical performance of friend leukemia virus integration 1 (FLI1) methylation as a biomarker for colorectal cancer (CRC).

Methods

The datasets of UALCAN, GEPIA, cBioPortal, STRING, TIMER2.0, GEO, and KOBAS were utilized in this present study. In order to testify the methylation function of FLI1 in CRC, we studied 6 CRC cell lines and 20 pairs of tumor tissues. The transcriptional levels of FLI1 were tested by reverse transcription PCR quantitatively and western blot. Cell viability, transwell assays and plate clone assay were utilized to assess the cell function.

Results

FLI1 was up-regulated in the GBM, KIRC, LAML, etc. Meanwhile, it was down-regulated in BLCA, BRCA, CESC, etc ($p < 0.05$). The CPTAC dataset showed higher total protein in the primary tissues of KIRC, lower protein in the BRCA, OV, LUAD, UCEC than normal tissues ($p < 0.05$). Highly expressed FLI1 was linked to poor prognosis of overall survival (OS) in LGG, UVM ($p < 0.05$). Low expression of FLI1 was associated with short OS in KIRC, LUAD, SKCM ($p < 0.05$). Compared with normal tissues, the methylation level of FLI1 was increased in BRCA, CESC, CHOL, COAD, etc ($p < 0.05$). In the contrary, it was decreased in KIRP, PCPG obviously ($p < 0.05$). Moreover, it showed a reduced phosphorylation for selected probes (BRCA: NP_002008.2:S39, NP_002008.2:S3241; OV: NP_002008.2:S39; LUAD: NP_002008.2:S79, NP_002008.2:S3241; all $p < 0.05$). Meanwhile, it showed an enhanced phosphorylation level of FLI1 in KIRC for selected probes (NP_002008.2:S241, $p < 0.05$). Besides, a statistical negative correlation was found between FLI1 and Treg cells, neutrophils, monocytes, macrophages, NK cells, cancer-associated fibroblasts, and common immune checkpoint gene levels ($p < 0.05$). Besides, in vivo experience showed that 5-Aza-2'-deoxycytidine diminished FLI1 methylation level and restored transcriptional levels ($p > 0.05$) in CRC. In vitro experiments demonstrated that the proliferation, colony formation, invasion and migration of CRC cells were inhibited by FLI1 overexpression through FMNL1 ($p < 0.05$).

Conclusions

This present study offers a comprehensive understanding of FLI1 in carcinomas with oncogenesis and immunotherapeutic implications. Moreover, FLI1 reduced the proliferation, colony formation, invasion and migration of CRC by FMNL1.

Introduction

Cancer is a common public health problem with high incidence worldwide. Despite great improvements in surgical techniques, chemotherapy, and radiotherapy interventions, the patients' survival still remains poor [1]. Moreover, there are some challenging cases with same tumor stage in which the patients' survival is heterogeneity [2]. Recently, the methylation has been outstanding with inspiring benefits. Unfortunately, identifying novel biomarkers for methylation are still poorly defined. Thus, it is important to conduct a clinical performance of interested gene with methylation implications for CRC.

The E26 transformationspecific gene family is a transcription factor and involved in the process of cell proliferation, transformation, apoptosis, and tumorigenesis [3]. Friend leukemia virus integration 1 (FLI1), one member of ETS gene family, was first identified as a proto-oncogene isolated in proviral integration in F-MuLV-induced erythroleukemias [4]. It was mainly expressed in hematopoietic cells and tissues and attributed to the self-renewal and differentiation of hematopoietic stem cell [5]. FLI1 was emerged as a transcriptional activator or repressor in the process of embryonic development, hematopoiesis, aberrant vasculogenesis [6]. Previous studies reported that FLI1 was involved in several human cancers, including hematological malignancies (erythroleukemias, acute myeloid leukemia, and B cell lymphomas) [7–9], and solid tumors such as UCEC [10], OV [11], BRCA [12], STAD [13], LIHC [14], SCLC [15], Ewing's sarcoma [16], metastatic melanomas [17]. Recent functional studies have demonstrated that it was engaged in hematological cell differentiation [18], tumor cell apoptosis, growth arrest through the activation of Caspase3 [15], and tumor metastasis [19]. Besides, FLI1 was reported to be hypermethylated in colorectal cancer (CRC) tissues [20], and the plasm testing showed good sensitivity in different stages by using a genome-wide highresolution array [21]. However, no pan-cancer evidence was reported on the relationship between FLI1 and various tumor types based on previous data. Hence, we aimed to conduct a clinical performance of FLI1 methylation as a biomarker for patients with CRC.

