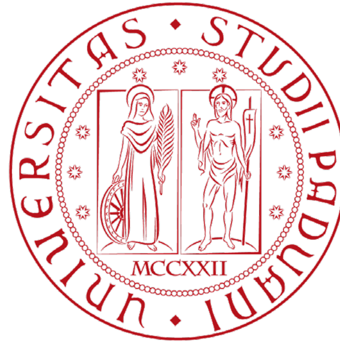

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TESI DI LAUREA

Locally Advanced Rectal Cancer: POU2F3 as a prognostic biomarker and potential therapeutic target

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ABSTRACT

Background: Rectal cancer (RC) represents about one-third of colorectal cancer (CRC), and most of them are Locally Advanced Rectal Cancer (LARC) at diagnosis. The gold standard approach for LARC patients is pre-operative chemoradiotherapy (pCRT) followed by surgery. However, response to pCRT varies considerably, with only 20% of patients achieving a pathological complete response (pCR). In responding patients, surgery might represent an over-treatment and a source of morbidity; on the other hand, non-responders are exposed to unnecessary toxicities and surgery delays.
Nowadays, clinicopathological features have been proposed to predict pCRT response, however, their utility is currently limited due to low sensitivity and specificity. Therefore, novel predictors of response to pCRT are urgently needed. To date, several studies have been conducted for this purpose, but most of them are limited due to their single-omic approach, often insufficient to investigate the entire complexity of cancer. To fill this gap, the NanoInspired Biomedicine Laboratory of Padova has employed a multi-omic approach to identify a potential novel biomarker of pCRT response: a gene named POU2F3.

Purposes of the study:

- To evaluate the prognostic value of POU2F3 in LARC patients.
- To determine *in vitro* whether POU2F3 is targeted by drugs used in clinical practice and whether they cause its up-regulation.
- To evaluate *in vitro* if an enhancing activity exists between 5-Fluorouracil and a chemical compound that upregulates POU2F3.

Materials and methods: 172 LARC patients' data were obtained from The Cancer Genome Atlas (TCGA). HCT-15, HCT-116, and SW480 CRC cell lines were maintained in a humidified atmosphere in a Growth Medium, that was changed every three days. Cell lines were seeded in culture plates and after 24 h they were washed in PBS and incubated with nadolol and entinostat. Then, total RNA was extracted and concentration and purity were determined. cDNA was obtained through reverse transcription PCR and real-time PCR was performed for

amplification and quantification. For cytotoxicity assay, cell lines were incubated with 5-FU, entinostat and a combination of both compounds. After 72 h resazurin dye was added to the wells, then cytotoxicity and IC50 were calculated through fluorescence assessment.

Results: In the TCGA cohort, high POU2F3 expression significantly correlated with higher overall survival (OS) compared to low-expressing LARC patients. Therefore, we analysed three independent databases to identify chemical compounds targeting POU2F3 and its paralogs, POU2F1 and POU2F2. The histone deacetylase inhibitor entinostat and the b-blocker nadolol targeted POU2F3 and its paralogs respectively. We exposed CRC cell lines to both chemicals, and we studied POU2F3 expression: entinostat-treated and nadolol-treated cell lines showed a significant up-regulation and down-regulation respectively. To test the cytotoxicity effect of entinostat we chose 5-FU as a comparison, and we exposed CRC cell lines to both compounds at different concentrations. 5-FU demonstrated concentration-dependent cytotoxicity, whereas entinostat showed poor to no effect on cells. Then, we investigated the effects of a combined approach with both chemicals, and we observed significantly enhanced cytotoxicity.

Conclusions: POU2F3 represents a potential prognostic biomarker for LARC patients and it is a promising therapeutic target. Dosing POU2F3 in clinical practice might identify patients that can truly benefit from pCRT, and spare others from unnecessary toxicity. The *in vitro* experiments suggested an empowering effect of entinostat on the classical 5-FU chemotherapy: this could lead to more efficient therapies and reduction of 5-FU dose, together with fewer side effects.

RIASSUNTO

Introduzione: Il cancro del retto rappresenta intorno a un terzo dei tumori colorettali, e la maggior parte di essi sono neoplasie localmente avanzate (LARC) alla diagnosi. L'approccio gold standard per i pazienti con LARC è la chemio-radioterapia preoperatoria (pCRT) seguita da chirurgia. Tuttavia, la risposta alla pCRT è molto variabile, solo il 20% dei pazienti raggiunge una risposta patologica completa (pCR). Nei pazienti responsivi, la chirurgia potrebbe rappresentare un sovratrattamento e una fonte di morbilità; d'altro canto, i non responsivi sono esposti a inutili tossicità e attese chirurgiche.

Ad oggi sono stati proposti dei fattori clinicopatologici per predire la risposta alla pCRT, tuttavia, la loro utilità è attualmente limitata a causa della bassa sensibilità e specificità. Di conseguenza, c'è urgente necessità di nuovi predittori di risposta alla pCRT. Numerosi studi sono stati condotti per questo scopo, ma molti di essi sono limitati a causa del loro approccio single-omic, spesso insufficiente per investigare la complessità del cancro. Per colmare questa lacuna, il NanoInspired Biomedicine Laboratory di Padova ha utilizzato un approccio multi-omico per identificare un nuovo potenziale biomarker di risposta alla pCRT: il gene POU2F3.

Obiettivi dello studio:

- Valutare il valore prognostico di POU2F3 nei pazienti con LARC.
- Determinare *in vitro* se POU2F3 è target di farmaci utilizzati nella pratica clinica e se essi ne causano up-regolazione.
- Valutare *in vitro* se esiste un'attività sinergica tra 5-FU e un composto chimico che upregola POU2F3.

Materiali e metodi: I dati di 172 pazienti affetti da LARC sono stati ottenuti dal The Cancer Genome Atlas (TGCA). Le linee cellulari HCT-15, HCT-116 e SW480 sono state mantenute in atmosfera umidificata in Growth Medium, che veniva cambiato ogni tre giorni. Le linee cellulari sono state seminate in piastre di coltura, dopo 24 ore sono state lavate in PBS e incubate con nadololo ed entinostat. Quindi veniva estratto l'RNA totale e determinata la concentrazione e la purezza. Con reverse transcription PCR è stato ottenuto il cDNA, che veniva amplificato e quantificato tramite real time PCR. Per il saggio di citotossicità, le linee cellulari

sono state incubate con 5-FU, entinostat e una combinazione di entrambi i composti. Dopo 72 h è stata aggiunta resazurina, quindi sono state calcolate citotossicità e IC50 tramite fluorescenza.

