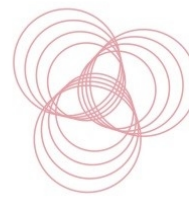


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NAALADL1 as a Key Modulator of Resistance to Tumor Treating Fields Therapy in Colorectal Cancer

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Abstract

Introduction: Colorectal cancer is the third most common malignancy worldwide. Tumor Treating Fields (TTF) therapy is an emerging non-invasive physical modality that delivers intermediate-frequency, low-intensity alternating electric fields to selectively disrupt mitosis in cancer cells. The objective of this study is to systematically evaluate the anti-tumor potential and underlying mechanisms of TTF in CRC, and to identify predictive biomarkers associated with treatment response.

Materials and Methods: Low-passaged CRC cell lines (N = 21) were treated with TTF for 72 hours using the NovoTTF-100A™ system, and cell viability was assessed via crystal violet staining. Anti-tumor mechanisms were explored using scanning electron microscopy, immunofluorescence, apoptosis assays, and proteomics. Transcriptomic profiling, panel-based next-generation sequencing, and weighted gene co-expression network analysis compared molecular and clinical features of TTF-sensitive and resistant lines. Candidate resistance genes were validated by shRNA knockdown, and virtual screening identified small molecules potentially synergistic with TTF. Additional functional assays preliminarily explored mechanisms of TTF resistance.

Results: TTF induced heterogeneous cytotoxic responses across the CRC cell lines. Multi-omic analyses revealed that TTF disrupted microtubule dynamics, induced mitotic abnormalities, metabolic stress, and DNA damage, ultimately triggering apoptosis. Transcriptomic analysis found N-acetylated alpha-linked acidic dipeptidase-like 1 (*NAALADLI*) as significantly upregulated in TTF-resistant cells,

which was further validated by weighted gene co-expression network analysis. Knockdown of NAALADL1 significantly sensitized resistant cells to TTF. Molecular docking and compound screening identified Lumacaftor and Bestatin as potential NAALADL1 inhibitors, showing synergistic anti-tumor effects with TTF. Bioinformatics analysis revealed NAALADL1 is underexpressed in CRC tissues and enriched in mitotic and cell cycle-related pathways. Empirical investigations demonstrated that NAALADL1 depletion regulates microtubule stability and the G2/M phase of the cell cycle, affecting the TTF response.

Conclusion : This study underscores the pronounced heterogeneity in CRC cell responses to TTF and identifies NAALADL1 as a pivotal regulator of TTF resistance. Inhibition of NAALADL1 has been demonstrated to enhance TTF efficacy by promoting microtubule stabilization and inducing G2/M phase arrest. Furthermore, the combination of TTF with Lumacaftor or Bestatin could be a promising therapeutic strategy. These findings offer mechanistic insights and potential molecular targets to inform personalized TTF-based therapies in CRC.

Keywords: Colorectal cancer, Tumor Treating Fields, N-acetylated alpha-linked acidic dipeptidase-like 1, Microtubule stabilization, G2/M cell cycle arrest, Patient-derived xenograft, Weighted gene co-expression network analysis, Lumacaftor, Bestatin

1. Introduction

Colorectal cancer (CRC) is a globally prevalent and highly lethal malignancy, ranking as the third most commonly diagnosed cancer and a leading cause of cancer-related mortality across diverse age groups and populations. Specifically, it has been identified as the primary cause of cancer-related deaths among men under the age of 50 and the second most frequent cause among young women [1]. Despite the considerable progress in diagnostic and therapeutic technologies over the past few decades—including comprehensive treatment approaches such as surgical resection, systemic chemotherapy, targeted therapy, immunotherapy, and radiotherapy—the prognosis for patients with advanced or metastatic CRC remains poor. The five-year survival rate for patients with late-stage disease is significantly lower than that for patients with early-stage disease, primarily due to the development of therapeutic resistance and distant metastasis [2–4].

The poor clinical outcomes observed in advanced CRC are intricately linked to several fundamental biological challenges. A multitude of factors must be taken into consideration when assessing the complexity of cancer. Among these factors are the spatiotemporal heterogeneity of tumors, which enables evasion of therapeutic pressure; the establishment of an immunosuppressive tumor microenvironment (TME) that impedes effective anti-tumor immune responses; and the epigenetically mediated persistence of cancer stem cells, which are implicated in tumor recurrence, resistance, and metastasis [5–8]. Furthermore, the limitations inherent in the prevailing therapeutic paradigm—including the dose-limiting toxicities of chemotherapeutic

agents, the suboptimal specificity of radiotherapy, and the controversial patient selection for targeted therapies—emphasize the pressing need to surpass conventional treatment approaches [9–10]. In light of these limitations, there is an imperative for the exploration of innovative therapeutic modalities with distinct mechanisms of action to improve clinical outcomes, particularly for patients with refractory or relapsed disease.

Tumor Treating Fields (TTF) therapy has emerged as a novel, non-invasive anti-cancer modality that employs low-intensity, intermediate-frequency (100–300 kHz) alternating electric fields to disrupt mitosis in rapidly dividing tumor cells. TTF has been shown to induce mitotic catastrophe, apoptosis, and ultimately tumor cell death by interfering with the alignment of mitotic spindles and perturbing chromosome segregation during cell division [11–13]. A distinguishing advantage of TTF is its ability to selectively target proliferating tumor cells while sparing quiescent normal tissues. This property offers a favorable safety profile and minimizes systemic toxicity. The therapeutic efficacy and tolerability of TTF have been validated in glioblastoma multiforme (GBM), with the U.S. Food and Drug Administration (FDA) approving its use in both recurrent and newly diagnosed cases. Clinical studies have consistently demonstrated that TTF confers significant improvements in progression-free survival (PFS) and overall survival (OS) among GBM patients, especially when combined with standard chemoradiation protocols [14–16].

The clinical success of TTF in GBM has prompted the expansion of its application to other malignancies, including malignant pleural mesothelioma, non-small cell lung

cancer (NSCLC), breast cancer, and pancreatic cancer [17–20]. These exploratory investigations have highlighted TTF’s potential to synergize with existing therapeutic agents and modalities, reduce treatment-associated adverse events, and enhance patient-reported outcomes. Notably, its non-invasive and localized nature positions TTF as an appealing addition to comprehensive cancer management, with the potential to transform the therapeutic landscape for difficult-to-treat cancers.

However, the translation of TTF into the clinical management of CRC remains in its nascent stages and is fraught with several obstacles. The most significant issue is the scarcity of CRC-specific mechanistic and clinical data. To date, only a limited number of in vitro studies have been conducted to investigate the anti-tumor potential of TTF in CRC cells. Furthermore, there is a conspicuous lack of early-phase clinical trials dedicated to CRC populations. The issue is further exacerbated by the pronounced inter- and intra-tumoral heterogeneity that is characteristic of CRC. A multitude of factors have been identified as determinants of varied responses to treatment, including variability in molecular subtypes (e.g., CMS1–CMS4), mutations in key oncogenes and tumor suppressor genes (e.g., KRAS, BRAF, TP53), and divergent TME compositions [21–23]. The aforementioned factors impede the generalizability of therapeutic outcomes and present significant challenges in predicting which patients are most likely to benefit from TTF-based interventions. In this context, the identification and validation of predictive biomarkers that correlate with TTF sensitivity are of paramount importance for the development of personalized therapeutic regimens. The incorporation of a biomarker-guided patient selection

approach into the design of clinical trials has the potential to enhance therapeutic efficacy, minimize unnecessary exposure to ineffective treatments, and streamline regulatory approval processes.

The low-passage human cancer cell lines, established from primary patient tumors or patient-derived xenograft (PDX) models, offer a clinically relevant and biologically representative platform for translational cancer research [24-26]. Conventional high-passage cell lines are susceptible to genetic drift and phenotypic alterations due to prolonged in vitro passaging. In contrast, low-passage models preserve key features of the parental tumors. The following aspects are of particular relevance in this context: genomic integrity, the presence of representative transcriptomic signatures, and the maintenance of phenotypic fidelity [27-30]. The objective is to minimize culture-induced artifacts and enhance the relevance of the results to human disease. These cell lines are systematically annotated with detailed molecular and clinical data at the time of derivation, enabling high traceability and direct linkage to patient-level information. The development of model systems that closely mirror the histopathological and molecular diversity observed in CRC patients is facilitated by this process. It is noteworthy that many of these cell lines represent distinct consensus molecular subtypes, thereby supporting investigations into subtype-specific therapeutic responses and resistance mechanisms. Furthermore, the existence of associated clinical metadata, including disease stage, treatment history, recurrence status, and metastatic profile, facilitates the correlation of in vitro findings with patient outcomes. This, in turn, enhances the translational robustness of preclinical

studies. Low-passage CRC cell lines have been shown to have significant utility in a variety of applications, including high-throughput drug screening, evaluation of chemoresistance and radiosensitivity, identification of predictive biomarkers, and mechanistic studies of tumor progression [31-33]. The utilization of these tools is of paramount importance in facilitating the transition from laboratory research to clinical implementation, thereby expediting the development of personalized treatment strategies in CRC.

Therefore, The primary objective of this study was to systematically evaluate the therapeutic potential of TTF in CRC, a disease marked by significant genetic and phenotypic heterogeneity. To achieve this, a panel of low-passage CRC cell lines, selected to represent a diverse range of molecular subtypes, was utilized in comprehensive in vitro experiments to assess the cytotoxic and anti-tumor effects of TTF treatment. In parallel with phenotypic assessments, a thorough suite of molecular and cellular techniques—including transcriptomic, proteomic, and functional analyses—was employed to uncover the underlying mechanisms driving both sensitivity and resistance to TTF. Special focus was placed on identifying tumor-killing mechanisms of TTF and predictive biomarkers that could aid in patient stratification and optimize therapeutic strategies. Ultimately, the goal of this research is to generate robust preclinical evidence to support the rational incorporation of TTF into precision medicine approaches for the management of CRC. Specifically, this study aims to inform future strategies for overcoming treatment resistance, thereby improving outcomes for patients who exhibit limited responsiveness to conventional

TTF therapy.

2. Materials and methods

2.1 Establishment of PDX models and cell lines

A total of 21 CRC low-passage cell lines were derived from PDX models or directly established from primary or metastatic tumor specimens of CRC patients. All patients provided informed consent for the use of their tumor specimens for research purposes and all procedures were approved by the Ethics Committee of the University of Rostock University Medical Centre (Reference numbers: II HV 43/2004 and A 45/2007). For the cell lines directly established from patient tumor specimens, specifically, the tumor specimens were immediately transported to the laboratory after surgical removal and processed under sterile conditions. The tumor tissue was meticulously mechanically minced to generate a single-cell suspension. After cultivation, the tumor cells began to grow in the medium and gradually formed cell lines. For cell lines from PDX, the tumor specimens were grown as xenograft subcutaneously in the flanks of 6-12-week-old NMRI-Foxn1^{nu/nu}, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, or NOD.Cg-Prkdc^{scid} (Central Facility for Animal Research and Care of Universitätsmedizin Rostock) mice. From the harvested tumors, single-cell suspensions were prepared for the establishment of CRC cell lines. Details were provided in prior studies [34-35].

Cells were maintained in a humidified incubator at 37 °C with 5% CO₂ with DMEM-F12 medium (Catalog No. DMEM-12-A, Capricorn-Scientific, Ebsdorfergrund, Germany), 2 mM L-glutamine, and 10% fetal calf serum. The medium was changed every 2-3 days, and cells were passaged once they reached

70-80% confluency. All CRC cell lines were validated by Short Tandem Repeat (STR) profiling and regularly tested for mycoplasma contamination. A comprehensive summary of the details can be found in Table 1.

2.2 Patient characteristics and pathological assessment

In this study, 21 CRC patients who underwent surgery at Rostock University Medical Center between January 2006 and December 2019 were enrolled. The patient's demographic information, including age and gender, was retrieved from the patient's medical records. A pathological examination was performed on resected tumor specimens to determine tumor location and histological grade (G grade). Tumors were staged according to the American Joint Committee on Cancer staging system. Molecular analyses were conducted using CRC classification methods to further characterize the tumor profiles including specific standard subtype (spSTD), microsatellite instability subtype (spMSI), Lynch syndrome-associated subtype (LS), and CpG island methylator phenotype-high (CIMP-H). Microsatellite instability (MSI) status was assessed using the Bethesda panel of five microsatellite markers: BAT25, BAT26, D2S123, D5S346, and D17S250 [36]. All pathological examinations were performed by the Institute of Pathology at Rostock University Medical Center. A comprehensive summary of the details can be found in Table 1.

Table 1. Information of the 21 cell lines and patient characteristics and clinical-pathological background.

Materials and methods

ID	Cell line full name in the biobank	Sample source	Patient age	Gender	Tumor location	G grading	Pathological staging	MSI status	Molecular subtype
HROC87	HROC87 T0 M2	PDX	76	Female	Right colon	3	II	MSI	spSTD
HROC212	HROC212	Patient	74	Female	Right colon	3	IV	MSI	spMSI
HROC24	HROC24 T1 M1	PDX	98	Male	Right colon	2	I	MSI	spMSI
HROC383	HROC383	Patient	83	Female	Transverse	3	II	MSI	spMSI
HROC257	HROC257	Patient	84	Female	Right colon	3	III	MSI	spMSI
HROC315	HROC315 T1 M2	PDX	42	Female	Left colon	3	III	MSI	LS
HROC113	HROC113	Patient	41	Female	Right colon	3	III	MSI	LS
HROC415MET	HROC415Met1 T0 M4	PDX	33	Male	Abdominal wall	3	IV	MSI	LS
HROC50	HROC50 T1 M5	PDX	67	Female	Right colon	2	II	MSI	spMSI
HROC80	HROC80 T1 M1	PDX	72	Male	Right colon	2	III	MSI	spMSI
HROC370	HROC370	Patient	77	Female	Right colon	2	I	MSI	spMSI
HROC131	HROC131 T0 M3	PDX	75	Female	Right colon	3	III	MSI	spMSI
HROC126	HROC126	Patient	58	Female	Rectum	2	III	MSS	spSTD
HROC159	HROC159 T2 M4	PDX	78	Female	Right colon	2	II	MSI	spMSI
HROC324	HROC324	Patient	55	Female	Right colon	3	IV	MSI	LS
HROC112MET	HROC112Met1 T0 M2	PDX	79	Female	Liver	2	IV	MSS	CIMP-H
HROC183	HROC183	Patient	59	Female	Right colon	3	III	MSS	CIMP-H
HROC285	HROC285 T0 M2	PDX	30	Female	Left colon	2	IV	MSI	LS
HROC252Tu2	HROC252Tu2 T2 M2	PDX	45	Male	Sigmoid	3	II	MSI	LS
HROC217	HROC217 T1 M2	PDX	38	Female	Left colon	2	IV	MSS	spSTD

2.3 TTF treatment and cell viability assessment

The coverslips (Catalog No. 83.1840.001, SARSTEDT, Nuremberg, Germany) were pre-wetted with culture medium prior to being carefully placed into a standard 6-well plate (Catalog No. 83.3920, SARSTEDT, Nuremberg, Germany). Cells were seeded

onto coverslips at a density of 2×10^6 cells/well on the first day of the experiment. The next day, the coverslips were transferred into the electric field disk (Novocure Ltd., Haifa, Israel). Cells were either left untreated (live control) or treated with TTF using the NovoTTF-100A™ System (Novocure Ltd., Haifa, Israel; Fig. 1) under the following conditions: 100 kHz frequency and 1–3 V/cm electric field intensity. TTF was applied continuously for 24 hours per day, over a total period of 3 days. Following TTF treatment, cell viability was evaluated using the crystal violet assay. After 72 hours, coverslips from both groups were transferred to new 6-well plates, and 1 mL of 0.2% crystal violet solution (Catalog No. C.I. 42555, Roche, Karlsruhe, Germany) was added to each well. The plates were placed on a shaker and incubated at room temperature for 1 hour to ensure even staining. After incubation, coverslips were gently washed three times with Dulbecco's Phosphate-Buffered Saline (DPBS), with each wash lasting 3 minutes, to remove excess dye. The coverslips were then air-dried overnight at room temperature before imaging. For dye extraction, the dried coverslips were returned to the 6-well plates, and 1 mL of 1% sodium dodecyl sulfate (SDS) (Catalog No. 0183.3, Roche, Karlsruhe, Germany) solution was added to each well. Plates were incubated on a shaker at room temperature for 2–3 hours until complete solubilization of the crystal violet stain. Finally, 100 μ L of the extracted solution from each well was transferred into a 96-well plate (Catalog No. 83.3924, SARSTEDT, Nuremberg, Germany), and absorbance was measured at 570 nm using a microplate reader (INFINITE M PLEX, Tecan, Switzerland). Cell viability (%) = (Absorbance of living control cells – Absorbance of blank) / (Absorbance of treated

cells – Absorbance of blank) $\times 100$.