Materials And Methods

Gene expression analysis of FLI1

The GEPIA2 (Gene Expression Profiling Interactive Analysis, version 2) web server (<http://gepia2.cancer-pku.cn/#analysis>) was allowed to explore the differential expression between tumor and adjacent normal tissues for FLI1 across all tumors. A p value of less than 0.05 was identified as significant computed by the Wilcoxon test. In addition, a violin chart of FLI1 expression was compared in different pathological stages (stage I-IV) through the "pathological stage map" module of GEPIA2. The expression data was showed by log2 [TPM (per million transcripts) + 1] to plots. The Clinical proteomic tumor analysis consortium of UALCAN (<http://ualcan.path.uab.edu/analysis-prot.html>), an interactive web resource for cancer data, was conducted to analyze protein expression. The FLI1 expression of phosphoprotein was also compared between primary tumor and normal tissues, respectively.

Survival analysis of FLI1 in pan-cancer

The module of survival analysis in GEPIA2 was performed to analyze the OS (Overall survival) and DFS (Disease-free survival) significance of FLI1 across tumors. A cutoff-value of 50% was used as the expression thresholds for total cohorts. The log-rank test was used for Kaplan-Meier curve as the hypothesis test.

Genetic alteration of FLI1 in pan-cancer

The cBioPortal website (<https://www.cbioportal.org/>) was utilized for genetic analysis. We selected FLI1 in the module of quick selection with section of TCGA Pan-Cancer Atlas Research. In the "Cancer Type Summary" module, we observed the genetic variation, mutation type and copy number change of TCGA tumors. Moreover, the survival data of overall, disease-free, progression-free, and disease-free survival were compared with the "comparison" module. Kaplan-Meier curves were plotted with p values as generated.

Immune infiltration analysis of FLI1

TIMER2.0 (<http://timer.cistrome.org/>) was a comprehensive resource for systematical analysis of immune infiltrates across diverse cancer types. This version of webserver provided the relationship analysis of FLI1 and immune infiltration estimated by de-convolution methods. The partial correlation with p value was obtained by the Spearman rank correlation test after purity adjustment. The data was shown with heat maps and scatter plots. In addition, we evaluated the relationship between FLI1 expression and immune checkpoint marker expression levels by Pearson's correlation analyses, respectively.

Gene enrichment analysis of FLI1

Firstly, we searched FLI1 in the STRING website (<https://string-db.org/>). The Species was set as homo sapiens. Subsequently, the minimum required interaction score was identified as "low confidence (0.15)". The meaning of the edge of the network was identified as "evidence", and the maximum number of interactor was displayed no more than 50. The experiments were originated from active sources of interaction. Afterward, the determined FLI1 binding protein was obtained.

The "Similar Gene Detection" module of GEPIA2 was applied to obtain the top 100 FLI1-correlated targeting genes based on TCGA datasets. A pairwise correlation analysis of FLI1 and selected genes was applied and plotted as dot. Moreover, the "Gene_Corr" module of TIMER2 was supplied to draw the heatmap in the selected genes.

The Jvenn (<http://bioinformatics.psb.ugent.be/webtools/Venn/>), an interactive Venn diagram viewer, was used to conduct an intersection analysis among interacted genes. Besides, we used KOBAS 3.0 (<http://kobas.cbi.pku.edu.cn/>) to perform KEGG (Kyoto encyclopedia of genes and genomes) pathway and GO (Gene ontology) analysis.

Methylation of FLI1 from Gene Expression Omnibus [GEO] datasets in colorectal cancer

In order to testify the significance of FLI1 methylation in CRC, the GEO datasets were selected for further analysis (GEO accession number: GSE42752 vs GSE77718 vs GSE101764). The number of RNA-Seq mappings per kilobase fragment of the exon model per kilobase map was identified to quantify RNA levels. All results were transformed by $\log^2$.

Clinical colorectal cancer samples and cell lines

From December 2015 to June 2017, twenty pairs of tumors and corresponding normal fresh frozen tissues were derived from the Sun Yat-sen University Cancer Center, Guangzhou, China. All samples should be naive patients with detailed follow-up data in our center. Human cancer cell lines (SW480 vs HCT116 vs HT29 vs SW1116 vs SW640 vs LOVO) were purchased from the cell bank of Chinese Academy of Sciences in Shanghai. The culture mediums of cells were McCoy's 5A (HCT116 vs HT29: HyClone, USA), incomplete Leibovitz L-15 (SW480: KeyGEN, China), and DMEM (SW640, SW1116, LOVO and HEK-293T: Gibco, USA), respectively. All cells were supported with 10% serum (Gibco, USA) in the right environment (temperature: 37°C; concentration of CO₂: 5%).

Ethical statement

The research was approved by the Ethics Committee of Sun Yat-sen University Cancer Center. Written informed consent was taken from the participants in the study

Sequenom EpiTYPER analysis

The Qiagen nucleic acid isolation kit (NW, Germany) and Zymo conversion kit (California, USA) were used to isolate and bisulfite genomic DNA from tissue samples and cell lines. Subsequently, the methylation of FLI1 was assessed by Agena Sequenom platform (CA, USA) quantitatively, which was based on the methods of ionization time-of-flight mass spectrometry. The FLI1 forward primer 5'-GGTTAATGTGTGGAGAATTGGG-3', methylated reverse primer 5'-CCGAACTCCACTTTAAAACTAA-3' and unmethylated reverse primer 5'-CCAAAACCTCCACTTTAAAACTAAATTTC-3' were shown above. Altogether, 38 CpG sites were examined. The methylation results were analyzed by the software of EpiTYPER (Agena, USA).