Risultati: Nella coorte TCGA, un'alta espressione di POU2F3 correlava significativamente con una più alta overall survival (OS) rispetto ai pazienti con scarsa espressione. Abbiamo quindi analizzato tre database indipendenti per identificare un composto chimico con target POU2F3 ed i paraloghi POU2F1 e POU2F2. L'inibitore delle istone-deacetilasi entinostat e il beta-bloccante nadololo avevano come target POU2F3 ed i paraloghi rispettivamente. Per testare la citotossicità di entinostat abbiamo scelto 5-FU come paragone e abbiamo esposto le linee cellulari a differenti concentrazioni di entrambi i composti. 5-FU ha dimostrato citotossicità concentrazione-dipendente, mentre entinostat ha mostrato scarso o nessun effetto sulle cellule. Abbiamo quindi investigato l'effetto di una combinazione di entrambi i farmaci e abbiamo osservato un aumento significativo della citotossicità.

Conclusioni: POU2F3 rappresenta un potenziale biomarker per i pazienti con LARC ed è un promettente target terapeutico. Dosare POU2F3 nella pratica clinica potrebbe identificare pazienti che beneficiano realmente della pCRT, e risparmiare altri soggetti da inutili tossicità. Gli esperimenti condotti in vitro hanno suggerito un effetto potenziante di entinostat sulla classica chemioterapia con 5-FU: questo potrebbe condurre a terapie più efficienti e riduzione della dose di 5-FU, con minori effetti collaterali.

1. INTRODUCTION

Colorectal cancer (CRC) ranks third in terms of incidence and second in terms of mortality worldwide (Figure 1). In 2020, more than 1.8 million new CRC cases and 915.880 deaths were estimated to occur, representing nearly 10% of all cancer cases and deaths. (1)

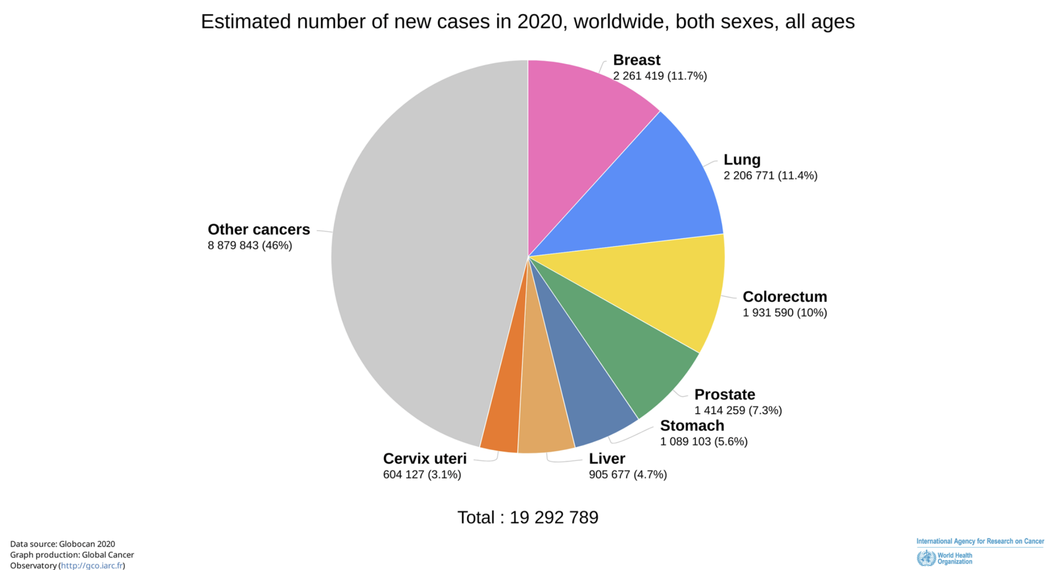


Figure 1: *Estimated number of new cancer cases in 2020 worldwide from Cancer Today.* (2)

Among CRCs, about 35% are rectal cancers (RCs) and most of them are Locally Advanced Rectal Cancers (LARC) at diagnosis, commonly defined as T3-4 and/or N+. (3,4)

Stage at diagnosis is the most important predictor of survival. The 5-year relative survival rate for CRC is 90% and 14% for patients affected by localized and distant-stage diseases respectively. RC patients have a higher overall 5-year survival (67% vs 63%) compared to colon cancers (CC). This is partly explained by the earlier appearance of symptoms in RCs and consequent diagnosis of a localized tumor. (5)

In 2003, the European Council acknowledged the effectiveness of fecal occult blood test (FOBT) screening and recommended a population-wide CRC screening for patients aged 50-74 years in European countries. (6) Most CRCs have a long development time (10-15 years), and that allows for the detection and removal of

precancerous lesions and early-stage cancers that can be successfully treated. (7) According to literature, screening has the potential to reduce CRC mortality by up to 30%, depending on the investigated test; however, it still remains elevated. (6)

Diagnosis of RC is based on digital rectal examination (DRE) and endoscopy with biopsy for histopathological confirmation. RCs are localized within 15 cm from the anal margin and classified as low (up to 5 cm), middle (from > 5 to 10 cm) or high (from > 10 to 15 cm). (8)

RC staging is assessed mainly through the Union for International Cancer Control (UICC) TNM staging classification, which is based on the depth of primary tumor invasion (T), involvement of regional lymph nodes (N), and the presence or absence of metastatic disease (M) (Figure 2). (8,9) The prefixes c, p and y represent clinical, pathologic and post neoadjuvant therapy, respectively. (10) Several imaging modalities, including endorectal ultrasound (EUS), computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) or PET-CT, are used to assess preoperative staging and therapeutic response. (11)

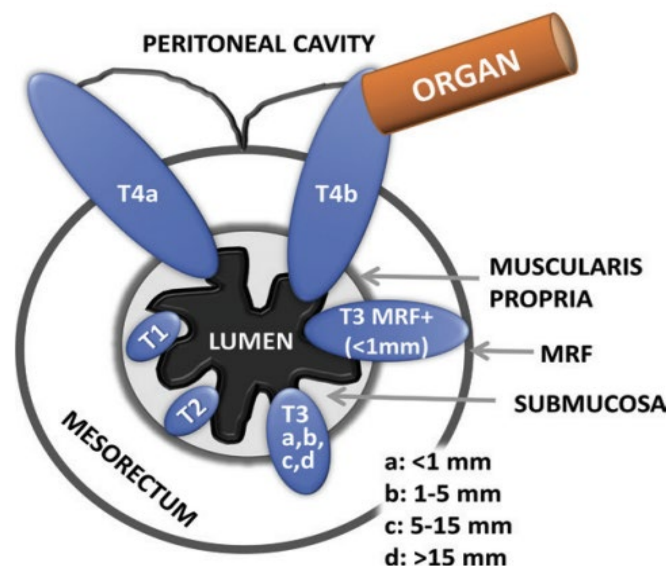


Figure 2: *Illustration of the anatomy of the rectum and the possible locations of rectal cancer, along with corresponding T categories and potential tumor sizes for each location. T1: Tumor invades submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades subserosa or perirectal tissues. The subclassification of T3 category (depth of invasion beyond the muscularis propria) is based on MRI evaluation and is recommended in the European guidelines for treatment decision. MRF: Mesorectal fascia; T4a: Tumor perforates visceral peritoneum; T4b: tumor directly invades other organs or structures.* (8,10)