Figure 1. NovoTTF-100A™ System for TTF treatment.



The NovoTTF-100A™ system includes a TTF generator and electrode discs.

2.4 Scanning electron microscopy (SEM) analysis of ultrastructural changes in CRC cells following TTF treatment

CRC cells were seeded onto sterile coverslips and divided into two groups: the control group, without TTF treatment, and the TTF-treatment group, which was exposed to TTF (100 kHz, 1-3 V/cm) for 48 hours. After treatment, the cells were digested with 0.25% trypsin-EDTA solution at 37°C for 3-5 minutes. The digestion was terminated by adding complete culture medium, and the cells were then resuspended to prepare a cell suspension containing 1×10^6 cells. Next, the cell suspension was centrifuged at 1400 rpm for 7 minutes to collect the cells. The cell pellet was then resuspended in DPBS and centrifuged again at 1400 rpm for 7 minutes to wash and collect the cells. Then, the cells were fixed with 2.5% glutaraldehyde at 4°C for 24 hours and stored at 4°C until further processing for embedding. Next, the cells were washed twice in 0.1 M phosphate buffer and then mixed with pre-warmed 3% low melt agarose (Fluka / Sigma-Aldrich, Taufkirchen, Germany) in 0.05 M HEPES

buffer at 40°C. In the liquefied agarose, the cells were collected by centrifugation and thus enclosed as a pellet in the agarose after hardening. The specimens were then post-fixed in a solution of 1% osmium tetroxide for 2 hours, and after washing in distilled water, were dehydrated in an ascending series of acetone. Infiltration with Epon resin (Serva, Heidelberg, Germany) was initiated with an overnight incubation in a 1:1 mixture of acetone and resin, followed by pure resin for 4 hours the following day. Specimen blocks were transferred to silicone rubber molds with fresh resin and were cured in an oven at 60°C for 2 days. Further processing included trimming of the resin blocks (Leica EM Trim2, Leica Microsystems, Wetzlar, Germany) and subsequent sectioning on an ultramicrotome (Ultracut S, Reichert, Wien, Austria) using a diamond knife (Diatome, Nidau, Switzerland). Semithin sections with a thickness of 0.5 µm were stained with toluidine blue to visualize and select areas for ultrastructural inspection. Thin sections of approximately 50-70 nm thickness were collected on copper mesh grids. After staining with uranyl acetate and lead citrate, the sections were coated with a 5 nm carbon layer (CCU-010-HV, Safematic, Zizers, Switzerland) to enhance conductivity.

Finally, the morphological characteristics of the cells were observed and imaged in a field emission scanning electron microscope (Zeiss Merlin VP compact, Carl Zeiss Oberkochen, Germany) using the scanning transmission electron microscopy (STEM) mode with dedicated combined dark field and bright field detection at an acceleration voltage of 15 kV and a working distance of approximately 2.7 mm. This section was completed with the assistance of Dr. Marcus Frank from the Department of Medical Biology and the Electron Microscopy Center, University Medical Center Rostock.

2.5 Cell doubling time determination

21 CRC cells in the logarithmic growth phase were seeded into each well of a 24-well

plate at a density of 1×10^4 to 1×10^5 cells / mL, with 100 μ L of the cell suspension added per well. After seeding, the cells were incubated at 37°C with 5% CO₂. At predetermined time points (0, 24, 48, and 72 hours), cell counts were performed. The cell numbers were recorded and used to plot a growth curve. The doubling time (Td) was calculated using the formula: $Td = (t \times \log 2) / (\log(N_t) - \log(N_0))$, where t is the time interval in hours, N_t is the cell count at the given time point, and N₀ is the initial cell count.

2.6 Next generation sequencing (NGS) for mutation analyses

Genomic DNA was extracted from cultured CRC cell lines utilizing the Precellys Tissue DNA Kit (PEQLAB Life Science, Erlangen, Germany), following the manufacturer's protocol. DNA quality control measures mandated a minimum concentration of 25 ng/ μ L, and integrity was assessed using the DNA Integrity Number (DIN), with a threshold set at ≥ 5 to ensure downstream sequencing reliability. High-throughput next-generation sequencing (NGS) was subsequently carried out by Centogene AG (Rostock, Germany) using a clinically validated solid tumor gene panel optimized for oncogenic profiling. Sequencing was performed on the Illumina NextSeq500 platform (San Diego, CA, USA), targeting the full coding sequences of 105 well-characterized cancer-associated genes alongside hotspot regions from an additional 146 oncogenes and tumor suppressor genes [37]. The sequencing protocol was optimized to achieve deep coverage, with >97% of targeted loci attaining a minimum read depth of 200 $\times$, ensuring robust variant calling. The pipeline demonstrated high analytical specificity (>99.9%) and was calibrated to detect somatic single nucleotide variants (SNVs) with a lower limit of detection set at 5%

variant allele frequency (VAF), thus allowing for sensitive detection of subclonal mutations.

2.7 RNA-seq

Total RNA was extracted from CRC cell lines using the RNeasy Plus Mini Kit (Catalog No. 74136, Qiagen, Hilden, Germany) according to the manufacturer's instructions. After RNA extraction, the concentration and purity were measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA), with an A260/A280 ratio between 1.8 and 2.1 indicating acceptable RNA purity. RNA quality was then assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), and RNA integrity was determined by the RNA Integrity Number (RIN), with $RIN \geq 7.0$ considered suitable for downstream library preparation and sequencing. Library preparation was performed using the Illumina TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA), following key steps including poly(A)+ mRNA enrichment (using Oligo (dT) magnetic beads to remove rRNA and non-coding RNA), mRNA fragmentation, cDNA synthesis, end repair and A-tailing, adapter ligation, and PCR amplification of the library. The constructed cDNA libraries were quantified using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and analyzed for fragment size distribution on an Agilent 2100 Bioanalyzer. Qualified libraries were sequenced on the Illumina NovaSeq 6000 platform using a 150 bp strategy, generating approximately 50M reads per sample. This section was completed with the technical and analytical support of Novogene Europe and the Candiolo Cancer Institute, Italy.

FastQC (v0.11.9) was used to assess raw data quality, and Trimmomatic (v0.39) was applied to remove low-quality reads and adapter sequences. For alignment, HISAT2 (v2.2.1) was used to map clean reads to the reference genome (GRCh38). Transcriptome assembly and quantification were performed using StringTie (v2.1.7), and gene expression levels were calculated with featureCounts (v2.0.1). The transcriptomic data of this study have been uploaded to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (Table 2).

Table 2. Accession details for transcriptomic data.

Cell line	BioSample ID
HROC87	BioSample: SAMEA5820600; SRA: ERS3609190
HROC212	BioSample: SAMEA115337558; SRA: ERS18339074
HROC24	BioSample: SAMN47449425; SRA: SRS24413664
HROC383	BioSample: SAMN47449426; SRA: SRS24413665
HROC257	BioSample: SAMEA115337560; SRA: ERS18339076
HROC315	BioSample: SAMN44335383; SRA: SRS22934986
HROC113	BioSample: SAMEA115337552; SRA: ERS18339068
HROC324	BioSample: SAMEA115337566; SRA: ERS18339082
HROC112MET	BioSample: SAMEA5820589; SRA: ERS3609179
HORC183	BioSample: SAMEA115337557; SRA: ERS18339073
HROC285	BioSample: SAMEA5820595; SRA: ERS3609185
HROC252Tu2	BioSample: SAMN44335382; SRA: SRS22934985
HROC217	BioSample: SAMN44335381; SRA: SRS22934984
HROC300	BioSample: SAMEA115337564; SRA: ERS18339080

2.8 Lentiviral transduction

To determine the appropriate puromycin selection concentration, 2×10^4 cells of each cell line (HROC217 and HROC300) were seeded evenly into multiple wells of a 96-well plate, ensuring that the cells were in the logarithmic growth phase. After 24 hours, a gradient of puromycin concentrations (0, 0.5, 1.5, 1.8, 2.0, 2.5, and 3.0

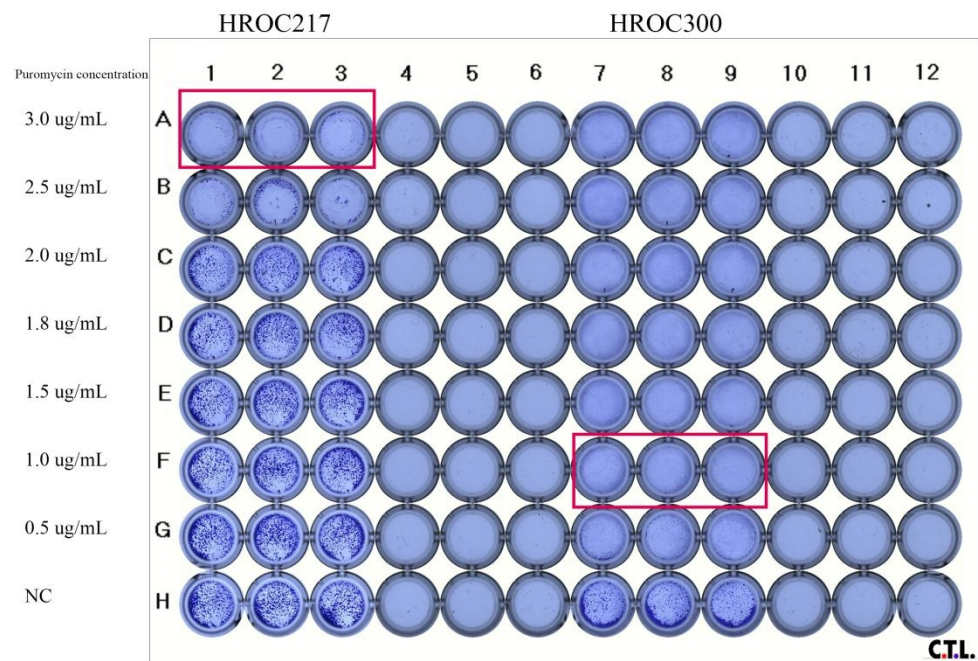
µg/mL) was added to the respective wells. Cell viability was observed and recorded daily over a period of 3 days. Following the observation period, crystal violet staining was performed to visualize surviving cells, after which the wells were rinsed with DPBS to remove excess stain. The lowest puromycin concentration that completely eliminated untransduced cells within 3 days was selected for use in subsequent experiments (Fig. 2).

Commercially available lentiviral particles targeting NAALADL1 knockdown (Catalog No. TL311278V, OriGene Technologies GmbH, Herford, Germany; A-D mix in equal volumes) and corresponding control vectors (Catalog No. TR30021V, OriGene Technologies GmbH), which contain random sequences of the same length as the shRNA but without human targets, were used to infect the HROC217 and HROC300 cell lines (HROC217shRNANC, HROC217shNAALADL1, HROC300shRNANC, and HROC300shNAALADL1).

Cells were seeded in a 48-well plate at a density of 1×10^5 cells/well in complete culture medium and incubated at 37°C with 5% CO₂ overnight to allow proper attachment. On the following day, the culture medium was carefully aspirated and replaced with 0.5 mL of fresh medium containing 6 µg/mL polybrene (Catalog No. TR-1003, Merck, Darmstadt, Germany) to enhance viral transduction efficiency. An appropriate volume of lentiviral suspension was then added to each well at a multiplicity of infection (MOI) of 50, followed by gentle mixing to ensure even distribution. The plate was maintained at 37°C with 5% CO₂ for 24 hours to allow efficient viral entry.

After 24 hours of infection, the virus-containing medium was removed, and cells were gently washed with DPBS to eliminate residual virus. 1 mL of complete culture medium was added to support cell recovery and continued growth. At 72 hours post-infection, transduction efficiency was assessed by observing green fluorescent protein (GFP) expression under a fluorescence microscope. If necessary, puromycin selection was performed to ensure high transduction efficiency.

Figure 2. Determination of optimal puromycin selection concentration for HROC217 and HROC300 cell lines.



The optimal puromycin concentration was determined to be 3.0 $\mu\text{g/mL}$ for HROC217 and 1.0 $\mu\text{g/mL}$ for HROC300.

2.9 Western Blot (WB)

WB was used to detect target protein expression. A total of 1×10^6 cells were lysed in 500 μ L RIPA buffer (Catalog No. 89900, Thermo Fisher Scientific, Schwerte, Germany) with protease inhibitor (Catalog No. 11836170001, Roche, Basel, Switzerland). Protein concentration was measured using Nanodrop. Twenty to 100 μ g of protein per well was loaded. Samples were mixed with SDS-PAGE loading buffer (1:4 ratio) and heated at 95°C for 5 min to denature proteins. SDS-PAGE was performed using 10% polyacrylamide gel (TGX™ FastCast™, Catalog No. 1610173, Bio-Rad, Munich, Germany) at 100 V for 1–1.5 h. Proteins were transferred to a nitrocellulose membrane (Catalog No. 1704270) using Bio-Rad's semi-dry transfer system (Trans-Blot Turbo) at 1 A for 30 min. Membranes were blocked with 5% skim milk for 1 h, followed by overnight incubation at 4°C with diluted primary antibodies. The next day, membranes were washed three times with TBST (5 min each), incubated with secondary antibody for 1 h at room temperature, and washed again (3×5 min). Detection was performed using a fluorescent imaging system, and band intensity was analyzed to quantify target protein expression. GAPDH and Vinculin were used as internal controls. Antibody details and dilutions are listed in Table 3.

Table 3. Detailed information and dilution concentrations of antibody.

