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Clinical Management of Colon Cancer

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1. **INTRODUTION**

According to the Global Burden of Disease Cancer Collaboration, colorectal cancer is the third cancer incidence globally and the second most common in terms of mortality, for both sexes combined, in western countries [1](Fig.1).

The therapeutic management of these oncologic pathologies is, for different reasons, extremely important. While specific causes of CRC insurgence have not been identified, different factors have been implicated in colorectal tumorigenesis, including genetic background, age, and different lifestyle factors, such as alcohol consumption [2], smoking [3], obesity [4], physical inactivity [5], and a dietary regimen comprising low fiber intake and high consumption of red/processed meat [6,7].

Despite improvements in treatment, the prognosis of CRC remains unfavorable. Although several important side effects can occur, negatively affecting patient quality of life [13], radiotherapy has been reported to significantly promote the downsizing and downstaging of large CRCs in neoadjuvant settings [14]. Notably, only a limited number of patients affected by CRC respond positively to a neoadjuvant treatment regimen, likely due to tumor heterogeneity.

Cancer Stem Cells (CSCs) are a small subset of cells that possess self-renewal potential and persistent tumorigenic capacity. Indeed, it has been widely demonstrated that CSCs are able to initiate and sustain tumor growth and, moreover, to recapitulate cancer cell heterogeneity. The existence of CSCs was first demonstrated in acute myeloid leukemia [15] and was successively proved in other hematological and solid tumors [16–25]. CSCs normally represent 0.1–10% of all tumor cells, and their identification is based on the expression of specific surface markers [26,27]. CSCs from colon cancer have been widely studied thanks to the identification of specific markers, including Lgr5 [28–30], CD133 [22,31], and CD44 [32,33]. In such studies, CSCs were shown to be responsible for tumor maintenance and propagation upon xenotransplantation [22,34,35].

Conventional anti-cancer therapies target only differentiated and fast proliferating cells, while sparing CSCs that are quiescent and undifferentiated. For this reason, CSCs are often drug-resistant, leading to tumor recurrence and metastasis [36]. Consequently, CSCs represent the primary therapeutic targets to abrogate the minimal residual disease and impede the reappearance of tumor lesions.

In this study, we developed an *in vitro* and *in vivo* model of radiotherapy, based on patient-derived CSCs, for the prediction of treatment efficacy to support clinical decisions. Our data indicate a concordance between *in vitro* and *in vivo* CSC sensitivity to radiotherapy, suggesting that the CSC model may be sufficient to assess the suitability of therapeutic regimens, thus providing a feasible translational approach for the prediction of efficacy. The proposed approach may be useful for the

timely identification of resistant patients who can be spared from side effects of ineffective radiotherapy, thereby supporting more appropriate clinical decisions for therapeutic options.