It has been suggested that CC and RC are two different tumor entities, as they differ in terms of molecular carcinogenesis, pathology, surgical topography and procedures, and multimodal treatment. (12) The therapeutic management of LARC has deeply evolved in the last decades. Before 2004, LARC patients were treated with adjuvant radiotherapy (RT) with or without chemotherapy (CT), an approach that led to significant morbidity. (9) Pre-operative chemoradiotherapy (pCRT) with 5-fluorouracil (5-FU) was adopted as the standard of care in the United States and Europe after 2004, and it currently represents the standard approach of LARC followed by total mesorectal excision (TME). (3,9) TME is a surgical technique introduced in 1980, defined as complete removal of the lymph node bearing mesorectum along with its intact enveloping fascia (Figure 3). (13)

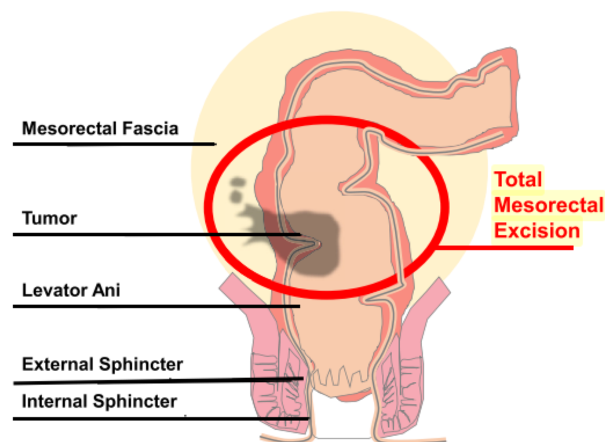


Figure 3: *Total mesorectal excision is the standard of care surgical procedure for rectal cancer that completely removes the rectum, surrounding mesorectal fat, perirectal lymph nodes and the thin sheath called the mesorectal fascia (MRF).* (9)

Various methods categorize tumor response after neoadjuvant therapy, including down-staging and Tumor Regression Grading (TRG). Downstaging is based on the clinical stage of the tumor before the start of the neoadjuvant treatment (cTNM) in comparison with post-treatment stage, either on imaging or endoscopic evaluation (ycTNM) or in resection specimens (ypTNM). Since the tumor response does not always result in downstaging, a more morphological approach was adapted: TRG. There exist many TRG systems which aim to categorize the amount of regressive changes after cytotoxic treatment, mostly they refer to the amount of therapy-induced fibrosis in relation to residual tumor or the estimated percentage of residual tumor in relation to the previous tumor site. The basis of most TRG systems is the

Mandard classification, which was first described in oesophagus carcinomas, then it was adapted by Dworak for rectal cancer. (14,15) Response to pCRT is a predictor of survival, and it varies considerably among RC patients. After neoadjuvant treatment, up to 40% of patients show a poor pathological response, while about 20% achieve a pathological complete response (pCR). (3,16) pCR is defined as the absence of any remaining viable cancer cells (not including acellular mucin pools) in all the resected specimens (including the primary rectal lesion and regional lymph nodes) after pCRT (i.e., ypT0N0M0). (17) Patients who achieve pCR have higher 5-year disease-free survival, and the necessity of radical surgery for those patients has been questioned. (16) TME is associated with relatively high morbidity and mortality rates and usually requires the creation of a temporary or permanent stoma, affecting patients' quality of life (QoL). Therefore, rectum-sparing strategies have been proposed, such as transanal local excision and the "Watch and Wait" approach. (18) On the other hand, patients who do not respond to pCRT will be exposed to unnecessary toxicities and surgery delay. Hence, it is crucial to predict patients' treatment response and outcomes before initiating pCRT. (19)

Several factors are associated with response to pCRT in rectal cancer, and in recent years, novel methods to improve the prediction of sensitivity to therapies have been proposed. Clinical factors, including tumor size, clinical T and N stage, distance of the tumor from the anal verge, and interval from pCRT to surgery are associated with response to pCRT in RC. In addition, some pathological features such as tumor differentiation, circumferential tumor, mucinous histology, and macroscopic ulcerations have been shown to predict poor response to pCRT. Imaging modalities used for pre-treatment staging are also instruments for assessing response to pCRT restaging. Findings from these imaging modalities, including tumor regression rates and circumferential resection margin, can potentially predict response to pCRT in rectal cancer. However, the utility of these clinicopathological and radiological features is currently limited due to low sensitivity and specificity. (20)

Molecular biomarkers, either tissue- or blood-based, have the potential to predict response to pCRT at an early time point. Some proposed blood-based biomarkers are proteins and metabolites, miRNAs, circulating tumor cells, cell-free nucleic acids, and factors involved in host immune response; the most used biomarker for prognosis and disease monitoring in CRC is the carcinoembryonic antigen (CEA).

(20) CEA is a glycoprotein measurable in blood that can become elevated (> 5 ng/mL) in various diseases such as CRC, and remains the only blood-based biomarker suggested by the NCCN guidelines for the workup, management, and surveillance of rectal cancers. (21) MicroRNAs (miRNAs) are a class of small, non-coding RNAs and act as post-transcriptional regulators of gene expression. miRNAs are involved in a variety of biological processes and some of them are deregulated in cancer. It has been shown that miRNAs are associated with pCRT tumor response, notably, D'Angelo et al. demonstrated that high miR-125b expression in tissue and serum was associated with poor treatment response in LARC patients. (22)

In the tissue-based biomarkers field, research has been focusing on DNA mutation and methylation, proteins, metabolites, and Tumor Micro Environment (TME). In addition, global gene expression profiling of tumor tissues has shown the potential to identify gene signatures associated with response to pCRT. (20)

TME is composed of cancer cells, fibroblasts, immune cells, and endothelial cells, that participate in the evolution of cancer (Figure 4). However, the most abundant component of TME is the Extracellular Matrix (ECM), which consists of a set of structural proteins and non-structural secreted enzymes; in the past years, ECM has demonstrated a critical role in tumor progression and diffusion. (23) Decellularization, a widely used tissue engineering technique, has recently been employed to investigate the role of ECM in cancer. Decellularization aims to remove cellular components from tissue and organs through chemical and enzymatic treatments while preserving the ECM integrity. (24) It allows the development of 3D culture models and proteomic analysis to identify key ECM components. (24,25) The composition of ECM can vary in rectal cancers, and it has been shown that altered levels of specific components are associated with specific responses to pCRT. (26)