Reagents for WB	Catalog Number	Manufacturer	Country	Dilution concentration
Prestained Protein Marker	PL00001	Proteintech	UK	5 μ L
	PL00002	Proteintech	UK	5 μ L

Primary Antibodies					
GAPDH Rabbit Polyclonal Antibody	TA890003	OriGene Technologies GmbH	Germany	1:2000	
NAALADL1 Recombinant Rabbit Monoclonal Antibody	MA5-29434	Thermo Fisher Scientific Inc	Germany	1:1000	
Caspase 3/p17/p19 Rabbit Polyclonal Antibody	19677-1-AP	Proteintech	UK	1:500	
Vinculin Rabbit Polyclonal Antibody	26520-1-AP	Proteintech	UK	1:50000	
Acetyl- α -Tubulin (Lys40) Antibody	3971	Cell Signaling Technology	Germany	1:500	
Secondary Antibody					
StarBright Blue 520 Goat Anti-Rabbit	12005870	BIO-RAD	Germany	1:2000	

2.10 Cellular functional assays: proliferation, cell cycle, and apoptosis

Cell Proliferation: Log-phase cells were seeded in 96-well plates at $1-2 \times 10^4$ cells/well in 100 μ L culture medium and incubated at 37°C with 5% CO₂ for 1 to 5 days. At each time point, medium was removed, and cells were washed with DPBS to eliminate residual medium and non-adherent cells. Cells were stained with 0.2% crystal violet for 15 min at room temperature, washed with distilled water, and air-dried. Stained wells were imaged, and 100 μ L of 1% SDS was added to solubilize the dye. After 30 min shaking at room temperature, absorbance at 570 nm was measured using a microplate reader. OD values were used to generate cell growth curves.

Cell Cycle Analysis: Log-phase cells (1×10^6) were harvested, centrifuged at 1400 rpm for 7 min, washed with DPBS, and fixed in 70% methanol at 4°C for 30 min.

Cells were centrifuged again, washed with DPBS, and resuspended in 500 μ L DPBS. RNase A (100 μ g/mL) treatment was performed at 37°C for 30 min, followed by staining with 50 μ g/mL PI in the dark for 30 min. DNA content was analyzed by flow cytometry (NovoCyte Quanteon, Agilent, San Diego, USA) to determine cell cycle distribution (G0/G1, S, G2/M).

Apoptosis Analysis: For non-transfected cells, log-phase cells (1×10^6) were washed with DPBS and resuspended in 500 μ L Annexin V binding buffer. Cells were stained with 10 μ L Annexin V-APC and 10 μ L PI per kit instructions, incubated in the dark for 15 min at room temperature, and analyzed by flow cytometry. Annexin V-positive cells indicated apoptosis; PI distinguished early vs. late stages. For transfected cells, cleaved Caspase-3 was detected by Western blot to avoid GFP interference, with Vinculin used for normalization.

2.11 Immunofluorescence experiment

Target cells were seeded onto pre-treated coverslips and cultured under optimal conditions until reaching the logarithmic growth phase. According to the experimental design, cells were either treated or not treated with TTF to assess its effects on microtubule structures. Fixation was performed using pre-chilled 70% methanol (-20 °C) for 30 minutes to preserve cell membrane integrity and cytoskeletal structures, preventing morphological alterations during staining. After fixation, cells were washed three times with PBS for 5 minutes each to remove residual methanol and minimize interference with antibody binding. Immunofluorescence staining for tubulin was conducted using Tubulin Tracker™ Green kit (Catalog No. T34075, Thermo Fisher Scientific, Berlin, Germany), with incubation at 4°C to ensure optimal binding to the target protein. Cells were then washed three times with PBS, each for 5

minutes, to remove unbound antibodies and reduce nonspecific background signals. Nuclear staining was performed using DAPI (1:5000), incubated at room temperature for 30 minutes, followed by PBS washes to remove excess dye and minimize nonspecific fluorescence. After staining, excess liquid was removed with absorbent paper, and an anti-fade mounting medium was applied to preserve fluorescence signals. Finally, fluorescence microscopy (Zeiss Axio Observer 7 with axiocam 506 mono and the ZenPro software, version 2.3) was used to detect tubulin (green fluorescence) and DAPI-stained nuclei (blue fluorescence) to analyze the effects of TTF treatment on the microtubule structure. This section was completed with the assistance of Dr. Olga Hahn from the Institut für Zellbiologie, University Medical Center Rostock.

Tubulin Tracker™ Green kit was also used for staining polymerized microtubules. Quantitative analysis of microtubule polymerization was conducted using the Tubeness plugin in Fiji. Raw images were initially converted to 8-bit grayscale to standardize processing. Tubular structures were subsequently enhanced via Tubeness filtering ($\sigma = 0.1$) to highlight microtubule-like features. After image enhancement, the "Mean Intensity" analysis was performed to quantify the average gray value of polymerized microtubule signals within each cell, serving as a relative measure of microtubule polymerization levels.

2.12 Mass spectrometry analysis of protein expression levels

Proteins from cell pellets were processed using the Mini MS EasyPep Kit (Thermo Fisher, MA). Briefly, cell pellets were resuspended in lysis buffer, and their protein concentration was determined by BCA assay. Equal amounts of protein were reduced

with DTT, alkylated with IAA, and subsequently subjected to enzymatic digestion with trypsin to generate peptides. Peptides were desalted using C18 solid-phase extraction. Two hundred ng of the purified peptides were loaded onto C18 EvoTip disposable trap columns (EV1137, 15 cm x 150 μ m, 1.5 μ m) and chromatographically separated using the extended 15 SPD method (88 min gradient, 220 nl/min) with an EvoSep One (EvoSep, Denmark). Subsequently, peptides were measured using the timsTOF fleX mass spectrometer (Bruker, Germany) in a label-free data-independent acquisition mode. This section was completed with the assistance of Dr. Timo Gemoll and Dr. Jasmin Ostermann from the Translational Surgical Oncology & Biobanking, Department of Surgery, University of Lübeck.

2.13 Prediction of protein tunnel structures and virtual screening of small molecule inhibitors via molecular docking

To investigate potential binding sites and channels of the NAALADL1 protein, its 3D structure (PDB ID: 4TWE) was obtained from the Protein Data Bank (<https://www.rcsb.org/>). The structure was analyzed using CaverWeb 2.0 (<https://loschmidt.chemi.muni.cz/caverweb/>, accessed on 2024.10.1) [38], which employs the Fpocket algorithm for pocket detection. Fpocket, based on Voronoi diagrams and alpha spheres, identifies candidate drug-binding pockets by analyzing geometric features of the protein surface. It provides detailed pocket attributes such as volume, depth, and surface area, aiding in drug design. Following pocket detection, the Caver algorithm was used to identify potential channels within the protein. Also based on geometric principles, Caver detects internal pathways that may serve as

access routes or binding sites for small molecules, providing parameters like radius, depth, and accessibility. After identifying these structural features, virtual screening was performed using an FDA-approved drug library from the ZINC database (<https://zinc.docking.org/>, accessed on 2024.10.1). Molecular docking with AutoDock Vina evaluated binding affinities of library compounds with the identified channels. Binding energies and interaction maps were used to rank compounds and select top candidates for further analysis and validation.

2.14 Drug sensitivity assay and TTF drug combination experiment

Logarithmic phase cells were seeded in 96-well plates at a density of 2×10^4 cells per well in 100 μ L of medium. After 24 hours, the cells were treated with drugs—Bestatin, Doxepin N-Oxide Glucuronide, Estradiol Benzoate, and Lumacaftor—at gradient concentrations of 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 3.125 μ M, 1.5625 μ M, and 0.78125 μ M, respectively. The living control was complete medium containing 0.5% DMSO. All conditions were set up in triplicate. The plates were then incubated for 72 hours, followed by staining with 0.2% crystal violet. After air-drying, the dye was solubilized with 1% SDS, and the absorbance was measured at 570 nm. The IC₅₀ values were calculated using Prism 9.0 software.

To evaluate the combined effects of TTF and drug treatments, four experimental groups were established: (1) NC group: Cells were treated with 0.5% DMSO without TTF exposure for 72 hours. (2) Drug alone group: Cells were treated with either Bestatin, Doxepin N-Oxide Glucuronide, Estradiol Benzoate, or Lumacaftor at the respective concentrations based on the drug sensitivity assay for 72 hours. (3) TTF

alone Group: Cells were exposed to TTF while being maintained in a 0.5% DMSO-containing medium for 72 hours. (4) Combination Group: Cells were treated with the respective drugs while simultaneously being exposed to TTF for 72 hours.

2.15 Bioinformatics analysis

The expression of the *NAALADL1* gene in colon adenocarcinoma (COAD) was analyzed using R-based bioinformatics tool Xiantao Academic (<https://www.xiantaozi.com/>, accessed on 2024.10.1). Whole transcriptome sequencing data from 647 COAD and 51 normal tissues were obtained from TCGA and GTEx databases. Unpaired analyses were performed to assess *NAALADL1* mRNA expression. Additionally, immunohistochemical data from the Human Protein Atlas (HPA, <https://www.proteinatlas.org/>) were used to compare expression in CRC versus normal colonic mucosa. To explore *NAALADL1* function in CRC, GSEA was performed on the transcriptomic data using the R package clusterProfiler (v4.4.4) with the MSigDB c2.cp.all.v2022.1.Hs.symbols.gmt gene set (via msigdb), identifying significantly enriched pathways associated with high and low *NAALADL1* expression.

To assess the impact of TTF on CRC cell function, mass spectrometry (MS) was used to profile protein expression before and after 48 hours of treatment. Data preprocessing included normalization and filtering of low-abundance proteins. Differential expression analysis (DEA) was conducted using limma (v3.63.10), calculating log₂ fold changes (log₂FC) and adjusted p-values (adj.P.Val). DEPs were identified using thresholds of adj.P.Val < 0.05 and |log₂FC| > 1. Results were

visualized in a volcano plot (ggplot2), highlighting upregulated ($\log_2FC > 1$) and downregulated ($\log_2FC < -1$) proteins. All proteins were ranked by $\log_2FC$, and KEGG pathway enrichment was conducted using clusterProfiler, with significance defined as $\text{adj.P.Val} < 0.05$ and $|\text{NES}| > 1$.

RNA-seq data were normalized using DESeq2 (v1.32.0). Weighted Gene Co-expression Network Analysis (WGCNA, v1.70-3) was applied to construct gene co-expression networks and identify functional modules. Prior to network construction, PCA was used to assess global patterns and detect batch effects; hierarchical clustering removed outliers. Pearson correlation was calculated between gene pairs, and a soft threshold of $\beta = 7$ was applied to ensure scale-free topology. The expression matrix was transformed into an adjacency matrix and then into a Topological Overlap Matrix (TOM), followed by average linkage hierarchical clustering. Modules were identified using the dynamic tree-cutting algorithm ($\text{minModuleSize} = 50$) and merged using a cut height of 0.25 and $\text{deepSplit} = 3$. Modules significantly associated with TTF sensitivity ($p < 0.05$) were further analyzed. DEA between high- and low-efficacy TTF groups was performed with limma, identifying DEGs using the same criteria ($\text{adj.P.Val} < 0.05$, $|\log_2FC| > 1$). Upregulated genes in the low-efficacy group were intersected with resistance modules, and those in the high-efficacy group with sensitivity modules, yielding candidate biomarkers and therapeutic targets.

2.16 Statistical analysis

All statistical analyses were performed with the SPSS 19.0 and R 4.0.3 software

programs. Quantitative data were summarized using mean and standard deviation. t-tests and ANOVA were used for analysis. Categorical data were described with frequencies and percentages and the Chi-Square test and Fisher's exact test were used. A two-sided $P < 0.05$ was considered significant. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3. Results

3.1 Antitumor effect of TTF on low-passage CRC cell lines

Twenty-one low-passage CRC cell lines established either directly from patient's tumors or derived from PDX models were treated with TTF for 72 hours (Fig. 3A). The clinical and pathological characteristics of the 21 CRC patients are summarized in Table 4. The results of the crystal violet assay demonstrated that the viability of cells following treatment with TTF ranged from 23.8% to 88.2%, as indicated in Table 5. The 21 cell lines were then categorized into three groups based on their response to TTF treatment, namely high efficacy, moderate efficacy, and low efficacy. A thorough statistical analysis was conducted, revealing that the cell viability in the low efficacy group exhibited a significantly higher value in comparison to the high efficacy group ($P < 0.001$, Fig. 3B).

Table 4. The clinical and pathological characteristics of 21 CRC patients.

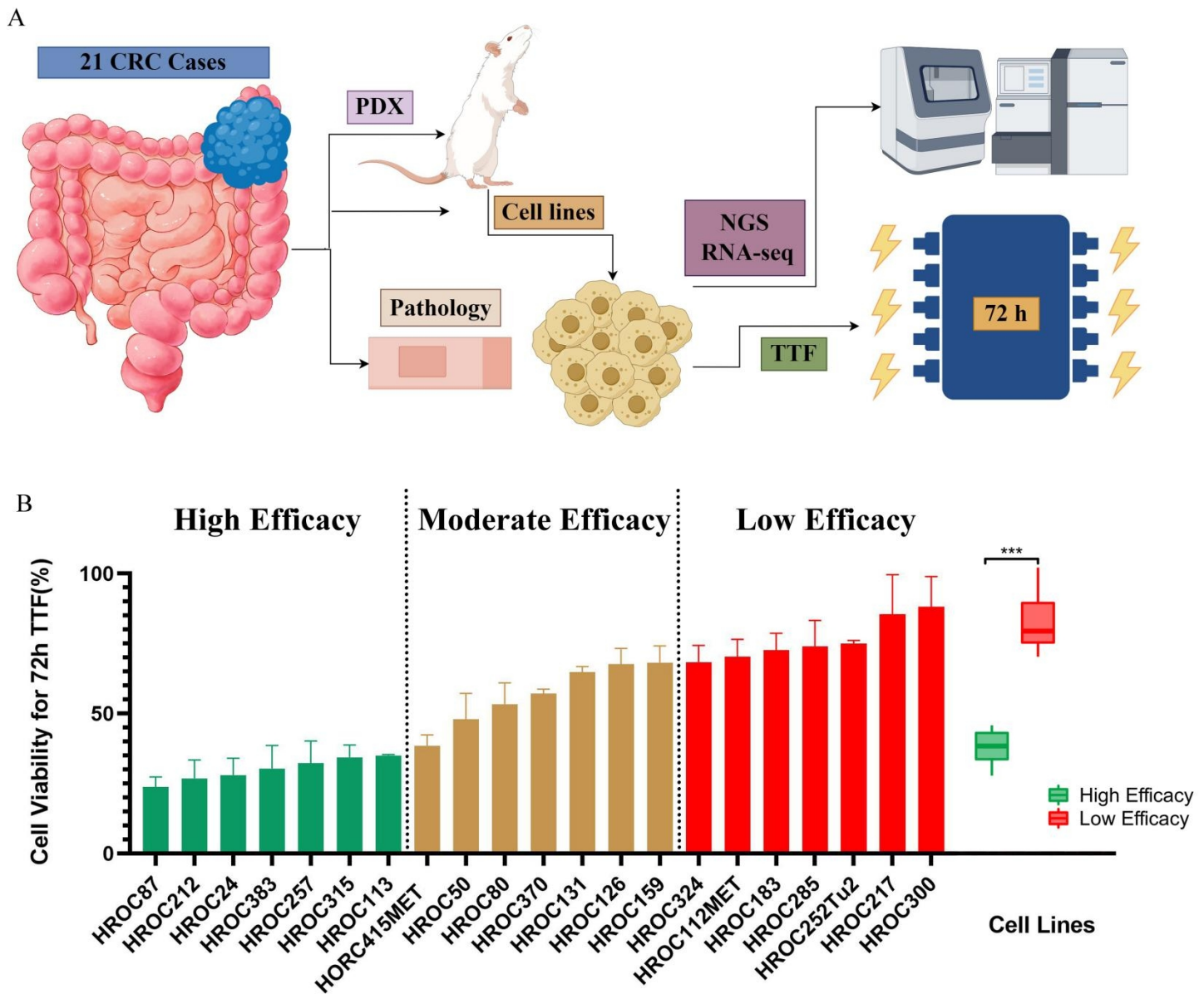
Characteristic	n = 21
Age (mean, SD)	63.7 (19.1)
Gender	
Male	5
Female	16
Tumor tissue site of origin	
Right colon	12
Transverse	1
Left colon	3
Rectum	2
Sigmoid	1
Liver	1
Abdominal wall	1
Differentiation	
G2	10

Results

G3	11
pTNM stage	
I	2
II	5
III	7
IV	7
Pathological molecular subtype	
spSTD	3
spMSI	9
LS	6
CIMP-H	3
MSI status	
MSI	16
MSS	5

Table 5. The cell viability of 21 CRC cell lines after 72 TTF treatment.