Bisulfite-specific PCR sequencing

The bisulfite-specific PCR (BSP) specific primer pair was used to amplify the bisulfite-treated DNA of the FLI1 gene (forward: 5'-GGTTAATGTGTGGAATATTGGG-3'; reverse: 5'-CCGAACTCCACTTTAAAACTAA-3'/5'-CCAAAACCTCCACTTTAAAACTAAATTTC-3'). The first synthetic process was denaturalized at 94°C for 3 minutes. Then the annealing step was recycled 40 times at 94°C for 30s, 48°C for 30s, 72°C for 30s. Finally, the DNA extension was set at 72°C for 5 minutes. The products were photographed by electrophoresis with ethidium bromide, which was separated and purified by PCR elution kit (Tiangen,

China). Then, the TOPO vector (Ruibo, China) was added into DNA step by step. Subsequently, six eligible clones were collected for next sequencing conducted by Ruibo.

Demethylation of 5-Aza-2'-deoxycytidine (5-AZC)

In order to verify the role of FLI1 epigenetic function in transcriptional regulation, HCT116 and SW480 were selected in vitro experiences. In short, 1×10^5 cells were co-cultured with 5-AZC (0 $\mu\text{mol/L}$ vs 5 $\mu\text{mol/L}$ vs 10 $\mu\text{mol/L}$), which were supplied with fresh medium every 24 hours for three days.

RNA extraction and qRT-PCR

Total RNA of cell lines was extracted by Trizol (Invitrogen, USA) and qualified by Nanodrop2000 (Thermo, USA). The SYBR Green qRT-PCR kit (Takara, Japan) was used to reverse RNA to cDNA, which was tested and analyzed by CFX96 RT-PCR (Bio-Rad, USA). The internal reference was set as GAPDH. The primers were proved to work effectively as follows: FLI1 (sense 5'-TAAGGACTGGAGCAAAGCGAGC-3' vs antisense 5'-CCGTCTGGGTCTGAAGTGCC-3'). Repeat the experiment in triplicate.

Western blot

Cells in an exponential growth period were collected, washed, and lysed in a mixture of RIPA and protease inhibitors (Beyotime, China). The BCA protein assay kit (Beyotime, China) was used to evaluate the protein concentration in samples. Then the protein lysate was suffered in sodium dodecyl sulfate sodium salt-polyacrylamide gel electrophoresis. Meanwhile, the target protein was electroblotted onto a polyvinylidene fluoride ultrafiltration membrane (Merck, USA). Subsequently, the membrane was blocked with 5% skim milk in buffer solution for 1 hour. The incubation concentration of primary antibodies were 1:500 (FLI1, Proteintech), 1:3000 (FNLI1, Proteintech) and 1:1000 (β -actin, Santa Cruz Biotechnology), respectively. The reaction of western blot was clear and easily judged by naked eyes in the Bio-rad imaging system (Bio-rad, USA).

Plasmid transfection and cells function

The pEZ-M35 was selected as a vector to human FLI1 cDNA (NM 002017), which was synthesized by Guangzhou GeneCopoeia Company. The FLI1 plasmids (pEZ-M35-FLI1 vs pEZ-M35-vector) were transfected with Lipofectamine 3000 (Invitrogen, USA) to CRC cell lines for 48 hours according to the manufacturer's instructions. The cell viability was measured at a wavelength of 490nm at 0 hours, 12 hours, 24 hours, 48 hours, and 72 hours through CCK8 kit (Dojindo, Japan) at three times. A two-chamber transwell system (Corning, USA) was used to evaluate cell invasion and migration analysis. Image J was performed to compare the difference in each group. Five hundred cells per dish in a 6-well plate were cultured for two weeks. The total cell clones were counted and photographed.

Statistical analysis

Statistical analysis was performed by R and SPSS 20.0 software. The comparisons of categorical or continuous variables were assessed by student's t test, and χ^2 , respectively. In addition, the Spearman correlation and ROC curve were utilized to analyze the linear relationship and the diagnostic role of FLI1 methylation. The prognostic significance of FLI1 methylation was evaluated by the methods of Kaplan-Meier analysis. Our tests were 2-sided, and the significance level is $p < 0.05$.