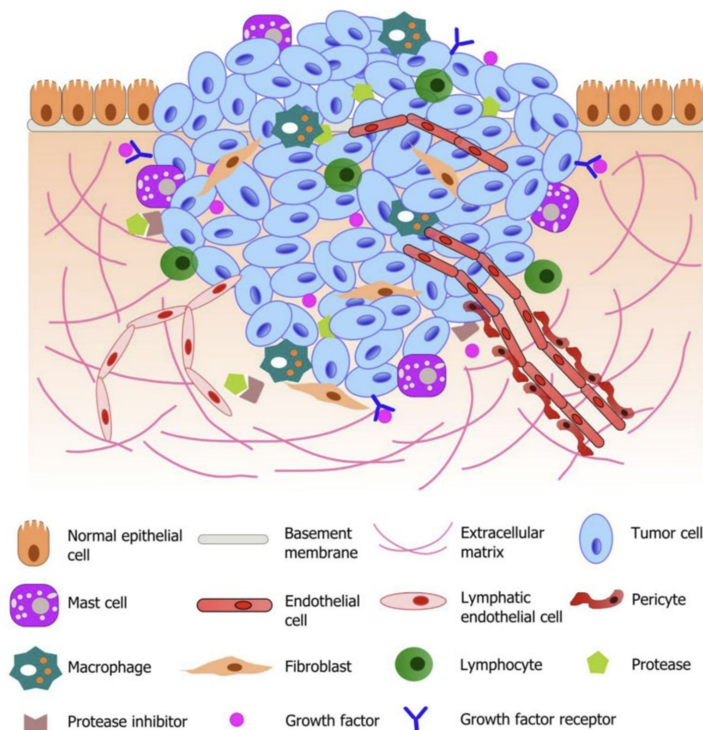


Figure 4: Schematic illustration of a typical tumor microenvironment. *Cancer cells reside in a complex microenvironment containing various supporting cells, extracellular matrix (ECM) and a suite of signaling molecules. These environmental components collectively contribute to the tumor-stromal interaction and tumor progression.* (27)

Lastly, rectal tumors can be categorized based on gene mutations, RNA expression, and epigenetic modifications. These genetic traits can also be used to identify patients that will respond favourably to neoadjuvant therapy. (28)

While the predictive value of some markers, such as the tumor stage, has been well characterized, others are yet emerging. In addition, only a few of these predictive markers are used in the clinical routine, as their potential utility is limited for several reasons. First, biomarkers studies in the field of rectal cancer are conceptually very heterogeneous. Second, validation by independent cohort is lacking or produces conflicting results for the majority of predictive markers. Third, it is questionable if a single biomarker will be sufficient for response prediction or if a combination of several markers is needed. (26)

Most diseases have extremely complex phenotypes, with confounding variables making it difficult to detect a clear causality. Therefore, through single-omic experiments, the interplay between the different molecular entities cannot be

identified. The term “-omics” describes a comprehensive quantitative characterization of a class of molecules in a given biological sample or specimen. There are numerous single-omics approaches, such as proteomics, genomics, and transcriptomics, but in order to understand the interaction between different molecular layers, a multi-omic approach is needed. (29)

In the NanoInspired Biomedicine Laboratory (NIB Lab) of Padova headed by Prof. Agostini, a multi-omic approach has been employed to identify a potential novel biomarker of pCRT response in LARC patients, integrating transcriptomic, post-transcriptomic, and proteomic expression data. A transcription factor called POU2F3 has been identified as the upstream regulator of the aforementioned network. POU2F3 binds to a specific octamer DNA motif and regulates cell type-specific differentiation pathways, it is primarily expressed in the epidermis, and it is a candidate tumor suppressor protein. (30) This thesis aims to further investigate the role of this TF in LARC and to set the base for future therapeutic strategies.

2. PURPOSES OF THIS STUDY

The purposes of this study are:

- To evaluate the prognostic value of POU2F3 in an independent cohort of LARC patients.
- To determine *in vitro* whether POU2F3 is targeted by drugs used in clinical practice and whether it causes its up-regulation.
- To evaluate *in vitro* if an enhancing activity exists between 5-Fluorouracil (the standard LARC chemotherapeutic agent) and a chemical compound that upregulates POU2F3.

3. MATERIALS AND METHODS

3.1 Patients

Patients' data were obtained from Genomic Data Commons (GDC) Data Portal, a data-driven platform that allows cancer researchers to search and download cancer data for analysis. (31) A cohort of 172 LARC patients from The Cancer Genome Atlas (TCGA-READ, Rectum Adenocarcinoma) was examined.

3.2 The Cancer Genome Atlas (TCGA) and Genomic Data Commons (GDC)

TCGA is a cancer genomics program that molecularly characterized 33 cancer types, analysing over 20,000 primary tumors and matched normal tissue samples. This landmark project was born in 2006, owing to the joint initiative of NCI (National Cancer Institute) and NHGRI (National Human Genome Research Institute), and included cancers with poor prognosis and overall public health impact, such as Colorectal Adenocarcinoma. Over the next dozen years, TGCA generated over 2.5×10^{15} bytes of genomic, epigenomic, transcriptomic, and proteomic data. In 2016, NCI launched the Genomic Data Commons, a unified data system that hosts TCGA and genomic data from other programs and studies, publicly available in the GDC portal. (32) Prognostic value of POU2F3 in the TCGA-READ cohort was investigated using KMPlotter pan-cancer selecting rectal adenocarcinoma only. (33)

3.3 Cell maintenance and expansion

Three human colon adenocarcinoma cell lines, HCT-15, HCT-116, and SW480 were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% L-Glutamine and 1% Penicillin/Streptomycin antibiotic solution (Growth medium, [GM]) in a humidified atmosphere at 37 °C in 5 % CO_2. The medium was changed every three days.

3.4 Databases

CTD (34), DrugBank (https://go.drugbank.com) and DrugTargetCommons (35) were used to identify possible drugs targeting and up-regulating POU2F3.

3.5 RNA isolation

HCT-15, HCT-116 and SW480 cells were seeded in a 24-well tissue culture plates at 3×10^4 cells/well. After 24 h, cells were washed three times in 1× PBS (Phosphate Buffered Saline) and incubated with nadolol (Merck) and entinostat (MS-275, Merck) both at concentration of 50 μM (this concentration has been chosen as it did not cause cytotoxicity). After 6 and 12 h of continuous drug exposure, the medium was aspirated, and total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of RNAs were determined by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The total RNA was considered suitable for the qPCR analysis if the following requirements were met: (1) the optical density (OD) ratio at 260 nm/280 nm was between 1.9 and 2.1; (2) the concentration was higher than 100 ng/μL.

3.6 Reverse transcription and quantitative real-time PCR (qPCR)

cDNA was synthesized from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol by the VerityTM 96-well Thermal Cycler instrument. qPCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystem) with HPRT1 gene as endogenous control. The amplification reaction was conducted in a final volume of 20 µl using 4 µl of cDNA, TaqMan ® Universal PCR Master Mix IX (Applied Biosystems) and a specific TaqMan ® Gene Expression Assay IX (Applied Biosystems): Hs00205009_ml for POU2F3. The thermal condition included one cycle at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60° for 1 min. Each example was run in duplicate and the threshold cycle (Ct) average was used for the calculations. The results from each sample were compared against one cDNA sample as a calibrator,

using the 2-ΔΔCt calculation method. The fold change was expressed as normalized relative quantification (nRQ).