Regione	Nazione	Mammella	Trachea, Bronchi e Polmone	Colon Retto	Prostata	Stomaco	Fegato	Cervice	Linfoma Non-Hodgkin	Esofago	Leucemia	Labbra E Cavità Orale	Vescica	Utero	Pancreas	Cervello	Rene	Melanoma	Ovaio	Tiroide	Cistifellea	Laringe	Altri Tumori Della Faringe	Mieloma Multiplo	Linfoma Di Hodgkin	Rinofaringe	Testicolo	Mesotelioma
Globale		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Paesi sviluppati		3	4	2	1	5	11	17	7	20	12	14	6	13	8	16	10	9	15	18	19	22	23	21	24	27	25	26
Paesi in via di sviluppo		1	2	4	6	3	5	7	11	8	10	9	16	13	14	12	20	23	18	15	19	17	21	25	24	22	26	27
Asia del Pacifico (High-Income)	Giappone	4	2	1	6	3	5	17	10	11	13	14	9	18	7	22	12	23	15	16	8	21	20	19	27	26	25	24
	Corea del Sud	5	2	1	8	3	4	13	10	15	14	18	11	19	9	16	12	23	17	6	7	21	22	20	25	24	27	26
Nord America (High-Income)	Canada	4	3	2	1	12	18	20	5	19	10	14	6	8	11	13	9	7	15	17	21	22	23	16	24	27	25	26
	USA	2	3	4	1	13	18	20	7	19	11	12	6	9	10	17	8	5	15	14	23	21	22	16	24	27	25	26
America Latina del sud	Argentina	1	4	3	2	5	18	6	8	14	12	16	10	7	9	19	11	15	17	21	13	20	25	23	24	26	22	27
Europa Occidentale	Francia	3	4	2	1	10	14	19	6	18	13	12	5	7	11	16	9	8	17	22	23	21	15	20	24	27	25	26
	Germania	3	4	2	1	6	16	21	9	18	11	15	5	12	8	14	7	10	13	22	17	23	19	20	26	27	24	25
	Italia	2	4	1	3	6	8	21	7	22	11	19	5	12	9	14	10	13	15	18	16	20	24	17	25	27	26	23
	Spagna	4	3	1	2	6	11	20	7	23	12	13	5	8	9	15	10	14	16	22	19	17	21	18	24	27	25	26
	UK	4	3	2	1	7	18	19	6	12	11	17	5	14	9	15	10	8	13	24	23	20	22	16	26	27	25	21
Asia Centrale	Uzbekistan	1	3	8	11	2	9	4	12	5	6	10	20	13	16	7	17	18	19	24	21	14	23	25	15	26	22	27
Europa Centrale	Polonia	3	1	2	4	6	19	11	14	20	12	16	5	9	8	13	7	17	10	21	18	15	23	22	25	26	24	27
Europa dell'Est	Russia	3	2	1	5	4	17	9	14	19	12	11	8	10	7	18	6	16	13	15	21	20	22	24	23	26	25	27
	Ucraina	2	3	1	4	5	21	9	15	20	11	8	10	17	7	14	6	13	12	16	19	18	22	24	23	27	26	25
America Latina Andina	Perù	3	6	4	1	2	9	5	7	21	8	18	20	10	12	15	14	17	16	11	13	24	25	19	23	27	22	26
America Latina Centrale	Colombia	2	6	4	1	3	10	5	9	17	7	16	23	13	12	11	20	18	14	8	15	19	25	22	21	26	24	27
	Messico	2	7	3	1	5	10	4	9	22	8	18	24	16	12	14	11	20	13	6	17	19	25	23	21	27	15	26
	Venezuela	2	4	5	1	6	13	3	7	19	8	16	22	10	11	17	12	20	15	9	18	14	25	21	23	26	24	27
America Latina Tropicale	Brasile	2	4	3	1	5	17	6	11	14	8	10	21	16	12	7	18	13	20	9	22	15	19	23	24	26	25	27
Asia Est	Cina	5	1	4	9	2	3	12	11	6	8	19	14	7	13	10	20	26	21	17	18	16	25	24	22	15	27	23
	Corea del Nord	4	1	5	11	3	2	7	12	6	9	14	17	8	13	10	22	23	21	20	19	16	25	24	18	15	27	26