ID	Cell viability after TTF for 72 hours (% , Mean $\pm$ SD)
HROC87	23.8 $\pm$ 3.58
HROC212	26.9 $\pm$ 6.57
HROC24	28.02 $\pm$ 6.04
HROC383	30.41 $\pm$ 8.18
HROC257	32.29 $\pm$ 7.92
HROC315	34.36 $\pm$ 4.41
HROC113	35.04 $\pm$ 0.369
HORC415MET	38.42 $\pm$ 3.96
HROC50	47.94 $\pm$ 9.23
HROC80	53.36 $\pm$ 7.63
HROC370	57.09 $\pm$ 1.64
HROC131	64.79 $\pm$ 2.03
HROC126	67.59 $\pm$ 5.71
HROC159	68.2 $\pm$ 6.02
HROC324	68.34 $\pm$ 6.02
HROC112MET	70.36 $\pm$ 6.18
HROC183	72.63 $\pm$ 6.06
HROC285	74.04 $\pm$ 9.25
HROC252Tu2	75.08 $\pm$ 1.02
HROC217	85.55 $\pm$ 14.06
HROC300	88.2 $\pm$ 10.75

Figure 3. Antitumor effect of TTF on low-passage CRC cell lines.

(A) Schematic workflow of the experimental design for assessing the antitumor effect of TTF on 21 low-passage CRC cell lines. (B) Crystal violet assay results showed the viability of 21 low-passage CRC cell lines after 72 hours of TTF treatment. Cell viability ranged from 23.8% to 88.2%, leading to classification into three groups: high efficacy, moderate efficacy, and low efficacy. Statistical analysis indicates that the low efficacy group exhibited significantly higher

cell viability compared to the high efficacy group ($P < 0.001$).

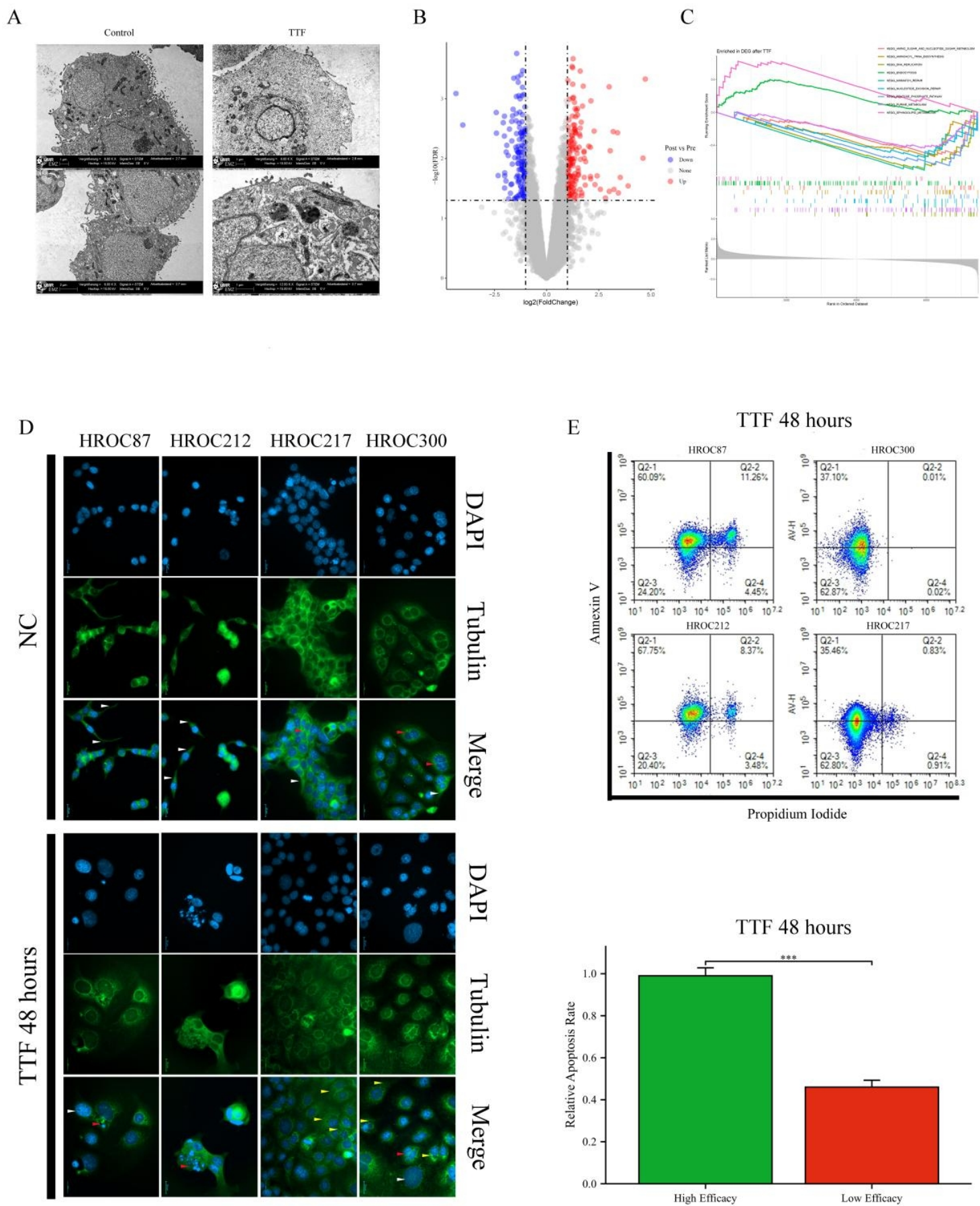
3.2 TTF induces apoptosis in CRC cells by disrupting mitosis and metabolism, and causing DNA damage

Subsequent SEM analysis revealed varying degrees of ultrastructural changes in CRC cells following 48 hours of TTF treatment compared to the NC group, which did not receive TTF treatment. The NC group exhibited cells with intact morphology and well-preserved organelle structures. Additionally, pseudopodia were clearly visible. However, following TTF treatment, there was a regression of the pseudopodia, accompanied by the occurrence of chromatin condensation and nuclear morphology becoming irregular. In addition, the presence of mitochondrial swelling and vacuolization was observed, suggesting the occurrence of cellular stress and organelle dysfunction (Fig. 4A). To further investigate the antitumor effects of TTF on CRC cells, two cell lines were selected which were most sensitive to TTF treatment, HROC87 and HROC212, as well as two cell lines that were most resistant, HROC217 and HROC300. I analyzed protein expression profiles, microtubule structure (using immunofluorescence), and apoptosis rates before and after 48 hours of TTF treatment in these selected cell lines. A subsequent mass spectrometry analysis of the HROC87 and HROC212 cell lines revealed that 150 proteins were significantly overexpressed and 131 proteins were significantly underexpressed after 48 hours of TTF treatment (Fig. 4B). Functional enrichment analysis revealed that these differentially expressed proteins were enriched in nine distinct pathways:

“KEGG_AMINOACYL_TRNA_BIOSYNTHESIS”, “KEGG_ENDOCYTOSIS”, “KEGG_MISMATCH_REPAIR”, “KEGG_PENTOSE_PHOSPHATE_PATHWAY”, “KEGG_NUCLEOTIDE_EXCISION_REPAIR”, “KEGG_DNA_REPLICATION”, “KEGG_PURINE_METABOLISM”, “KEGG_SPHINGOLIPID_METABOLISM”, and “KEGG_AMINO_SUGAR_AND_NUCLEOTIDE_SUGAR_METABOLISM” (Fig. 4C). Immunofluorescence results demonstrated that in the control group not subjected to TTF treatment, microtubule structures exhibited well-organized and intact characteristics, accompanied by discernible polarity. The nuclei appeared normal, and mitosis proceeded as expected. However, in the TTF-treated group, varying degrees of microtubule disruption and fragmentation were observed. The nuclei displayed atypical morphologies and exhibited indications of mitotic arrest or apoptosis (Fig. 4D). The results of the FC analysis demonstrated that the apoptosis rate in the most sensitive cell lines was significantly higher than that in the resistant cell lines ($P = 0.001$, Fig. 4E).

Figure 4. TTF triggers apoptosis and alters ultrastructure, protein expression, cellular pathways, and microtubule structure in CRC cells.

Results



(A) SEM images of CRC cells with and without 48 hours of TTF treatment. (B) Volcano plot showed differentially expressed proteins in the most TTF-sensitive CRC cell lines (HROC87 and HROC212) after 48 hours of treatment. A total of 150 proteins were significantly upregulated, and 131 proteins were significantly downregulated. (C) Functional enrichment analysis of differentially expressed proteins in TTF-sensitive cell lines. The affected pathways included nucleotide metabolism, DNA replication and repair, amino sugar and nucleotide sugar metabolism, and microtubule-associated processes. (D) Immunofluorescence analysis of microtubule structures in CRC cells before and after TTF treatment. Control cells showed well-organized microtubules with clear polarity (white arrow) and normal mitosis (red arrow), while TTF-treated cells exhibited disrupted and fragmented microtubules (yellow arrow), abnormal nuclear morphology, and mitotic arrest or apoptosis (red arrow). (E) FC analysis of apoptosis rates in CRC cells after 48 hours of TTF treatment. The most TTF-sensitive cell lines (HROC87 and HROC212) exhibited significantly higher apoptosis rates compared to the resistant cell lines (HROC217 and HROC300).

3.3 Comparison of cell division rate, mutations, and clinicopathological features between high- and low-efficacy TTF treatment groups

To investigate potential factors influencing TTF treatment efficacy in CRC cells, cell division rates, genetic mutations, and clinicopathological characteristics between the high (n = 7) and the low efficacy (n = 7) groups were compared. Correlation analysis between cell viability after TTF treatment and cell doubling time did not reveal a significant correlation ($R = 0.304$, $P = 0.291$, Fig. 5A).

Utilizing a panel NGS mutation analysis, a total of 16 distinct gene mutations were identified in these 14 HROC cell lines. The mutated genes were: *FGFR4*, *BRAF*, *TP53*, *PTPRJ*, *KRAS*, *MSH6*, *PIK3CA*, *APC*, *KMT2D*, *FLCN*, *MLH1*, *MSH2*, *SMAD4*, *CDKN2A*, *ERBB3*, and *NFI*. However, no statistically significant differences in the frequency or distribution of specific mutations including nonsense, missense, insertions, deletions, and multi-hit mutations were observed between high and low efficacy groups (Fig. 5B).

Subsequent analysis of clinicopathological characteristics, encompassing patient age, gender, tumor tissue site of origin, differentiation grade, pathological TNM stage, and molecular subtypes, failed to identify substantial disparities between the groups. The proportion of MSI status was higher in the high efficacy group compared to the low efficacy group; but the difference was not significant ($P = 0.075$, Fig. 5C and Table 6).

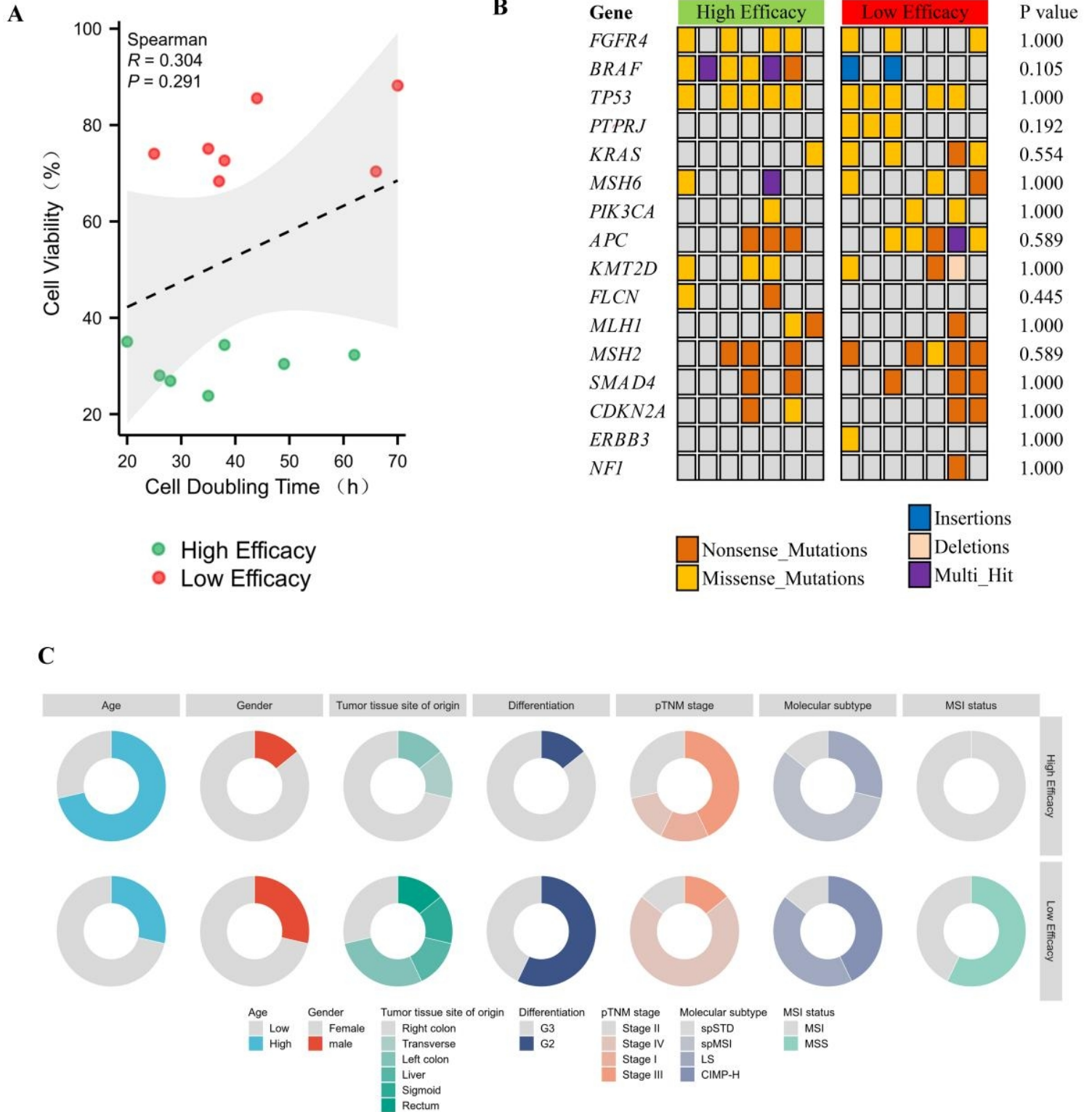
Table 6. Univariate analysis of clinical and pathological characteristics for TTF sensitivity.