3.7 Drug treatment and cytotoxicity assay

HCT-15, HCT-116, and SW480 cells were seeded in a 24-well tissue culture plates at 3 x 10^4 cells/well. After 24 h, cells were incubated with nadolol or entinostat for 6 and 12 h. At every time point medium was removed, cells were washed twice with 1 x PBS and trypsinazed for RNA extraction and gene expression analysis. In parallel, the same cell lines were seeded in a 96-well tissue culture plates at 1 x 10^4 cells/well. After 24 h, cells were incubated with 5-Fluorouracil (5-FU), entinostat and entinostat combined with 5-FU (entinostat concentration was kept constant at 100 μM and 5-FU concentration was modulated in the previous range). After 72 h of continuous drug exposure, the medium was aspirated from the wells, and 20 μl of resazurin dye (Abcam) were added to the wells and incubated for 2 hours. Fluorescence was read at 530/590 nm using the Tecan Microplate Reader Spark (Tecan LifeScience). Cytotoxicity was determined as the percentage of fluorescence in exposed cells compared to the untreated cells. The Inhibitory Concentration 50% (IC50) index was used to indicate the drug cytotoxicity calculated using GraphPad Prism v.6 software (GraphPad Software, San Diego, CA, USA).

3.8 Statistical analysis

All graphs and statistical analysis were performed using GraphPad Prism Software v.6. Data are expressed as means ± SD. For comparison between coupled experimental groups, two-sided Student’s t-tests (for parametric dataset) and Mann-Whitney test (for non-parametric dataset) were used. One-way ANOVA with Bonferroni's post-test (for parametric dataset) and Kruskal-Wallis test with Dunn’s post-test (for non-parametric dataset) were performed for multiple comparisons. A p-value<0.05 was considered statistically significant (*: p-value <0.05; **: p-value <0.01; ***: p-value <0.001; ****: p-value <0.0001).

4. RESULTS

4.1 POU2F3 and survival in LARC patients

As POU2F3 has been identified in the NIB Lab as a key factor in LARC response to pCRT, and since responsiveness to therapy is usually correlated with an increased patients' survival, we investigated the association between POU2F3 expression and overall survival (OS) in LARC patients. For this purpose, 172 patients from the TCGA-READ cohort were stratified according to the median POU2F3 expression level in high-level or low-level group. Kaplan-Meier analysis (Figure 5) showed that high POU2F3-expressing LARC patients have a significantly higher OS compared to low POU2F3-expressing patients (HR of 0,34, interval 0,13-0,91; p-value = 0.024).

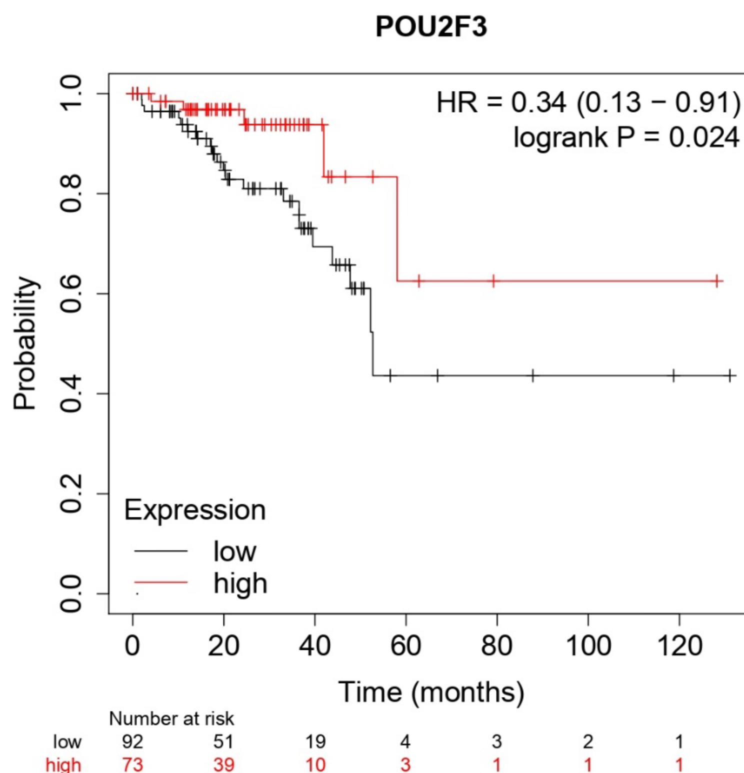


Figure 5: *Prognostic analysis of POU2F3 in LARC patients of TCGA cohort. Kaplan-Meier curves for OS of patients in the high- and low-expression groups (HR of 0,34, interval 0,13-0,91; p-value = 0.024).*

4.2 Targeting POU2F3 as an upstream regulator

As POU2F3 has shown to be associated with higher OS in LARC patients, we searched for therapeutic strategies able to up-regulate this key gene. To do this, we investigated three independent databases (DrugTargetCommons, DrugBank and CTD): we identified 33 molecules targeting POU2F3, mostly toxic, and 13 compounds that upregulate it. According to PubChem, an open chemistry database at NIH (National Institute of Health), only 5 of them have anticancer activity: pictilisib, belinostat, entinostat, trichostatin A and valproic acid. (36) Among those agents, entinostat (previously known as MS-275) is a class I and IV histone deacetylase inhibitor (HDACi), primarily HDAC 1 and 3, that demonstrated antitumoral activity in multiple preclinical studies. As it was the first HDACi to enter clinical trials and it has shown promising therapeutic potential in both solid and hematologic malignancies, as well as a low toxicity profile, entinostat was chosen as the POU2F3-upregulating compound for the following *in vitro* experiments. (37,38) To strengthen our hypothesis, we looked for a chemical that downregulated POU2F3, but the search in the same three databases has not produced results. Therefore, we conducted the same search for POU2F3 paralogs, POU2F1 and POU2F2. Both POU2F1 and POU2F2 were found to be targeted by nadolol, a non-selective beta-adrenoreceptor blocker, that is as well a substrate of OCT1 and OCT2 (Organic Cation Transporter), respectively encoded by POU2F1 and POU2F2 in liver and kidneys. (39) As paralogs are the result of gene duplication and they can retain the same function, we hypothesized that nadolol targeted POU2F3 as well as POU2F1 and POU2F2. (40) Therefore, we used nadolol as a methodological control, as we evaluated entinostat as an upregulating compound, and nadolol as a downregulating one, analysing gene expression in two opposite directions. Thus, we exposed HCT-15, HCT-116 and SW480 CRC cell lines to both compounds and through qRT-PCR we reported POU2F3 expression in non-treated cells (NT), after 6 h and 12 h from exposition. Cell lines treated with entinostat and nadolol showed respectively a significant up-regulation and down-regulation of POU2F3 compared to the corresponding non-treated cell-lines (p value < 0,0001, < 0,0098 and < 0,001 respectively for entinostat; p-value <0,035, < 0,0001 and < 0,001 respectively for nadolol) (Figure 6). As nadolol down-regulated POU2F3, it was not further considered.