Results

Gene expression data of FLI1

As shown in Figure 1A, the transcriptional level of FLI1 was up-regulated in the GBM, KIRC, LAML, LGG, PAAD, TGCT. Meanwhile, it was down-regulated in BLCA, BRCA, CESC, LUAD, LUSC, UCEC, UCS ($p < 0.05$). However, no significant difference was found between FLI1 and others. The CPTAC dataset showed higher total protein in the primary tissues of KIRC, lower protein in the BRCA, OV, LUAD, UCEC than normal tissues (Figure 1B, $p < 0.05$). The pooling analysis of FLI1 and pathological stages found that a significant correlation was found in BRCA, KIRC, SKCM, STAD, THCA (Figure 1C, $p < 0.05$).

Survival data of FLI1 in pan-cancer

As shown in Figure 1D, highly expressed FLI1 was linked to poor OS in LGG, UVM and better OS in KIRC, LUAD, SKCM ($p < 0.05$). Moreover, it was shown that there was no correlation between FLI1 expression and DFS across all listed tumors (Figure 1E, $p > 0.05$).

Genetic alteration of FLI1 in pan-cancer

The genetic alteration of FLI1 across tumor samples was analyzed in the TCGA cohort. As depicted in Figure 2A, the highest alteration frequency of FLI1 ($> 8\%$) was TGCT with “deep deletion” as the major type. The “amplification” type of CNA was mainly in the uterine carcinosarcoma cases, which showed an alteration frequency of almost 3.51% (Figure 2A). The “mutation” type was in the UCEC with frequency of 4.16% (Figure 2A). We found that missense and truncating mutation of FLI1 were the main type of genetic alteration, and A25V/G alteration was detected in LIHC, UCEC, STAD (Figure 2B).

Additionally, the potential association between genetic alteration of FLI1 and the survival prognosis of colorectal adenocarcinoma was showed in Figure 2D. However, FLI1 alteration was linked to poor OS and DSS in BRCA (Figure supplement, $p < 0.05$). Additionally, FLI1 alteration was associated with poor OS, DSS and PFS in adrenocortical carcinoma (Figure supplement, $p < 0.05$).

DNA methylation and protein phosphorylation

Compared with normal tissues, the methylation level of FLI1 was increased in BRCA, CESC, CHOL, COAD, ESCA, BLCA, HNSC, KIRC, LIHC, LUAD, LUSC, PAAD, PRAD, READ, THCA, and UCEC (Figure 3A, $p < 0.05$). In the contrary, it was decreased in KIRP, and PCPG (Figure 3A, $p < 0.05$). Furthermore, we analyzed the phosphorylation level of FLI1 in CPTAC datasets of UALCAN (Figure 3B). Only five types of tumors

(BRCA, KIRC, LUAD, OV and UCEC) were calculated. It showed a reduced phosphorylation in cancer for selected probes (BRCA: NP_002008.2:S39, NP_002008.2:S3241; OV: NP_002008.2:S39; LUAD: NP_002008.2:S79, NP_002008.2:S3241; all $p < 0.05$). Meanwhile, enhanced phosphorylation level of FLI1 was found in KIRC tissues for selected probes (NP_002008.2:S241, $p < 0.05$).

Immune infiltration of FLI1

The algorithms of TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, XCELL, MCPOUNTER and EPIC were utilized to investigate the relationship between FLI1 gene expression and different immune cells in TCGA. After a series of analyses, we observed a statistical negative correlation between cancer-associated fibroblasts (Figure 4A, $p < 0.05$), Treg cells ($p < 0.05$), neutrophils ($p < 0.05$), monocytes ($p < 0.05$), macrophages ($p < 0.05$), NK cells ($p < 0.05$), and FLI1. Moreover, a significant relationship was found between FLI1 expression and common immune checkpoint gene levels (Figure 4D, $p < 0.05$).

Gene enrichment analysis of FLI1

In order to investigate the potential molecular mechanism of FLI1 in tumor, we used the STRING tool to obtain a total of 50 FLI1-binding proteins, which were based on experimental evidence (Figure 5A). Meanwhile, the GEPIA2 was used to explore the top 100 genes correlated with FLI1 expression. As shown in Figure 5B, the FLI1 expression was found to be correlated with CARD8, DOCK2, DOCK8, IKZF1, NCKAP1L. The corresponding heat map data was shown in Figure 5C, which indicated a positive correlation between FLI1 and the five genes. The above two groups shared a common gene, namely FMNL1 (Figure 5D). The KOBAS 3.0 was used to perform KEGG and GO enrichment analyses (Figure 5E-5F). This suggested that transcriptional dysregulation was involved in the effect of FLI1 on tumor development.

Bioinformatics of FLI1 methylation and transcriptional level

All target data of FLI1 were extracted and analyzed in the TCGA dataset. It was found that the methylation level of FLI1 in CRC was significantly higher than normal tissues (Figure 6A, $p < 0.05$). In the contrary, the mRNA expression of FLI1 was obviously lower in tumor (Figure 6C, $p < 0.05$). Other methylation datasets were derived from GEO to further validate FLI1 methylation in CRC (GSE42752, GSE77718 and GSE101764). Similarly, the results implied that FLI1 methylation was significantly augmented in tumors (Figure 6C, $p < 0.01$).