Characteristics	High Efficacy	Low Efficacy	<i>P</i> value
n	7	7	
Age, mean $\pm$ sd	71.143 $\pm$ 21.667	73.286 $\pm$ 13.913	0.829
Gender, n (%)			0.514
Female	6 (85.7%)	5 (71.4%)	
male	1 (14.3%)	2 (28.6%)	
Tumor tissue site of origin, n (%)			0.388
Right colon	5 (71.4%)	2 (28.6%)	
Transverse	1 (14.3%)	0 (0%)	
Left colon	1 (14.3%)	2 (28.6%)	

Results			
Liver	0 (0%)	1 (14.3%)	0.266
Sigmoid	0 (0%)	1 (14.3%)	
Rectum	0 (0%)	1 (14.3%)	
Differentiation, n (%)			
G3	6 (85.7%)	3 (42.9%)	0.236
G2	1 (14.3%)	4 (57.1%)	
pTNM stage, n (%)			
Stage I/II	3 (42.9%)	1 (14.3%)	0.073
Stage III/IV	4 (57.1%)	6 (85.7%)	
Pathological molecular subtype, n (%)			
spSTD	1 (14.3%)	1 (14.3%)	0.076
spMSI	4 (57.1%)	0 (0%)	
LS	2 (28.6%)	3 (42.9%)	
CIMP-H	0 (0%)	3 (42.9%)	
MSI status, n (%)			
MSI	7 (100%)	3 (42.9%)	
MSS	0 (0%)	4 (57.1%)	

Figure 5. Cell division rate, mutations, and clinicopathological features between high and low efficacy TTF treatment groups.

Results



(A) Correlation analysis between cell viability and cell doubling time did not reveal a significant correlation ($R = 0.304$, $P = 0.291$). (B) No significant differences in the frequency or distribution of specific mutations were observed between high and low efficacy groups. (C) Clinicopathological characteristics, including patient age, gender, tumor tissue site of origin, differentiation grade, pathological TNM stage, molecular subtypes, and the proportion of MSI did not reveal significant differences between the two groups.

3.4 N-acetylated α -linked acidic dipeptidase-like 1 (NAALADL1) expression level as a biomarker of resistance to TTF treatment

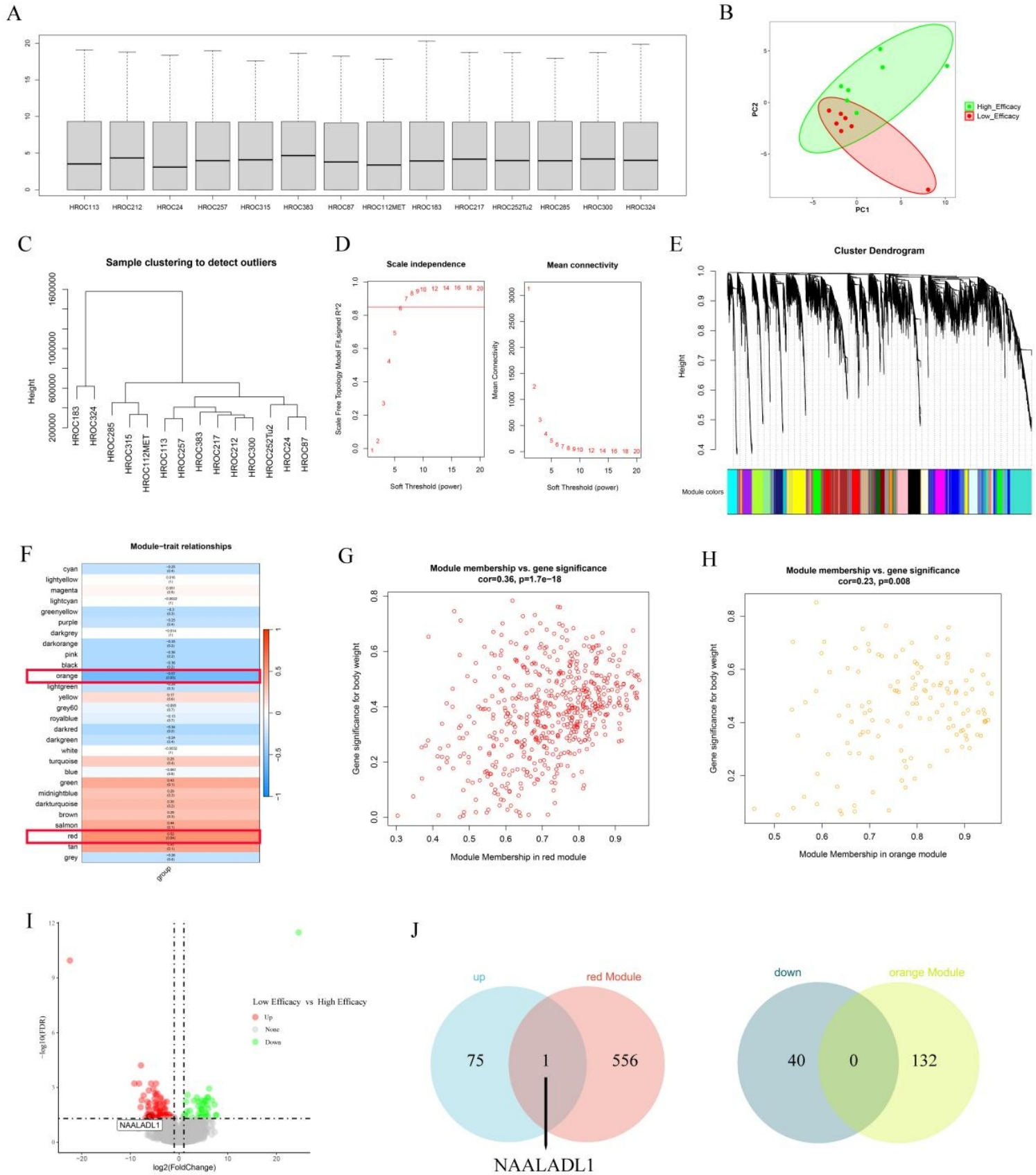
Subsequent analysis of RNA-seq data from the 14 HROC cell lines, including the seven with high and the seven with low efficacy in response to TTF treatment, was then conducted. In order to guarantee the reliability of the gene expression data, a series of quality control procedures were executed. The aforementioned procedures encompassed normalization and outlier detection. As demonstrated in Fig. 6A, the boxplot of gene expression levels across samples indicated that the data underwent proper normalization, exhibiting comparable distributions across all samples. PCA was performed to evaluate overall expression patterns and detect batch effects. The PCA plot (Fig. 6B) exhibited a discernible divergence between the high efficacy and low efficacy groups, suggesting the existence of discrete transcriptional disparities. To further identify potential outliers, a hierarchical clustering analysis was performed on all samples based on their gene expression profiles (Fig. 6C). No extreme outliers were detected, confirming the dataset's integrity for subsequent network construction.

The utilization of the WGCNA package facilitated the construction of a weighted gene co-expression network, thereby enabling the identification of gene modules associated with treatment efficacy. To ensure scale-free network topology (Fig. 6D), a soft thresholding power of $\beta = 7$ was selected. The hierarchical clustering dendrogram of genes was generated based on the TOM (Fig. 6E), and modules were detected using the dynamic tree-cutting algorithm, with a minimum module size of 50. Consequently, a total of 28 co-expression modules were identified, each assigned a unique color for representation. In order to identify modules that are correlated with the efficacy of TTF treatment, the association between each ME and treatment outcome was calculated. As illustrated in Figure 6F, the module-trait heatmap revealed a substantial correlation between the red module ($r = 0.56$, $P = 0.002$) and the orange module ($r = -0.54$, $P = 0.003$) and treatment response. The red module demonstrated a positive association with the low efficacy group, while the orange module exhibited a negative association. Further analysis of module membership (MM) and gene significance (GS) revealed a robust correlation in the red module ($\text{cor} = 0.36$, $P = 0.001$, Fig. 6G) and the orange module ($\text{cor} = 0.23$, $P = 0.008$, Fig. 6H), confirming the functional relevance of genes within these modules. To this end, a differential gene expression analysis was conducted between the high efficacy and low efficacy groups using the limma package. Volcano plot analysis (Fig. 6I) revealed that *NAALADL1* was significantly overexpressed in the low efficacy group. In order to further refine potential biomarkers, the DEGs were intersected with the module genes. Venn diagram analysis (Fig. 6J) showed that *NAALADL1* was the only gene

common between the upregulated genes in the low efficacy group and the red module. This finding suggests that NAALADL1 may serve as a significant biomarker associated with resistance to TTF treatment.

Figure 6. *NAALADL1* expression as a biomarker of resistance to TTF treatment.

Results

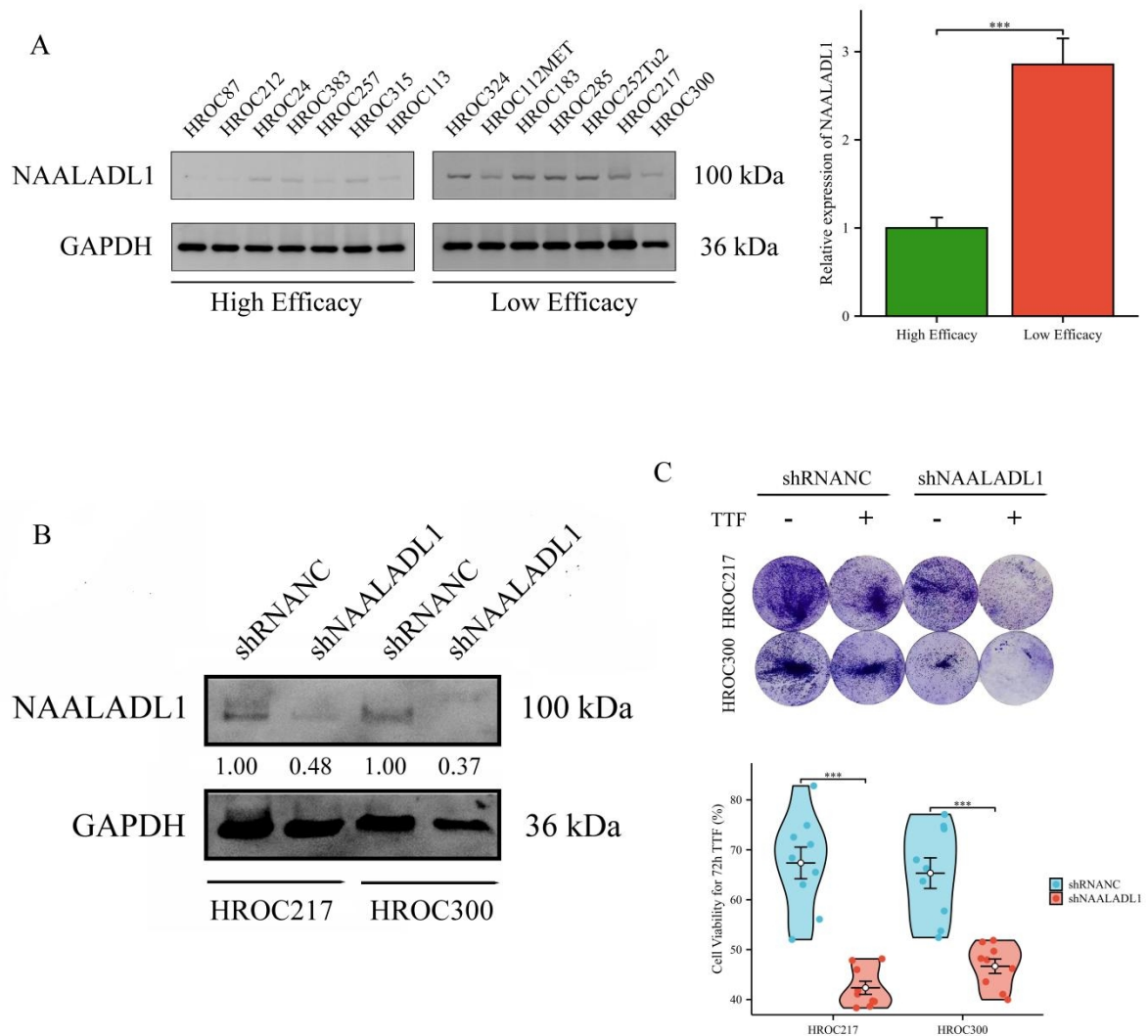


(A) Boxplot analysis of gene expression levels across all samples confirmed successful normalization. (B) PCA revealed a clear separation between the high efficacy and low efficacy groups. (C) Clustering analysis did not detect extreme outliers. (D) A soft thresholding power of $\beta = 7$ with scale-free topology fit index (R^2) > 0.85 was selected to construct a scale-free co-expression network. (E) A hierarchical clustering dendrogram based on the TOM identified a total of 28 co-expression modules. (F) The red module ($r = 0.56$, $P = 0.002$) was positively associated with the low efficacy group, while the orange module ($r = -0.54$, $P = 0.003$) was negatively associated. (G) Significant correlation of MM and GS were detected in the red module ($\text{cor} = 0.36$, $P < 0.001$) (H) and in the orange module ($\text{cor} = 0.23$, $P = 0.008$). (I) Differential gene expression analysis found *NAALADL1* significantly upregulated in the low efficacy group. (J) Venn diagram analysis found *NAALADL1* was the only gene overlapping between the upregulated genes in the low efficacy group and the red module.

3.5 Knockdown of NAALADL1 enhances CRC sensitivity to TTF treatment

Subsequent WB results demonstrated that NAALADL1 protein expression differed among the 14 HROC cell lines, with lower expression correlating with higher TTF treatment efficacy compared to cell lines with higher NAALADL1 expression ($P < 0.001$, Fig. 7A). Taking advantage of shRNA knockdown technology, NAALADL1 protein expression levels could be significant reduced in HROC217 and HROC300 cells (Fig. 7B). Viability assays further revealed that NAALADL1 knockdown enhanced sensitivity to TTF treatment in both cell lines (Fig. 7C).

Figure 7. NAALADL1 knockdown enhanced CRC sensitivity to TTF treatment.



(A) WB analysis of NAALADL1 expression in 14 HROC cell lines. NAALADL1 expression in the high-efficacy group was statistically lower than that in the low-efficacy group ($P < 0.001$). (B) WB analysis showed successful knockdown of NAALADL1 in HROC217 and HROC300 cells using shRNA lentivirus. (C) Viability assays demonstrated that NAALADL1 knockdown enhanced sensitivity to TTF treatment, as indicated by decreased cell viability in both HROC217 ($P < 0.001$) and HROC300 ($P < 0.001$).