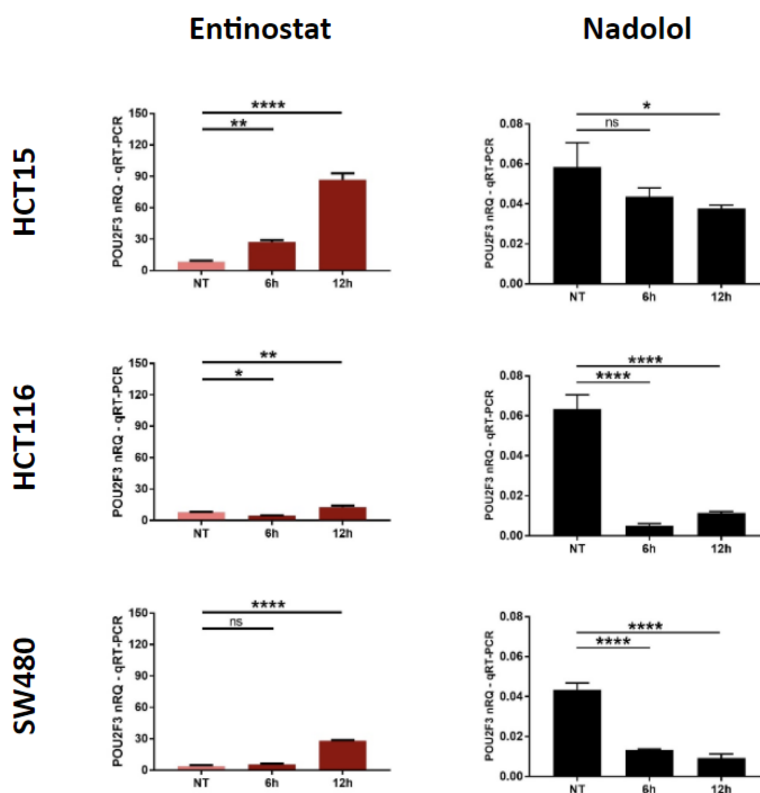


***Figure 6:** Up-regulation of POU2F3 after entinostat treatment (50 µM) in HCT-15, HCT-116 and SW480. nRQ, normalized Relative Quantity. *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001; ****: p-value<0.0001. Data were expressed as means ± SD.*

4.3 Cytotoxicity evaluation of 5-Fluorouracil (5-FU) and entinostat on CRC cell lines

To test the cytotoxicity effect of entinostat and compare it with classic LARC chemotherapeutics, 5-FU was chosen as it represents the backbone of LARC chemotherapy approach. (9) Thus, HCT-15, HCT-116 and SW480 CRC cell lines were either treated with 5-FU or entinostat at 0.1, 1, 10 and 100 µM. As portrayed in Figure 7, 5-FU demonstrated a concentration-dependent cytotoxicity, significantly inhibiting all three CRC cell lines proliferation (****: p-value < 0.0001). IC-50 values calculated from the dose-response curves were 6.935, 8.147 and 10.51 for HCT-15, HCT-116 and SW480 CRC respectively (Figure 8). On the other hand, entinostat showed poor to no cytotoxicity effect, as represented in Figure 7. In HCT-15 cell line, entinostat significantly inhibited cell proliferation

only at the highest concentration (100 μM) (**: p-value<0.01). In HCT-116 cell line no cytotoxicity has been recorded, indeed at 100 μM cells were able to proliferate (*: p-value<0.05). Lastly, in SW480 cell line, 0,1 μM entinostat showed no effect, at 1 and 10 μM cell proliferation has been recorded (*: p-value<0.05 for both concentrations), finally 100 μM entinostat caused a significant cell cytotoxicity (***: p-value<0.001). Remarkably, the IC50 of entinostat was not determined due to the lack of anti-proliferative effect in these cells.

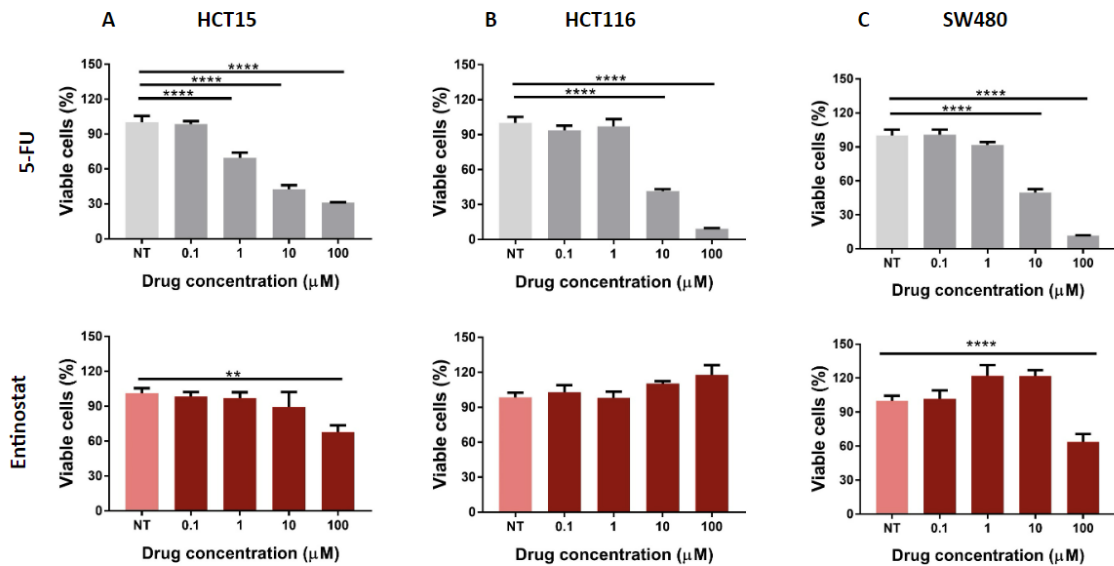
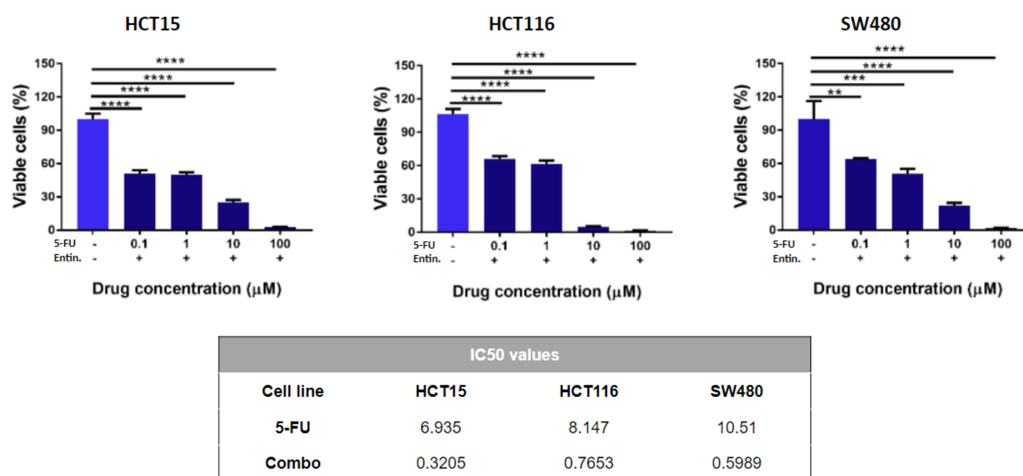