Sud-est Asiatico	Indonesia	1	2	4	7	5	10	3	8	21	11	6	18	13	15	12	19	23	14	9	16	20	22	25	24	17	26	27
	Malesia	1	2	3	4	7	6	10	5	19	8	14	15	12	17	18	16	22	11	9	23	20	21	25	24	13	26	27
	Birmania	1	2	5	11	12	4	3	9	17	7	6	22	8	15	14	23	24	10	13	16	19	20	25	21	18	26	27
	Filippine	1	2	4	3	12	7	5	11	23	6	10	20	9	15	14	18	21	13	8	19	17	22	26	25	16	24	27
	Tailandia	3	1	4	5	7	2	6	10	19	11	8	13	17	16	14	18	23	15	12	9	20	21	26	25	22	24	27
	Vietnam	4	2	5	12	3	1	8	6	10	11	7	18	13	17	9	21	25	20	14	19	15	16	26	24	22	23	27
Asia del Sud	Afghanistan	3	2	8	10	1	9	4	12	17	5	14	11	13	18	6	20	23	22	16	21	15	25	24	7	19	26	27
	Bangladesh	3	4	7	11	6	2	8	5	10	9	1	19	17	22	14	23	24	12	26	18	15	13	25	20	16	21	27
	India	1	6	4	15	5	8	3	11	7	10	2	19	24	22	12	21	16	14	17	20	13	9	25	18	23	26	27
	Nepal	1	4	6	13	7	9	3	12	5	8	2	21	22	17	14	23	25	11	20	16	15	10	24	18	19	26	27
	Pakistan	1	3	6	8	13	12	15	5	4	7	2	9	17	24	14	22	25	11	18	20	10	16	23	19	21	26	27
Nord Africa e Medio Oriente	Algeria	1	3	2	10	4	15	7	6	23	5	21	14	20	16	8	22	24	17	12	9	18	25	19	13	11	26	27
	Egitto	1	6	7	3	9	2	11	13	20	4	14	8	15	10	5	16	23	19	12	18	17	24	25	22	26	21	27
	Iran	2	5	6	3	1	9	15	10	4	7	14	12	24	17	8	18	21	19	13	16	11	26	23	20	25	22	27
	Iraq	1	2	5	8	6	7	10	11	19	3	15	14	9	12	4	16	26	17	13	20	18	24	23	21	25	22	27
	Marocco	1	2	6	3	5	9	4	10	20	8	13	15	11	12	7	21	23	17	16	14	19	25	26	18	22	24	27
	Arabia Saudita	1	4	2	6	9	3	16	5	17	8	13	14	22	11	7	15	26	18	10	12	19	21	23	24	25	20	27
	Sudan	1	2	4	5	3	8	11	9	15	6	14	10	18	17	7	19	22	20	12	21	16	26	24	13	23	25	27
	Turchia	2	1	3	4	5	13	18	7	24	6	20	10	11	9	8	16	22	14	12	19	15	27	21	23	25	17	26
	Yemen	1	2	5	8	3	9	6	10	19	4	15	11	12	18	7	22	23	20	13	16	17	26	24	14	21	25	27
Africa Centrale sub-sahariana	Congo	1	7	5	3	4	6	2	9	8	11	10	16	17	15	12	19	14	20	24	18	21	23	22	13	25	26	27
Africa sub-sahariana dell'est	Etiopia	1	8	4	3	7	6	2	9	5	18	11	21	15	16	10	12	13	19	22	17	25	24	20	14	26	23	27
	Kenya	1	10	5	4	6	9	3	8	2	14	7	15	23	13	12	20	18	11	17	24	16	25	19	21	22	26	27
	Mozambico	2	7	4	1	5	6	3	8	10	14	11	19	18	16	9	13	15	20	25	17	24	23	21	12	26	22	27
	Tanzania	1	10	4	2	8	5	3	7	6	14	11	21	18	17	9	13	15	19	23	16	25	24	20	12	26	22	27
	Uganda	3	9	6	1	8	7	2	4	5	12	11	16	15	19	21	18	20	10	13	25	24	22	23	14	17	26	27
Africa sub-sahariana sud	Sudafrica	2	4	3	1	10	11	6	7	5	13	8	15	14	9	21	17	12	16	20	23	18	22	19	24	27	25	26
Africa sub-sahariana ovest	Ghana	3	10	5	1	6	4	2	8	13	12	17	15	7	9	11	20	19	14	21	18	25	26	22	16	24	23	27
	Nigeria	3	9	5	4	6	1	2	7	16	11	17	13	8	12	14	18	20	10	21	19	23	25	22	15	26	24	27