3.6 Inhibition of NAALADL1 enhances CRC cell sensitivity to TTF treatment

In order to explore potential binding sites and channel structures of NAALADL1, its three-dimensional structure was obtained from the Protein Data Bank. The structural features of NAALADL1 were analyzed using CaverWeb 2.0, including its pockets and tunnels. The Fpocket algorithm identified multiple binding pockets based on geometric features such as volume, depth, and surface area, which could serve as potential drug-binding sites. Furthermore, the Caver algorithm identified accessible channels that may influence ligand interactions and molecular transport. Among the identified binding pockets, the highest-ranked pocket was selected for further investigation. This pocket contains key catalytic residues (A:L227, B:L227, A:E394, B:E394, A:D623, B:D623) and achieved a relevance score of 100%, with a volume of 3,462 Å³ and a druggability score of 0.153. In addition to the identified binding pocket, the tunnel structure within NAALADL1 was also analyzed for this pocket. The selected tunnel features a bottleneck radius of 1.6 Å, a total length of 24.1 Å, and a distance of 19.1 Å from the protein surface. Further analysis revealed a curvature of 1.3 and a throughput value of 0.62, indicating its potential role in ligand accessibility. This tunnel comprises 34 residues and contains a single bottleneck, which may influence molecular transport and interaction dynamics within the protein (Fig. 8A). Subsequently, virtual screening was conducted using the FDA-approved drug library from the ZINC database (<https://zinc.docking.org/>). The virtual screening process incorporated molecular docking simulations to assess the binding affinity of small molecules from the FDA library with the NAALADL1 protein channels. AutoDock

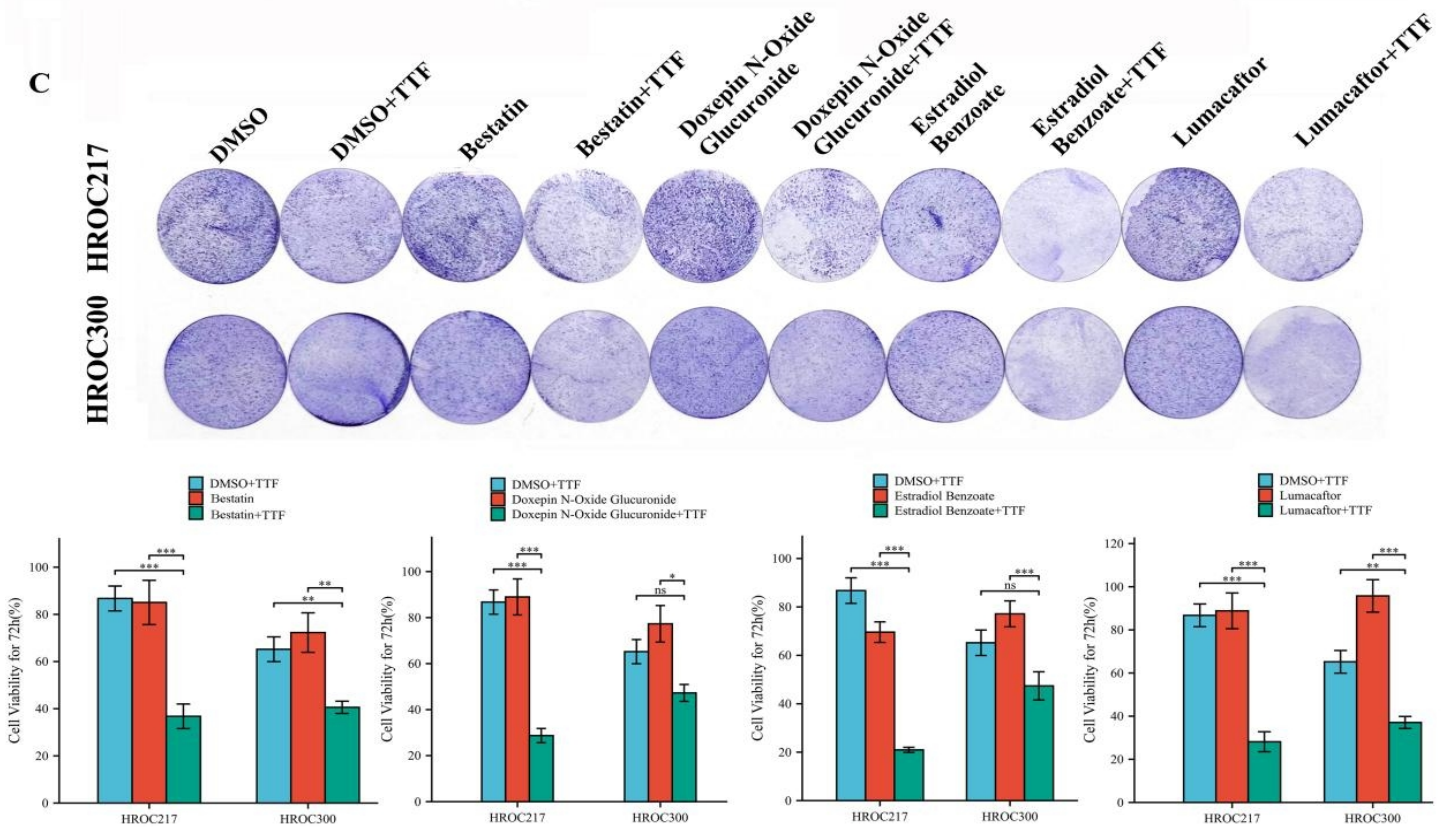
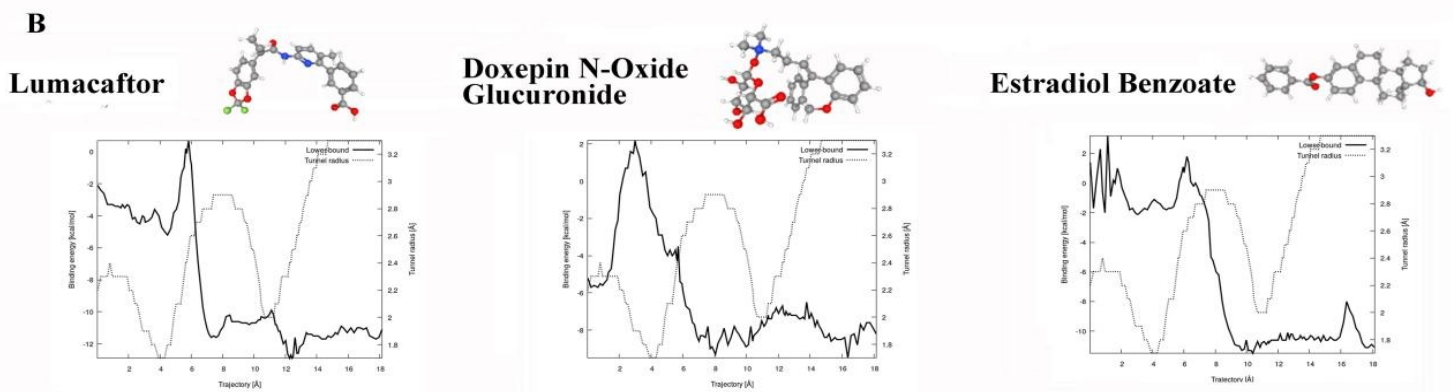
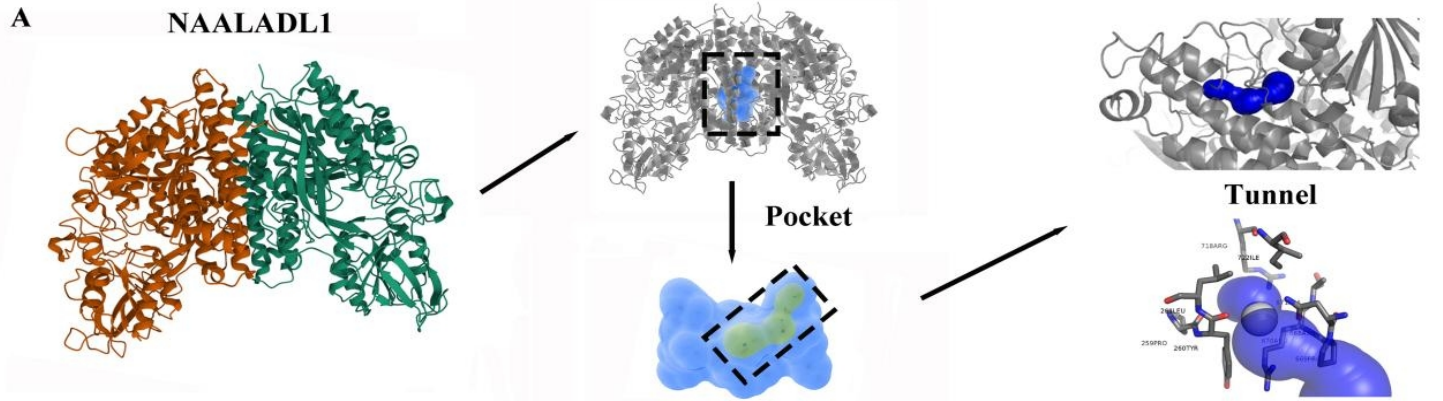
Vina, a widely used molecular docking software, was employed to calculate the binding energy between the small molecules and the protein channels, generating detailed interaction maps. These computational results were used to rank the compounds based on their binding affinity, thereby enabling the selection of the most promising candidates for further analysis and experimental validation.

Based on the molecular docking results, 12 compounds from the FDA-approved drug library exhibited strong binding affinities with the NAALADL1 protein channel, with binding energies ranging from -10.6 to -10.0 kcal/mol (Table 7). The top three compounds were selected based on binding energy (Fig. 8B): Doxepin N-Oxide Glucuronide (ZINC000095618886), Estradiol Benzoate (ZINC000003881345), and Lumacaftor (ZINC000064033452), along with Bestatin, a previously reported NAALADL1 small-molecule inhibitor [39]. Then the IC₅₀ values of these four compounds were evaluated in the cell lines HROC300 and HROC217. The results showed that the IC₅₀ values for all four compounds in both cell lines were above 50 μ M (Fig. 9).

Cell viability assays were conducted to evaluate the synergistic effects of Bestatin, Doxepin N-Oxide Glucuronide, Estradiol Benzoate, and Lumacaftor in combination with TTF on HROC217 and HROC300 cells. Among them, Lumacaftor and Bestatin showed statistically significant combined effects in both cell lines (Fig. 8C).

Figure 8. Inhibition of NAALADL1 enhances CRC sensitivity to TTF treatment.

Results



(A) Structural analysis of NAALADL1. Left: Ribbon diagram of NAALADL1 dimer. Middle: Identification of a major binding pocket (highlighted in blue) based on geometric parameters. Right: Tunnel detection within the protein structure using CaverWeb; a key tunnel connecting the protein surface to the binding pocket is shown with a bottleneck highlighted. (B) Molecular docking results for top-ranked FDA-approved compounds—Lumacaftor, Doxepin N-Oxide Glucuronide, and Estradiol Benzoate. Each panel shows the chemical structure of the compound and the corresponding docking affinity plot, indicating potential binding strength and trajectory characteristics within the NAALADL1 tunnel. (C) Cell viability assays of HROC217 and HROC300 treated with DMSO, TTF, NAALADL1 inhibitors alone, or in combination with TTF.

Table 7. Top 12 FDA-approved compounds with high binding affinities to the NAALADL1 protein channel identified by molecular docking.

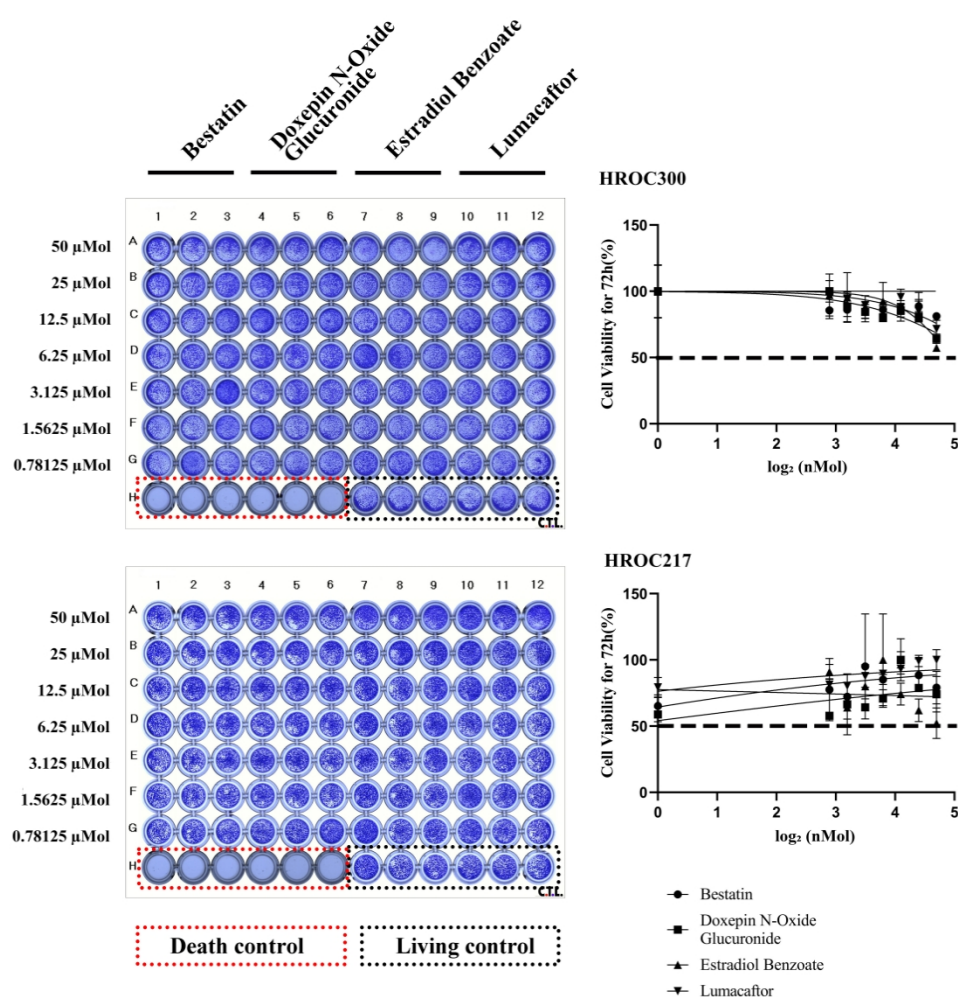
ZINC ID	binding energy (kcal/mol)
ZINC000064033452	-10.6
ZINC000003881345	-10.4
ZINC000095618886	-10.3
ZINC000116473771	-10.3
ZINC000000897408	-10.2
ZINC000003779720	-10.2
ZINC000031425361	-10.2
ZINC000089224316	-10.2
ZINC000000577115	-10.1

ZINC000003821234 -10.1

ZINC000012494489 -10.1

ZINC000001539579 -10

Figure 9. IC₅₀ values of Bestatin, Doxepin N-Oxide Glucuronide, Estradiol Benzoate, and Lumacaftor in the cell lines HROC300 and HROC217.



The IC₅₀ values for Doxepin N-Oxide Glucuronide, Estradiol Benzoate, Lumacaftor along with Bestatin in HROC217 and HROC300 were above 50 μ M.