*Figure 7: Dose-response curves of 5-FU (top) and Entinostat (bottom) in drug-exposed HCT-15 (A), HCT-116 (B) and SW480 (C). *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001; ****: p-value<0.0001. Data were expressed as means ± SD.*

4.4 Combining entinostat and 5-FU: a possible enhanced effect?

Next, we investigated the effects of a combined approach with both 5-FU and entinostat on the same three cell lines. To study this pharmacological interaction, we treated CRC cell lines with the four doses of 5-FU discussed in the previous paragraph, in combination with the highest concentration of entinostat evaluated in the single-treatment setup (100 μM), since it was the only to show a significant cytotoxic effect on HCT-15 and SW480. As represented in Figure 8, combination treatment has demonstrated significantly enhanced cell cytotoxicity compared to 5-FU and entinostat alone in all CRC cell lines. IC50 was 0.3205, 0.7653 and 0.5989 for HCT-15, HCT-116 and SW480 respectively, showing a stronger response in the

first cell line. This in vitro experiment suggested that entinostat plays an empowering effect on 5-FU chemotherapy, leading to an enhanced pharmacological activity.



IC50 values			
Cell line	**HCT15**	**HCT116**	**SW480**
5-FU	6.935	8.147	10.51
Combo	0.3205	0.7653	0.5989

Figure 8: *Dose-response curve of 5-FU and entinostat combined. "+" indicates a constant concentration of 100 μM for entinostat. Calculated IC50 index are summarized in the table (as entinostat did not demonstrate a sufficient cytotoxicity in cell lines, relative IC50 is not available for this compound). *: p-value<0.05; **: p-value<0.01; ***: p-value<0.001; ****: p-value<0.0001. Data were expressed as means ± SD.*

5. DISCUSSION

CRC is the third diagnosed cancer and ranks third in terms of cancer-related mortality, accounting for nearly 10% of all cancers and deaths worldwide. (1)

Among CRCs, about 35% are RCs and the majority of them are LARC at diagnosis. (3) At this stage, commonly defined as T3-4 and/or N+, the therapeutic management consists in pCRT with 5-FU regimen followed by surgery. (3,9) However, response to pCRT is extremely variable among patients: only 20% of them achieve a pCR and it has a considerable impact on their 5-year disease-free survival. (3,9) On the other hand, 40% of patients are poor- or non-responding and are then exposed to unnecessary chemotherapy toxicity. (3,19)

Therefore, it is of paramount importance to find novel prognostic biomarkers to predict pCRT response at an early time point, allowing to identify LARC patients who can take advantage of neoadjuvant therapy. (19)

Several studies have been conducted for this purpose, and different predictors of pCRT response have been proposed. (20) However, only a few of them are used in clinical practice as their prognostic potential has various limitations. (26) Cancer is an extremely complex disease and ignoring the intricacy of its underlying molecular mechanisms could lead to wrong assumptions. To date there are numerous single-omics approaches, investigating how distinct molecular layers contribute to the development of various diseases. Nevertheless, none of these strategies can clearly identify the interplay between the aforementioned levels. (29) To fill this gap, the NanoInspired Laboratory of Padova has employed a multi-omic approach, simultaneously investigating the transcriptomic, post-transcriptomic and proteomic fields of RC. As a result, POU2F3 transcription factor has been identified as a potential hidden player in LARC molecular network.

To further investigate the role of this TF in LARC, we demonstrated that an increased expression of POU2F3 in the TCGA cohort correlates with a higher OS. POU2F3 (POU class 2 homeobox 3; also known as SKN-1a/OCT-11) is a TF encoded by the homonymous gene, it is required for the generation of Tuft cells, a rare chemosensory cell type found in the gastrointestinal and respiratory tract.

(30,37) Like neuroendocrine cells, Tuft cells respond to external stimuli by releasing bioactive substances to regulate local epithelial and immune cell functions. (37) It has been shown that POU2F3 is involved in different types of tumors, e.g. small cell lung cancer (37), CRC (38), cervical cancer (40) and cutaneous squamous cell carcinoma (41). Furthermore, in some of these malignancies Tuft-cell like cancer cells have been identified. (37,38)

Lastly, we searched for chemical compounds able to up-regulate POU2F3 expression, as we demonstrated that high POU2F3 levels are correlated to better prognosis. We investigated three different databases and we proposed entinostat as a novel pre-operative therapy in combination with 5-FU, the backbone of LARC chemotherapy. (9) Entinostat (MS-275) is a synthetic benzamide derivative and a HDAC-inhibitor; as HDAC expression and histone hypoacetylation have been noted in tumors in the setting of transcriptional repression of genes, those compounds have been investigated as therapeutic agents in cancer. Entinostat leads to inhibition of cell-proliferation, terminal differentiation and apoptosis and its selectivity for class I and IV HDACs results in better safety and efficacy profiles compared with non-selective pan-HDAC inhibitors. (42) Entinostat has demonstrated promising antitumor activity both *in vitro* and *in vivo* models of human malignancy and it has been evaluated in multiple trials as a therapy for advanced and/or refractory solid tumors (e.g. melanoma, lung and breast cancer (43)) and haematological malignancies. (42,44)

In accordance with our data, previous works have demonstrated promising findings when combining entinostat to chemotherapeutics. Marx et al. showed that the combined application of entinostat with irinotecan, a topoisomerase I inhibitor, synergistically kills CRC cells *in vitro* and *in vivo*, triggering mitochondrial damage and apoptosis. (45) Moreover, Flis et al. evaluated the effects of entinostat and another HDACi (suberic bishydroxamate) in combination with 5-FU on human CRC cell lines (SW48, HT-29, Colo-205). The obtained results indicated that HDACi, especially entinostat, synergistically potentiated cytotoxic effects of the classical cytostatic agent 5-FU. The authors proposed that synergism between the two drugs is related to the increase of apoptotic signal. (46) In our study, we took a step forward evaluating the underlying molecular mechanism of entinostat's enhancing effect on 5-FU cytotoxicity. The correlation of POU2F3 with better

survival together with entinostat-driven upregulation of POU2F3 highlights a fundamental role of this gene in LARC prognosis and response to therapy.