FLI1 is hypermethylated in CRC

In order to study the epigenetic function of FLI1 methylation in specific tumor, we included 20 pairs of CRC tissues. Table 1 summarized the total clinic-pathological characteristics in detail. Compared with adjacent normal tissues, FLI1 was significantly hypermethylated in tumors (0.23 ± 0.09 vs 0.08 ± 0.02 , $p < 0.05$, Figure 7A). Besides, all target CpG sites showed the same statistical differences ($p < 0.05$). In addition, the BSP results were in line with the above results (Figure 7B). However,

no obvious correlation was found between FLI1 methylation and tumor differentiation, CEA, TNM staging, etc. (Table 1, $p>0.05$).

ROC curve analysis and prognostic role of FLI1 methylation in CRC

The diagnostic sensitivity and specificity of FLI1 methylation for CRC were evaluated by ROC. The value of 0.11 was identified as the best cutoff of the average FLI1 methylation ratio according to the Youden index. As depicted in Figure 7C, the sensitivity was 90.0%, and the specificity was 95.0%, respectively. The area under the curve was 0.96. In order to assess the prognostic value of FLI1 methylation, patients were divided into two cohorts. The median follow-up was 878 days (range: 222 days to 1341 days). According to Kaplan-Meier analysis, FLI1 methylation level was not independently predict OS, but it showed a trend in patients with CRC ($p=0.53$, Figure 7D)

Regulation of FLI1 methylation in CRC cell lines

The FLI1 methylation level was further explored in cancer cells (SW480 vs HCT116 vs SW640 vs HT29 vs LOVO vs SW1116). The results found that cancer cells showed hypermethylation than control one (Figure 8A, $p<0.05$). All cells were divided into three groups and treated with 5-AZC for 72 hours (0 μ mol/L vs 5 μ mol/L vs 10 μ mol/L). The FLI1 methylation was gradually declined after three days (Figure 8B, $p<0.05$). At the same time, the mRNA and protein levels were found to be ameliorated obviously (Figure 8C-8D, $p<0.05$). The existing data indicates that FLI1 methylation may be the reason for the dysregulation of FLI1 transcription expression in CRC.

FLI1 inhibited proliferation, invasion and migration via FMNL1 in CRC cells

As showed in Figure 8E-8F, the expression of FLI1 in the vector and FLI1 transfected HCT116 and SW480 cells were tested by RT-PCR and Western blot. After restoring the expression of FLI1, cell viability was significantly reduced (Figure 8G, $p<0.05$). The clones were 172.00 ± 10.58 and 246.33 ± 14.57 in the HCT116 group (pEZ-M35-FLI1 and pEZ-M35-Vector), respectively. The number of clones in the SW480 group (pEZ-M35-FLI1 and pEZ-M35-vector) were 102.33 ± 18.55 vs. 300.33 ± 13.31 after overexpression of FLI1 (Figure 8H, all $p<0.05$). After re-expression of FLI1 in the HCT116 and SW480 cell group, the expression of FMNL1, the number of invasion and migration cells was significantly restrained (Figure 8I-8J, $p<0.05$).

Discussion

Cancer is a highly complex disease with heterogeneous prognosis and the immunotherapy is promising [1]. Herein, we reported that (1) FLI1 was obviously dysregulated in the mRNA and total protein levels for various cancers. (2) High FLI1 expression was linked to poor OS in LGG, UVM and better OS in KIRC, LUAD, SKCM. (3) Compared with normal tissues, the FLI1 methylation was increased in BRCA, CESC, et al. and decreased in KIRP, PCPG. Moreover, it showed low phosphorylation level in BRCA and high phosphorylation level in KIRC for selected probes. (4) A statistical negative correlation was found

between FLI1 and common immune checkpoint gene levels / immune cells (cancer-associated fibroblasts, Treg cells, neutrophils, monocytes, macrophage, NK cells). (5) In vivo experience revealed that 5-AZC reduced FLI1 methylation level and restored its transcriptional expression in CRC. In vitro experiments demonstrated that the proliferation, colony formation, invasion and migration of CRC cells were inhibited by FLI1 through FMNL1.

The expression and survival data of FLI1 was different among tumors. Mhawech-Fauceglia et al. performed an immunohistochemical analysis on 4323 tumor samples to determine the expression of FLI1 in various malignant neoplasms. It was shown that FLI1 could be seen in a variety of solid tumors, which was considered to be highly sensitive and specific in distinguishing EWS/PNET from other tumors in general [16]. Some scholars put up that FLI1 was over-expressed in OV [22], UCEC [10], BRCA [12], and LIHC [14]. It was associated with advanced tumor stage, poor OS and DFS in OV [22]. Up-regulated FLI1 was associated with a high differentiation grade, mutated P53 expression, histological subtype, and short prognosis in UCEC [10]. Moreover, the abundance of FLI1 protein was strongly correlated with advanced stage, poor differentiation, and lymph node metastasis in BRCA [12]. However, others demonstrated that FLI1 was down-regulated in BRCA, which was associated with short survival and aggressive phenotypes [23]. Low FLI1 was associated with poor OS in LUAD [24]. These conflicting results suggested that FLI1 was involved in a complex pathway, which needs to be explored further. FLI1 was reduced in CRC cell line [25], which was consistent with our study, indicating the potential role of FLI1 in CRC.