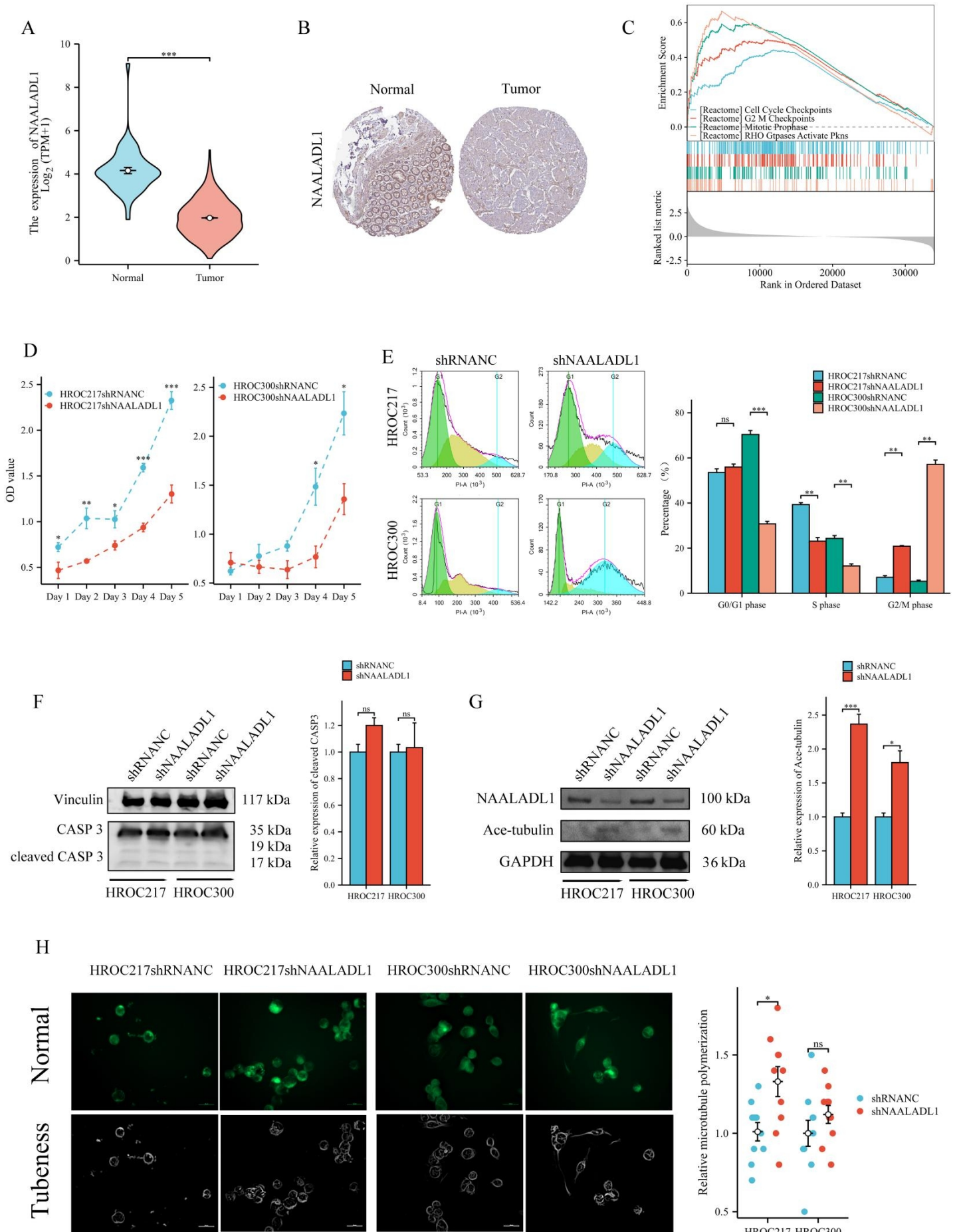
3.7 Downregulation of NAALADL1 induces G2/M arrest via microtubule stabilization in CRC cells

To investigate the functional role of NAALADL1 in CRC, its gene and protein expression levels were firstly analyzed in normal and tumor tissues using public databases. The results showed that *NAALADL1* gene expression was significantly lower in tumor tissues compared to normal tissues (Fig. 10A). The result could be further validated at the protein level by immunohistochemical staining (Fig. 10B). GSEA revealed that *NAALADL1* was significantly enriched in several cell cycle and mitosis-related pathways, including “REACTOME_CELL_CYCLE_CHECKPOINTS”, “REACTOME_G2_M_CHECKPOINTS”, “REACTOME_MITOTIC_PROPHASE”, and “REACTOME_RHO_GTPASES_ACTIVATE_PKNS” (Fig. 10C). Functional crystal violet proliferation assays demonstrated that knockdown of NAALADL1 significantly suppressed the proliferation of HROC217 and HROC300 (Fig. 10D). FC analysis further revealed that the proportion of cells in the G2/M phase was significantly increased in both HROC217 and HROC300 cell lines after NAALADL1 knockdown (Fig. 10E). WB analysis showed that knockdown of NAALADL1 did not significantly alter the levels of cleaved caspase-3 (Fig. 10F). Notably, NAALADL1 silencing markedly increased the expression of α -tubulin which reflects microtubule stability (Fig. 10G). Immunofluorescence staining further supported this observation, showing that the relative level of microtubule polymerization was significantly higher in NAALADL1 knockdown HROC217 compared to the

respective control group cells (Fig. 10H).

Figure 10. Downregulation of NAALADL1 induces G2/M phase arrest via enhanced microtubule stabilization in CRC cells.

Results



(A) *NAALADL1* was significantly downregulated in tumor tissues compared with normal tissues based on data from public databases ($P < 0.001$). (B) Representative immunohistochemical staining showing reduced NAALADL1 protein expression in CRC tissues compared to normal tissues. (C) GSEA revealed *NAALADL1* was significantly enriched in “REACTOME_CELL_CYCLE_CHECKPOINTS”, “REACTOME_G2_M_CHECKPOINTS”, “REACTOME_MITOTIC_PROPHASE”, and “REACTOME_RHO_GTPASES_ACTIVATE_PKNS” pathways. (D) Crystal violet assays showed that NAALADL1 knockdown significantly suppressed the proliferation of HROC217 and HROC300. (E) FC analysis revealed a significant increase in the proportion of G2/M phase cells upon NAALADL1 knockdown in both HROC217 ($P < 0.01$) and HROC300 ($P < 0.01$). (F) WB analysis of cleaved caspase-3 expression in NC and NAALADL1 knockdown cell lines, revealed no significant difference. (G) WB analysis demonstrated that NAALADL1 knockdown upregulated Ace-tubulin levels in HROC217 ($P < 0.001$) and HROC300 ($P < 0.05$). (H) Immunofluorescence staining showed significant increased microtubule polymerization in HROC217 cells following NAALADL1 knockdown ($P < 0.05$).

4. Discussion

TTF constitutes a novel, non-invasive physical therapy that utilizes intermediate-frequency, low-intensity alternating electric fields to disrupt cancer cell division [40–44]. The clinical potential of TTF was initially demonstrated in GBM. The pivotal Phase III EF-14 trial demonstrated that TTF, when combined with temozolomide, significantly improved both PFS and OS compared to standard chemotherapy alone [45]. Following its initial FDA approval in 2004 for recurrent GBM, TTF has steadily expanded into the treatment of other solid tumors. Notably, the STELLAR trial [46] in malignant pleural mesothelioma reported a median OS of 18.2 months, while the ongoing Phase III LUNAR trial is assessing its efficacy in NSCLC [18]. In addition, in vitro and early-phase clinical studies are currently exploring its use in pancreatic and ovarian cancers [11, 47-49].

While TTF have garnered increasing attention for their potential in treating various solid tumors, research into their application in CRC remains in its early, investigational stages. The first in vitro study demonstrating TTF-induced growth inhibition in CRC cell lines was published in 2019 [50]. In this study, researchers explored how TTF affect the growth of CRC cells, particularly in the context of 5-fluorouracil sensitization. The researchers treated human HCT116 and SW480 CRC cell lines with 5-FU and/or TTF, and examined the effects on cell viability, apoptosis, autophagy, and metastatic potential. The findings indicated that the combination of 5-FU and TTF treatment enhanced the efficacy of TTF therapy in CRC cells. This combination treatment downregulated key signaling pathways involved in cell

proliferation, survival, invasion, and migration, while simultaneously upregulating pathways that mediate apoptosis and autophagy-mediated cell death. However, as of 2025, there remains a paucity of clinical trials, and a definitive translational pathway has yet to be established. The optimal therapeutic strategies and patient indications remain subjects of active investigation.

Despite these challenges, TTF holds considerable therapeutic promise for CRC. TTF is a non-systemic and non-invasive approach to cancer treatment that has been shown to circumvent the toxicities commonly associated with traditional chemotherapy and radiotherapy [11, 18, 48]. These toxicities include myelosuppression and gastrointestinal adverse effects. Therefore, TTF is particularly well-suited for patients with advanced or metastatic disease, especially those with poor performance status. The mechanistic actions of TTF are multifaceted, including the induction of mitotic disruption through microtubule destabilization, metabolic reprogramming via mitochondrial dysfunction, and immune activation through the release of damage-associated molecular patterns and tumor-associated antigens [42, 51-52].

The evidence presented herein indicates that the utilization of a combination of mechanisms has the potential to provide a particularly promising strategy for targeting refractory CRC subtypes, including those characterized by KRAS or BRAF mutations. In the future, the incorporation of multi-omics data has the potential to facilitate the creation of an "electric field sensitivity scoring system." When combined with liquid biopsy markers (e.g., KRAS mutations, MSI status, and expression levels of certain proteins), this system could allow for the real-time personalization of field parameters

(e.g., frequency, intensity, and duration) to optimize therapeutic outcomes. Additionally, TTF may enhance vascular permeability, potentially facilitating the delivery of nanoparticle-based therapies or CAR-T cells, and could synergize with immune checkpoint inhibitors to transform immunologically "cold" tumors into "hot" ones, thereby boosting the efficacy of immunotherapy [40, 53].

For patients with advanced rectal cancer, particularly those with stage III low-position rectal cancer, neoadjuvant therapies, including radiation therapy, are often required to ensure clear surgical margins in subsequent procedures [54-58]. However, radiation therapy can lead to tissue edema and scarring in the pelvic floor, significantly increasing the difficulty of subsequent surgical interventions, as well as other adverse effects [59-63]. The characteristic of TTF to have minimal impact on normal tissues may position it as a promising adjunctive treatment. Due to its non-invasive nature and low toxicity to healthy tissues, TTF holds potential as an effective strategy in neoadjuvant therapy, potentially alleviating the side effects caused by radiation therapy, improving postoperative recovery, and reducing the incidence of surgical complications.

However, several significant challenges must be addressed before TTF can be widely implemented in the clinical treatment of CRC. First, the molecular heterogeneity of CRC [64-68], coupled with the absence of reliable predictive biomarkers, complicates patient selection. Distinct molecular subtypes, such as MSI versus MSS tumors, may exhibit varying sensitivities to TTF, and mutations in key oncogenes like KRAS or BRAF may further modulate treatment response. Second, unlike GBM and lung

cancer, peritoneal metastases are common in advanced CRC and are often characterized by diffuse and irregularly distributed lesions [69-73], which pose significant technical challenges for achieving uniform electric field coverage and accurately targeting multiple lesions within the anatomically complex abdominal cavity. Third, the scheduling of combination therapies remains suboptimal. Preclinical studies suggest that the G2/M phase cell cycle arrest induced by TTF may conflict with the S-phase-specific cytotoxicity of agents such as oxaliplatin and 5-FU, leading to asynchronous cell cycle effects that may diminish the overall therapeutic efficacy. Consequently, optimizing combination treatment strategies—particularly through dynamic metabolomic profiling to identify optimal treatment windows—will be critical.

In view of these considerations, it is imperative to further investigate the precise mechanisms by which TTF exerts cytotoxic effects on CRC cells, to identify molecular biomarkers that predict sensitivity to TTF, and to elucidate the mechanisms underlying resistance to treatment. Multi-omics approaches, encompassing transcriptomics, metabolomics, and phosphoproteomics, have the potential to facilitate the discovery of predictive and pharmacodynamic biomarkers. Consequently, this approach may facilitate the identification of patient populations that are most likely to benefit from TTF. Moreover, the acquisition of mechanistic insights into resistance mechanisms has the potential to inform the development of strategies to overcome or reverse treatment resistance. This, in turn, could maximize the clinical potential of TTF in the treatment of CRC.

In this study, the antitumor efficacy of TTF was systematically assessed for the first time in a large panel of low-passage CRC cell lines derived from primary patients' tumors and PDX tissues. A wide range of surviving cell fractions was observed in the aftermath of TTF treatment, exhibiting survival rates ranging from 23.8% to 88.2%. Based on these results, CRC cell lines were stratified into high-, moderate-, and low-response groups, with statistically significant differences in cell viability observed between the high- and low-responders. This marked heterogeneity reflects the molecular complexity of CRC and suggests that responsiveness to TTF is likely governed by multifactorial determinants. These determinants include, but are not limited to, cell cycle status, genetic background, metabolic state, and activity of oncogenic signaling pathways. As a non-invasive physical therapy that interferes with mitotic progression through alternating electric fields, TTF may exert differential efficacy depending on intrinsic biophysical properties such as membrane potential, microtubule behavior, and cytoskeletal structure [42, 43, 51]. Overall, these findings underscore the importance of elucidating the specific mechanisms underlying TTF response and resistance in CRC. The identification of predictive molecular biomarkers is critical for effective patient stratification and for optimizing the clinical application of TTF. Furthermore, understanding the mechanisms associated with low responsiveness or resistance may inform the development of rational combination strategies to improve therapeutic outcomes, particularly in refractory CRC.

Therefore, a series of experimental approaches were employed to further elucidate the biological mechanisms by which TTF exerts its antitumor activity in CRC cells. A

thorough examination of the cells using SEM revealed substantial alterations in the morphology of TTF-treated cells. These alterations included the loss of pseudopodia, the condensation of chromosomes, the swelling of mitochondria, and the formation of vacuoles. These structural changes are indicative of significant cellular stress and damage [74, 75]. These observations suggest that TTF disrupts cellular integrity, initiating a cascade of molecular events that ultimately lead to cell death. A proteomic analysis of TTF-sensitive cell lines revealed extensive alterations in protein expression, involving nine key biological pathways, primarily related to DNA damage and metabolic dysregulation. The results provide compelling evidence that TTF impinges on multiple essential cellular functions, thereby impairing cell survival and propagating its antitumor effects. Furthermore, immunofluorescence staining further confirmed significant microtubule depolymerization and nuclear deformation before and after TTF treatment, accompanied by mitotic arrest. These findings lend support to the hypothesis that TTF exerts its therapeutic effects primarily through interference with mitotic processes. FC analysis revealed a significant increase in apoptotic cell populations following TTF treatment, particularly in the highly responsive CRC cell lines. This finding further substantiates the apoptotic mechanism of action. The collective analysis of the results indicates that TTF induces apoptosis through a multifaceted mechanism, involving cytoskeletal destabilization, metabolic disruption, and genomic instability. A comprehensive understanding of these fundamental molecular events supports the therapeutic potential of TTF in CRC treatment and lays the groundwork for the development of combination strategies that could enhance its

clinical efficacy.