Entinostat is a promising future treatment option for cancer. It is administered orally and systemically distributed and it also displays an extremely favourable toxicity profile. The most frequent adverse events consist of fatigue, nausea and electrolyte disturbances. All of these are easily correctable or reversible. (44) On the other hand, 5-FU is an antimetabolite administered intravenously, it is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen and it exerts its anticancer effects through the inhibition of thymidylate synthase and incorporation of its metabolites into RNA and DNA. 5-FU is widely used for the treatment of a variety of cancers, including colorectal, breast, head and neck malignancies. (47,48) 5-FU has many adverse effects when used systemically. The most common adverse event according to FDA Adverse Event Reporting System (AERS) is diarrhea. Other common adverse effects include vomiting, nausea, and dehydration. More concerning side effects include neutropenia, pyrexia, pulmonary embolism, thrombocytopenia, and leukopenia. (48) In addition, infusion administration causes infections, bleeding and thromboembolism, as a consequence, research has moved forward in the study of oral fluoropyrimidines, e.g. the pro-drug capecitabine. (48,49)

According to AIOM 2021 guidelines, 5-FU and capecitabine are the two recommended drugs for LARC chemoradiotherapy. In greater detail, the most common treatment of non-metastatic resectable rectal cancer (stage II-III: cT3-T4a N0 or every T cN+) consists of pCRT, TME within 6-8 weeks from the end of pCRT, then different adjuvant chemotherapy regimens based on restaging (ypTNM). The duration of treatment (pre- and post-operative) must be six months. If cancer is localized in the high rectum and it is staged as cT3 early (infiltration of mesorectum < 2 mm) cN0, TME with no pCRT (TME upfront) can be considered. Staging with high-quality RMN and a highly experienced surgeon are mandatory in this case. On the other hand, non-metastatic non-resectable tumors (cT4b) are first treated with CRT with or without induction chemotherapy, then, if resecability is achieved, patients undergo surgery. Patients who achieve complete clinical response (cCR) after neoadjuvant therapy can be addressed to simple observation, an approach called Non-Operative Management (NOM). The potential advantage

of NOM is the possibility to avoid surgical morbidities without affecting the oncological outcome. To date, this method is still discussed as the rate of local recurrences after NOM is between 20-30% and rescue surgery is hardly applicable unless the patient is strictly monitored with follow-up. (4)

Overall, the current LARC management has many downsides: fluoropyrimidines have a severe toxicity profile and TME can lead to post-operative morbidities and need of a permanent stoma, resulting in a significant reduction in quality of life. First, a novel pCRT response biomarker could spare non-responding patients from chemoradiotherapy toxicities, and evaluation of POU2F3 expression seems to be a viable solution. Second, the enhancing effect of entinostat on the classical 5-FU chemotherapy paves the way for new pCRT regimens. Combined to entinostat, lower doses of 5-FU may be sufficient, leading to fewer side effects. Similarly, it may be possible to reduce the number of chemotherapy cycles in the preoperative. Moreover, the empowered cytotoxic effect of combination therapy could lead to a greater pCRT response, increasing the chances of NOM for patients.

We are aware that this study has limitations. We studied *in vitro* the pharmacological response of RC to 5-FU and entinostat, and we based our experiments on CRC cell lines. Pre-clinical cancer research is usually performed in two-dimensional (2D) in vitro cancer cell culture models. However, it is increasingly acknowledged that 2D models do not provide an accurate *in vivo* phenotype. Cells grown in 2D lack the *in vivo* 3D tissue architecture and cell-cell interactions, essential for maintaining intracellular function and polarity. Culturing cells in 2D on hard plastic or glass is not replicative of the *in vivo* growth of cells, where the ECM maintains cell differentiation, normal growth, and homeostasis. Conversely, cells have an artificial polarity due to their orientation on the plastic surface and they are unable to migrate in response to chemical signals. Consequently, they often fail to adequately model normal tissue and disease. (50,51)

Before drugs can be tested in human clinical trials, they must first undergo pre-clinical testing in animals. As such, our results are still limited and further investigations could be conducted *in vivo* to accurately characterize POU2F3 expression and LARC response to entinostat, 5-FU and combination therapy.

Animal models provide a controllable experimental environment with complexities similar to those observed within human cells and organs. However, it is widely recognised that the use of animals in drug research is far from ideal. Such models are expensive and limited by availability, feasibility and ethical issues. (51) As a consequence, to overcome the need of animal testing, in the past decades significant efforts have been made towards the development of three-dimensional cancer models, such as organoids. (52) Organoids are the most studied 3D patient-derived models, several culture methods have been established for healthy and diseased tissues from oesophagus, stomach, intestine, pancreas, bile duct and liver, and other non-gastrointestinal tissues. Because organoids can be generated with high efficiency and speed (14 days), they can serve as a personal cancer model. Samples are obtained from fine-needle aspirations, biopsies or resection specimens. For digestive tract organoids, tissue is either digested to release the crypts, subsequently plated in a basement membrane extract, and overlaid with organoid growth medium. When fully grown, organoids form hollow spheres with an outer layer of cells, an inner lumen and organ-like properties. (53,54) A different 3D CRC model has been obtained in the NIB Lab of Padova based on the essential role of ECM in cancer discussed in the introduction section. Decellularized patient-derived ECM and CRC cell lines (HCT-29 and HCT-116) have been combined to obtain a 3D construct, that organized in a rounded configuration characteristic of dysplastic colic crypts. After cytotoxicity assay with 5-FU, this model mimicked the effects observed in vivo in a xenogenic zebrafish model, therefore it could be a reliable preclinical patient-specific platform to bridge the gap between in vitro and in vivo drug testing and provide effective cancer treatment. (52)

6. CONCLUSIONS

In conclusion, we demonstrated a key role of POU2F3 in prognosis and treatment of LARC patients. First, POU2F3 correlates with better OS in LARC patients, and it represents a promising biomarker of pCRT response. Dosing POU2F3 in clinical practice could identify patients that can truly benefit from pCRT and spare non-responders to unnecessary toxicities and surgery delays. Moreover, POU2F3 is a promising therapeutic target for LARC, and we have therefore identified a chemical compound that could upregulate this pivotal transcription factor: the histone deacetylase inhibitor entinostat. We conducted *in vitro* experiments on CRC cell lines to test entinostat's effect on cells, we compared it to 5-FU, a classic LARC chemotherapeutic agent, then we combined both chemicals. These assays suggested an empowering effect of entinostat on the classical 5-FU chemotherapy.

We are still far away from introducing these findings into everyday medicine. Further investigations are needed to better understand the potential of POU2F3 in LARC prognosis and to use it as a biomarker in clinical practice, as well as entinostat and 5-FU combination therapy needs validation in clinical trials. However, we are confident that our results will pave the way for new prognostic-therapeutic approaches to LARC.

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