FLI1 was hypermethylated in most TCGA cancers. For example, FLI1 was methylated in adenomas and colorectal tumor tissues and its mRNA level could be recovered by vincristine in cells [20, 26]. This was in agreement with our findings, suggesting that FLI1 methylation might be a common event in the development of CRC. Besides, it was demonstrated to be a valuable biomarker for early diagnosis of CRC [27]. The plasma FLI1 was reported to be hypermethylated in CRC with good sensitivity in various stages by the method of a genome-wide high-resolution array [21]. Similar to previous studies, our study also revealed the diagnostic value of FLI1 methylation with good sensitivity and specificity, which deserves further validation in large cohorts. In the contrary, FLI1 methylation showed a trend without statistical significance for patients' OS. Some plausible explanations should be acknowledged as follows: The sample size of CRC cohort was insufficient, and the follow-up was short and censored. In the future, we should validate the result in a larger study.

The detail phosphorylation role of FLI1 in cancer was unclear. The TGF β /c-Abl/PKC δ /phospho-FLI1 pathway was involved in scleroderma therapy [28]. Mutation of FLI1 phosphorylation site led to a significant GM-CSF promoter activation, which suggested that FLI1 was a critical druggable regulator of inflammation and immunity [29]. Our study indicated that the phosphorylation of FLI1 was dysregulated in BRCA, OV, LUAD, KIRC for selected probes, which needs to be investigated in the future.

Previous studies suggested that FLI1 was associated with T cell activation. Improving effector activity of tumor-specific T cells was a major goal in cancer immunotherapy. Some scholars indicated that FLI1 safeguarded the developing CD8⁺ T cell transcriptional landscape from excessive ETS: RUNX-

driven effector T cell differentiation. Genetic deletion of FLI1 improved effector T cell differentiation and protective immunity in infections and cancer [30]. It was demonstrated that the FLI1 was enriched in immune biological processes with 28 immune cell infiltrations. Furthermore, the FLI1 was correlated with the immune-related genes in BRCA [31]. In the human T-cell line Jurkat, both isoforms of FLI1 (p51, p48) were phosphorylated on serine residues, which was increased by a Ca^{2+} -mediated process, and inhibited by protein phosphatase 2A [32]. Our study was in line with previous researches, which indicated a significant correlation between FLI1 and Treg cells, neutrophils, monocytes, macrophage, NK cells, cancer-associated fibroblasts. Besides, we found a statistic relationship between the FLI1 and common immune checkpoint gene levels. These suggested that FLI1 may participate in the immune progress and could be as a candidate biomarker for immunotherapy.

The detailed molecular and biological mechanism of FLI1 in tumor was misty. Some reported it as an oncogene in OV, SCLC, UCEC, LIHC, and BRCA. It was found to promote cell proliferation in OV [11] and clone formation and cell proliferation in UCEC [10]. Moreover, FLI1 was a driving factor that promoted tumor growth in SCLC through miR-17-92 [15]. FLI1 exon circular RNA was a new oncogenic driver through miR584-Rock1 pathway. Serum exosomal FLI1 exon circular RNA 1 was served as a promising biomarker to track disease progression of SCLC [33]. FLI1 promoted metastasis through MMP2 in LIHC [14]. It inhibited tumor apoptosis and promoted metastasis through Rho GTPase [12] and cancer stem cell properties of BRCA cells [34, 35]. FLI1 exon circular RNA 1 was acted as an upstream regulator to control BRCA tumor growth by coordinating the regulation of DNA methylated and demethylated enzymes [19]. In erythroleukemia, its overexpression promoted erythroid dedifferentiation, apoptosis and proliferation [36]. Besides, others reported FLI1 as a tumor suppressor in BRCA, STAD, and CRC. It was decreased or lost in BRCA with increased proliferation, invasion, migration, and metastasis, which was associated with short survival. FLI1 altered gene expression in several cellular pathways, such as the cytokine-cytokine receptor interaction and PI3K-Akt signaling pathways [23]. It was a suppressor of invasiveness and proliferation in STAD, specifically in the transition from pre-cancer lesions and dysplasia to invasive adenocarcinoma, suggesting that FLI1 may be a prognostic biomarker of survival in STAD [13]. FLI1 overexpression attenuated colony formation and invasion via Klotho signaling in CRC [25]. Our KEGG analysis indicated that transcriptional dysregulation was involved in the effect of FLI1 on tumor development. Hence, a plausible explanation was that FLI1 was emerged as a transcriptional activator or repressor in carcinomas through various pathways. Our in vivo and in vitro experiences demonstrated that FLI1 functioned as a tumor suppressor via FMNL1 in CRC due to methylation, which was consistent with previous studies, indicating that the methylation was devoted to the absence of FLI1 in CRC.