Mutation analysis identified 16 gene mutations across 14 CRC cell lines, including alterations in *KRAS*, *TP53*, and *BRAF*, etc. Nevertheless, no substantial disparities in the nature or frequency of mutations were identified between the high- and low-response groups. This finding indicates that the effectiveness of TTF is unlikely to be contingent on a specific oncogenic driver. Similarly, clinicopathological comparisons revealed no significant differences in patient age, sex, tumor location, histological differentiation, or TNM stage between the two groups. Despite the fact that the high-response group demonstrated a higher percentage of MSI cases, this tendency did not attain statistical significance, thereby suggesting a possible yet inconclusive association. This observation may be ascribed to the relatively modest sample size of the present study. The findings, when considered collectively, suggest that the response to TTF in CRC is governed by complex, multifactorial mechanisms. These mechanisms cannot be reliably predicted by proliferative capacity, mutational status, or conventional clinicopathological features alone.

Therefore, additional transcriptomic analyses were conducted to systematically evaluate the impact of TTF treatment on CRC cells and to identify key transcriptional features associated with therapeutic response. By comparing differentially expressed genes between the high- and low-efficacy groups, the objective was to uncover potential biomarkers capable of accurately predicting the efficacy of TTF therapy. The initial phase of the research involved the execution of a thorough quality control procedure for the transcriptomic data. This procedure encompassed the

implementation of essential analytical processes, including data normalization and the identification and removal of outliers. The boxplots of gene expression levels across samples indicated a consistent distribution, thereby confirming the successful normalization of the data. PCA and hierarchical clustering revealed a clear separation between high-efficacy and low-efficacy groups, suggesting the presence of distinct transcriptional profiles and the absence of significant outliers. Subsequently, I employed WGCNA to construct gene co-expression modules and identify those associated with treatment response. Employing a soft-thresholding power of $\beta = 7$ yielded the identification of 28 co-expression modules. Subsequent intra-module analysis revealed robust correlations between module membership and gene significance within the pertinent modules. The intersection of DEGs with module-associated genes revealed that the NAALADL1 gene was the sole gene that exhibited both upregulation in the low-efficacy group and inclusion in the TTF resistance module. This finding suggests that NAALADL1 may play a critical role in mediating resistance to TTF therapy and holds promise as a predictive biomarker.

WB analysis further confirmed that the expression level of NAALADL1 protein was significantly higher in TTF-resistant cell lines compared to TTF-sensitive cell lines. Subsequently, the functional validation of the role of NAALADL1 in mediating TTF resistance was conducted. The utilization of shRNA to knockdown NAALADL1 in the most TTF-resistant CRC cell lines (HROC217 and HROC300) resulted in a significant decrease in cell viability following TTF treatment, indicating an increased sensitivity to TTF and supporting the efficacy of the NAALADL1 function in

modulating TTF.

Enzymes are considered to be promising drug targets due to their well-defined active sites, which generally exhibit conserved three-dimensional structures. These structures are capable of accommodating small-molecule inhibitors with high specificity and affinity. These active sites often possess physicochemical properties—such as hydrophobic pockets, catalytic residues, and accessible grooves—that facilitate the binding of drug-like compounds [76, 77]. Furthermore, the catalytic function of enzymes signifies that even partial inhibition can result in substantial downstream biological effects, thereby enhancing therapeutic efficiency. Therefore, given that NAALADL1 is an enzyme protein with promising clinical and translational potential, an in-depth analysis of its potential drug-binding sites and channel structures was further conducted. A combination of structural biology tools was used to identify several promising drug-binding pockets and channels. Notably, one of the pockets with the highest score contained key catalytic residues and demonstrated strong drug-binding affinity. Moreover, structural analysis indicated that the protein channel within NAALADL1 may be implicated in ligand interactions and molecular transport. The present findings contribute to a more profound comprehension of the functional role of NAALADL1 and provide further evidence in support of its potential as a drug target.

Virtual Screening is a computer-aided drug screening method widely used in drug discovery and design. Unlike traditional experimental screening methods, virtual screening uses computer simulations to predict the interactions between compounds

and target proteins, screening potential drug candidates from large compound libraries. It can significantly accelerate the drug discovery process, particularly in the initial screening phase, helping researchers quickly identify promising candidate compounds [78-82]. Through virtual screening of the FDA-approved drug library, several small molecules with strong binding affinity to the NAALADL1 protein channels were identified. Although these compounds exhibited relatively elevated IC_{50} values at the cellular level, they showed significant synergistic effects when combined with TTF. Specifically, Lumacaftor and Bestatin exhibited statistically significant synergistic effects in the two most resistant cell lines to TTF therapy. This finding suggests that despite the limited pharmacological effects of these compounds when used alone, their combination with TTF may partially overcome CRC resistance to TTF treatment, thereby enhancing the therapeutic efficacy of TTF.

Due to the finding that both knockdown and inhibition of NAALADL1 lead to increased sensitivity to TTF treatment in TTF-resistant cell lines, I became interested in its function in CRC and the mechanism through which it influences TTF response. NAALADL1, a close homolog of glutamate carboxypeptidase II—a zinc-dependent metallopeptidase extensively characterized as a therapeutic and diagnostic target in solid malignancies and neuropathologies [83-87] —remains poorly understood in terms of its biological function. In this study, bioinformatic analyses, in conjunction with immunohistochemistry data, revealed consistently lower expression levels of NAALADL1 in CRC tissues compared to normal tissues across multiple public datasets. GSEA revealed that NAALADL1 is significantly associated

with several signaling pathways related to cell cycle regulation, particularly G2/M checkpoint control, mitotic prophase, and cytoskeletal remodeling mediated by Rho GTPases. These findings suggest a potential role for NAALADL1 in regulating cell cycle progression and microtubule dynamics.

To further investigate its biological function, shRNA-mediated knockdown of NAALADL1 in CRC cell lines with are most resistant to TTF was performed. Knockdown of NAALADL1 markedly suppressed cell proliferation and induced G2/M phase arrest, as confirmed by FC. Notably, cleaved caspase-3 levels remained constant following NAALADL1 knockdown, suggesting that the observed growth suppression was not attributable to apoptosis but rather to a cell cycle blockade. Mechanistically, the knockdown of NAALADL1 led to a substantial increase in the levels of Ace-tubulin, a marker of microtubule stabilization [88-90]. This finding was further substantiated by immunofluorescence imaging, which revealed enhanced microtubule polymerization. The results of this study suggest that NAALADL1 may play a regulatory role in mitotic progression by modulating microtubule stability.

Previous studies have demonstrated that the dynamic equilibrium of microtubules is crucial for proper mitotic progression. Microtubules must undergo continuous polymerization and depolymerization to facilitate spindle assembly and chromosome segregation [91]. Both excessive stabilization and destabilization of microtubules can disrupt this balance [92-93]. Such perturbations impair the formation of the mitotic spindle and interfere with the fidelity of chromosome alignment and segregation. Consequently, cells may activate the spindle assembly checkpoint, leading to arrest at

the G2/M phase of the cell cycle [94-95]. This mechanism ensures genomic integrity but also renders cells more vulnerable to agents that target mitotic phase.

Based on the above findings, we propose the following hypothesis: in TTF-resistant CRC cells, knockdown or inhibition of NAALADL1 leads to increased microtubule stability. Although the precise molecular mechanisms linking NAALADL1 to microtubule dynamics remain to be elucidated, we speculate that excessive stabilization of microtubules disrupts their intrinsic dynamic remodeling, thereby impairing proper spindle assembly. This disturbance in microtubule dynamics can interfere with mitotic progression and ultimately cause cell cycle arrest at the G2/M phase. Previous studies have demonstrated that TTF primarily exerts its antiproliferative effects by interfering with microtubule dynamics during mitosis. Therefore, when a greater proportion of cells are arrested at G2/M due to NAALADL1 inhibition, the therapeutic efficacy of TTF may be amplified. In other words, suppression of NAALADL1 enhances the antitumor effects of TTF in resistant CRC cells by altering microtubule stability and cell cycle distribution.

5. Conclusions

This study systematically evaluated the antitumor efficacy of TTF in low-passage CRC cell lines and identified marked heterogeneity in treatment response. From a mechanistic perspective, the cytotoxic effects of TTF are the result of a multifaceted mode of action, primarily involving the disruption of cellular architecture, mitotic disruption, the induction of DNA damage, and metabolic dysregulation. Transcriptomic analysis in conjunction with WGCNA identified *NAALADL1* as a potential resistance-associated gene, with significantly elevated expression observed in low-response cell lines. As demonstrated by functional assays, knockdown of NAALADL1 modulates TTF sensitivity by stabilizing microtubules and inducing G2/M cell cycle arrest. A structure-based virtual screening of FDA-approved drug libraries was conducted, revealing that Lumacaftor and Bestatin, while exhibiting limited efficacy as monotherapy, demonstrated synergistic antitumor effects when combined with TTF. This finding underscores their potential for further therapeutic development. The findings collectively substantiate the notion that NAALADL1 functions as both a predictive biomarker and a promising therapeutic target for overcoming TTF resistance. This conclusion is supported by a mechanistic rationale and translational basis for the personalized application of TTF in CRC.

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7. Appendix

7.1 List of Abbreviations

Abbreviation	Full Name
CIMP-H	CpG Island Methylator Phenotype-High
COAD	Colon Adenocarcinoma
CRC	Colorectal Cancer
DAPI	4',6-Diamidino-2-Phenylindole
DEGs	Differentially Expressed Genes
DEPs	Differentially Expressed Proteins
DMSO	Dimethyl Sulfoxide
FC	Flow Cytometry
FDA	Food and Drug Administration
GBM	Glioblastoma Multiforme
GFP	Green Fluorescent Protein
GSEA	Gene Set Enrichment Analysis
GTE _x	Genotype-Tissue Expression
HPA	Human Protein Atlas
IC ₅₀	Half-Maximal Inhibitory Concentration
KEGG	Kyoto Encyclopedia of Genes and Genomes
LS	Lynch Syndrome
MOI	Multiplicity of Infection
MS	Mass Spectrometry

Appendix

MSI	Microsatellite Instability
MSS	Microsatellite Stability
NAALADL1	N-acetylated α -linked Acidic Dipeptidase-like 1
NGS	Next-Generation Sequencing
NSCLC	Non-Small Cell Lung Cancer
OS	Overall Survival
PCA	Principal Component Analysis
PDX	Patient-Derived Xenograft
PFS	Progression-Free Survival
PI	Propidium Iodide
RIN	RNA Integrity Number
SD	Standard Deviation
SEM	Scanning Electron Microscopy
shRNA	Short Hairpin RNA
spMSI	Sporadic Microsatellite Instability Subtype
spSTD	Sporadic Standard Subtype
STR	Short Tandem Repeat
TCGA	The Cancer Genome Atlas
Td	Doubling Time
TME	Tumor Microenvironment
TOM	Topological Overlap Matrix
TTF	Tumor Treating Fields

WB	Western Blot
WGCNA	Weighted Gene Co-expression Network Analysis

7.2 Figure list

Figure 1. NovoTTF-100A™ System for TTF treatment.

Figure 2. Determination of optimal puromycin selection concentration for HROC217 and HROC300 cell lines.

Figure 3. Antitumor effect of TTF on low-passage CRC cell lines.

Figure 4. TTF triggers apoptosis and alters ultrastructural, protein expression, cellular pathways, and microtubule structure in CRC cells

Figure 5. Cell division rate, mutations, and clinicopathological features between high and low efficacy TTF treatment groups.

Figure 6. *NAALADL1* expression as a biomarker of resistance to TTF treatment.

Figure 7. *NAALADL1* knockdown enhances CRC sensitivity to TTF treatment.

Figure 8. Inhibition of *NAALADL1* enhances CRC sensitivity to TTF treatment.

Figure 9. IC₅₀ values of Bestatin, Doxepin N-Oxide Glucuronide, Estradiol Benzoate, and Lumacaftor in the cell lines HROC300 and HROC217.

Figure 10. Downregulation of *NAALADL1* induces G2/M phase arrest via enhanced microtubule stabilization in CRC cells.

7.3 Table list

Table 1. Information of the 21 cell lines and patient characteristics and

clinical-pathological background.

Table 2. Accession details for transcriptomic data.

Table 3. Detailed information and dilution concentrations of antibody.

Table 4. The clinical and pathological characteristics of 21 CRC patients.

Table 5. The cell viability of 21 CRC cell lines after 72 TTF treatment.

Table 6. Univariate analysis of clinical and pathological characteristics for TTF sensitivity.

Table 7. Top 12 FDA-approved compounds with high binding affinities to the NAALADL1 protein channel identified by molecular docking.

7.4 Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki. All patients gave informed written consent to participate in the study and all procedures were approved by the Ethics Committee of the University of Rostock University Medical Centre (Reference numbers: II HV 43/2004 and A 45/2007) in accordance with generally accepted guidelines for the use of human material.

8. Thesis statements

- ◆ In 21 colorectal cancer (CRC) cell lines, significant survival rate differences were observed after tumor treating fields (TTF) treatment.
- ◆ Proteomic analysis, scanning electron microscopy, immunofluorescence, and flow cytometry analyses revealed that TTF disrupted microtubule dynamics, induced mitotic abnormalities, caused metabolic stress, and induced DNA damage, ultimately triggering apoptosis.
- ◆ No significant differences in cell doubling time, gene mutation profiles, or clinical features were found between high- and low-efficacy TTF cell line groups.
- ◆ Transcriptomic analysis and weighted gene co-expression network analysis identified N-acetylated alpha-linked acidic dipeptidase-like 1 (*NAALADL1*) as the sole gene associated with both differential expression and resistance.
- ◆ Functional NAALADL1 knockdown increased TTF sensitivity in resistant CRC cell lines.
- ◆ Virtual screening of FDA-approved drugs targeting NAALADL1's crystal structure identified 12 high-affinity compounds, including Lumacaftor.
- ◆ Combination treatment of Lumacaftor or Bestatin together with TTF significantly enhanced in vitro treatment efficacy.
- ◆ Knockdown of NAALADL1 upregulated acetylated α -tubulin, enhancing microtubule stability and inducing G2/M phase arrest, thereby increasing the sensitivity of TTF-resistant cell lines to TTF treatment.

9. Eidesstattliche Versicherung

Ich versichere hiermit, dass ich die vorliegende Arbeit mit dem Thema: “NAALADL1 as a Key Modulator of Resistance to Tumor Treating Fields Therapy in Colorectal Cancer” unabhängig verfasst habe.

Ich erkläre an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel erstellt habe. Die aus anderer Literatur verwendeten Inhalte sind als solche kenntlich gemacht. Ich versichere, dass ich für die inhaltliche Erstellung der vorliegenden Arbeit nicht die entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten (Promotionsberater oder andere Personen) in Anspruch genommen habe.

Ich erkläre hiermit weiterhin, dass ich meine wissenschaftlichen Arbeiten nach den Prinzipien der guten wissenschaftlichen Praxis gemäß der gültigen „Satzung der Universität Rostock zur Sicherung guter wissenschaftlicher Praxis“ angefertigt habe.

Rostock 30.4.2025

Abgabedatum

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Vollständige Unterschrift

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Publication

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