There exist some limitations in this study. First, analysis of FLI1 on the transcriptional level, methylation, phosphorylation, and immune infiltrating cells could only reflect some aspects of immune status, which was just a tip of the iceberg. In the future, multicenter cohorts should be performed to validate our results.

Second, our team failed to conduct in-depth data mining, which needs to be confirmed. Finally, the sample size and survival data of our study was insufficient, which should be validated future.

Conclusions

In conclusion, our pan-cancer analyses suggested that FLI1 was involved in the oncogenesis and development across multiple tumors with respect to DNA methylation, protein phosphorylation, immunotherapy, and survival benefits. Moreover, we demonstrated that FLI1 reduced the proliferation, colony formation, invasion and migration of CRC by FMNL1.

Abbreviations

BRCA	Breast invasive carcinoma
COAD	colon adenocarcinoma
READ	rectum adenocarcinoma
ESCA	esophageal carcinoma
GBM	glioblastoma multiforme
KIRC	kidney renal clear cell carcinoma
LGG	brain lower grade glioma
LIHC	liver hepatocellular carcinoma
LUAD	lung adenocarcinoma
LUSC	lung squamous cell carcinoma
SCLC	small cell lung cancer
OV	ovarian serious cystadenocarcinoma
PAAD	pancreatic adenocarcinoma
PRAD	prostate adenocarcinoma

STAD
stomach adenocarcinoma
THCA
thyroid carcinoma
UCEC
uterine corpus endometrial carcinoma
CRC
colorectal cancer
TGCT testicular germ cell tumors
UCS
uterine carcinosarcoma
LAML
acute myeloid leukemia
BLCA
bladder urothelial carcinoma
CHOL
cholangio carcinoma
UVM
uveal melanoma
SKCM
skin cutaneous melanoma.

Declarations

Data Availability

All data in this manuscript would be accessed under the admission of corresponding author.

Author Contributions

HY conceived and modified the study. WJ and XCL conducted, performed, and drafted the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declared that they have no conflict of interest.

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Ethics Statement and Informed Consent

The research was approved by the Ethics Committee of Sun Yat-sen University Cancer Center. All patients were recruited with signed informed consent.

Data Availability

All data in this manuscript would be accessed under the admission of corresponding author.

Consent for publication

The consent for publication was approved by all authors.

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Tables

Table1: Relationship between FLI1 methylation and clinicopathological parameters in colorectal cancer

Clinical parameters	n(n%)	The average methylation of FLI1	
		mean±SD	p
Sex			
Male	12(60.0%)	0.21±0.10	0.23
Female	8 (40.0%)	0.26±0.06	
Age (years)			
≥50	10(50.0%)	0.26±0.10	0.21
<50	10(50.0%)	0.20±0.08	
Differentiation			
Well	13(65.0%)	0.23±0.08	0.88
Poor	7(35.0%)	0.23±0.08	
TNM stage			
I+II	12(60.0%)	0.23±0.08	0.95
III+IV	8 (40.0%)	0.23±0.11	
T			
3	11(55.0%)	0.22±0.07	0.59
4	9 (45.0%)	0.24±0.11	
Lymph node			
Positive	7(35.0%)	0.24±0.11	0.68
Negative	13(65.0%)	0.22±0.08	
Metastasis			
Positive	4 (20.0%)	0.24±0.14	0.75
Negative	16(80.0%)	0.23±0.08	
CEA (ng/ml)			
0-4.9	8 (40.0%)	0.21±0.06	0.46
≥5	12(60.0%)	0.24±0.10	
CA199			
>37ku / l	5 (25.0%)	0.24±0.10	0.83
≤37ku / l	15 (75.0%)	0.23±0.09	

Fecal occult blood			
Positive	16(80.0%)	0.24±0.09	0.31
Negative	4(20.0%)	0.19±0.10	

TNM: tumor node metastasis; CEA: carcino embryonic antigen; CA199: carcino antigen 199

Figures

Figure 1

(A) The expression status of FLI1 gene in different cancers was analyzed through GEPIA2. The cancer in red font was up-regulated ($p < 0.05$). The cancer in green font was down-regulated ($p < 0.05$). (B) Based on the CPTAC dataset, the expression level of FLI1 total protein was analyzed between normal tissue and primary tissue of LUAD, BRCA, OV, KIRC, and UCEC (* $p < 0.05$). (C) Based on the TCGA data, the expression level of FLI1 was analyzed by the pathological stages (stage I, stage II, stage III, and stage IV) of BRCA,

KIRC, SKCM, STAD, and THCA ($p<0.05$). (D) Correlation between FLI1 and overall survival prognosis of cancers in TCGA ($p<0.05$). (E) Correlation between FLI1 and disease-free survival prognosis of cancers in TCGA ($p>0.05$).

Figure 2

Mutation feature of FLI1 in different tumors of TCGA. The alteration frequency with mutation type (A) and mutation site (B) were displayed. We display the mutation site with the highest alteration frequency (A25V/G) in the 3D structure (C). We also analyzed the potential correlation between mutation status and disease-free, disease-specific, overall, and progression-free survival of colorectal adenocarcinoma (D) using the cBioPortal tool.

Figure 3

(A) Methylation analysis of FLI1 in different tumors (* $p < 0.05$). (B) Phosphorylation analysis of FLI1 protein in different tumors.

A

D

Figure 4

(A) Representative correlation analysis between FLI1 and immune infiltration of cancer-associated fibroblasts. Different algorithms were used to explore the potential correlation between the expression level of the FLI1 gene and the infiltration level of cancer-associated fibroblasts in COAD (B) and READ (C). A significant relationship was analyzed between FLI1 expression and common immune checkpoint gene levels (D).

Figure 5

FLI1-related gene enrichment analysis. (A) The available experimentally determined FLI1-binding proteins was derived from the STRING tool. (B) Using the GEPIA2 approach, we obtained the top 100 FLI1-correlated genes in TCGA projects and analyzed the expression correlation between FLI1 and selected targeting genes, including CARD8, DOCK2, DOCK8, IKZF1, NCKAP1L. (C) The corresponding heatmap data in the detailed cancer types were displayed. (D) An intersection analysis of the FLI1-binding and correlated genes was conducted. (E+F) Based on the FLI1-binding and interacted genes, KEGG pathway and GO analysis were performed.

Figure 6

Bioinformative evaluation of FLI1 methylation and mRNA Expression in TCGA and GEO databases: (A) The methylation level of FLI1 was significantly up-regulated in colorectal cancer compared to normal tissues ($p < 0.05$). (B) Three additional GEO methylation datasets demonstrated that the levels of FLI1 methylation were significantly higher in colorectal cancer samples compared to normal colorectal

mucosa samples (***p<0.0001). (C) The mRNA expression of FLI1 was significantly down-regulated in colorectal cancer than normal tissues (fold change ≥ 2 , *p<0.05).

Figure 7

(A) Sequenom EpiTYPER system analysis of the average methylation ratio of FLI1 in CRC tissues (p<0.05). (B) Representative FLI1 methylation levels between tumor and normal sample using BSP PCR based sequencing analysis. The tumor sample was higher and the normal sample was lower. Each line individually sequenced clone, and each square CpG residue. White and black squares were unmethylated and methylated cytosines, respectively (p<0.05). (C) ROC analysis of FLI1 methylation in CRC: the AUC, sensitivity and specificity of FLI1 methylation were 0.96, 90.0%, 95.0%, respectively. (D) Kaplan-Meier survival analysis of FLI1 methylation for overall survival in colorectal cancer (p=0.53).

Figure 8

(A) Sequenom EpiTYPER system analysis of the average methylation ratio of FLI1 in CRC cell lines (HCT116, SW480, SW640, HT29, SW1116, LOVO) and reference cell line (HEK-293T) (*p<0.05). (B) Sequenom EpiTYPER system analysis of the average methylation ratio of FLI1 in CRC cell lines treated with 5-Aza-2'-deoxycytidine (0 $\mu\text{mol/L}$ vs 5 $\mu\text{mol/L}$ vs 10 $\mu\text{mol/L}$) (*p<0.05). (C) SYBR analysis of the relative mRNA level of FLI1 in CRC cell lines treated with 5-Aza-2'-deoxycytidine (0 $\mu\text{mol/L}$ vs 5 $\mu\text{mol/L}$ vs 10 $\mu\text{mol/L}$) (*p<0.05). (D) Western blot analysis of the protein level of FLI1 in CRC cell lines treated with 5-Aza-2'-deoxycytidine (0 $\mu\text{mol/L}$ vs 5 $\mu\text{mol/L}$ vs 10 $\mu\text{mol/L}$) (p<0.05). (E+F) Expression of FLI1 by RT-PCR and western blot analysis in vector- and FLI1-transfected HCT116 and SW480 cells. (G) CCK8 assays showed that cell proliferation was significantly enhanced in vector-transfected HCT116 and SW480 cells than FLI1-transfected HCT116 and SW480 cells (*p<0.05). (H) Representative colony formation assay in vector- and FLI1-transfected HCT116 and SW480 cells (*p<0.05). (I) After re-expression of FLI1 in the HCT116 and SW480 cell group, the expression of FMNL1 was diminished

($p < 0.05$). (J) Cell invasion and migration assays revealed that the abilities of invasion and migration were weakened after transfected with FLI1 ($*p < 0.05$).

Supplementary Files

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