Figure 1 Most frequent tumors incidence in western countries

The other objective of this study is to identify prognostic factors related to adjuvant therapy in colon stage III tumors, in order to guide the therapeutic decision after surgery. The proposed strategy is to identify variations in molecular profiles detected in circulating Free DNA before and after surgery.

In this study we want to address the questions in the clinical management of cancer both in healthy subjects that carry an increased genetic risk for cancer as well as in subjects that already had a primary tumor. The area of intervention addresses recurrent tumors or metastasis of a previous tumor.

Early diagnosis is one of the most qualifying targets of innovative intervention in cancer management because it is linked to a significant increase in the likelihood of disease-free survival.

The concept liquid biopsy includes the analysis of circulating nucleic acids (DNA, RNA and miRNA), the analysis of exosomes and of Circulating Tumor Cells (CTC) which are thought to represent the tumor population responsible for relapse. The advantage of liquid biopsy over conventional diagnostic methods is due to its not invasive thus permitting monitoring the tumor progression, which is responsible for the therapeutic failure in cancer patients, over time. This line of research will address the question of appropriate second line therapy and beyond in colorectal tumors.

Another characteristic of liquid biopsy is the fact that the biological material collected can be analyzed with a variety of methods in order to identify the appropriate biomarker. For example, one can analyze circulating cell free DNA (cfDNA) for genomic alterations for the design of the best therapy. Other biomarkers present in liquid biopsies are circulating miRNA. An innovative approach is represented by the isolation of circulating exosomes from blood which contain DNA, RNA, miRNA and proteins which can all be profiled for biomarker detection. Finally, although only applicable to diagnosis at relapse, are Circulating Tumor Cells (CTC) which are thought to represent the population of the tumor which is responsible for relapse and/or metastasis.

Since liquid biopsy is non-invasiveness and can be applied repeatedly over longer time periods, it represents at the moment the only alternative method to current instrument-based diagnostic approaches used for the early detection of primary tumors as well as recurrent tumors. Hence the need to determine the molecular profile of the tumor that is to be cured through a molecular diagnostic approach that comes alongside traditional histopathological diagnostics.

However, in the face of these positive findings, it should be noted that the success of these new therapies is often mitigated by the onset of resistance phenomena, due to an intrinsic heterogeneity of the tumor composition that may contain cells with mutant variants that still support tumor growth but are no longer target-sensitive to previously used drugs. To cope with the challenge posed by this dynamic evolution of the cancer molecular profile useful to define appropriate therapy is choice liquid

biopsy to monitor molecular changes (and modularize therapeutic possibilities) throughout the course of the disease.

This study proposes an innovative solution, liquid biopsy, which should allow to improve the diagnostic results obtained by traditional instrumental diagnostics. Furthermore, the molecular characterization of the tumor, also at time of relapse, will allow a personalized and timely approach to therapy, improving quality of life and hopefully prolonging overall survival of patients.

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the third most common form of cancer and the second cause of cancer-related death in the Western world. Despite the development of new targeted agents and the use of different therapeutic combinations, the treatment options available are not all curative in patients with advanced cancer. Recently, several studies are supporting the idea that human cancers can be considered the stem cell disease. According to the cancer stem cell model, malignancies originate from a small fraction of cancer cells that display self-renewal and are capable of initiating and supporting tumor growth [37]. Experimental evidences suggested that tumors may be organized as a heterogeneous cell population having different self-renewal capacities, grades of differentiation, and clonogenic potentials. [38].

1.2 Cancer Stem Cell

A large number of recent studies investigated the role of a subpopulation of tumoral cells “cancer stem cells” (CSCs), which possess a limitless proliferative potential and the ability to reproduce the original human tumor in an *in vivo* model. This small population is responsible for tumor initiation, progression and diffusion. They have an asymmetric replicative modality: cellular division leads to the formation of two distinct cells, one retaining the parenteral phenotype and one destinated to differentiation. Stem cells are undifferentiated cells well-defined by an unlimited potential for self-renewal, multi-lineage differentiation, and long-life. Self-renewal is the ability to divide and produce a precise copy of itself [39].

The existence of CSCs was proved first in acute myeloid leukemia and thereafter in other solid tumors including breast, colon, brain, prostate, ovarian and melanoma [40-45].

Many recent studies showed that targeting cancer stem cells (CSCs) is a promising therapeutic approach, due to their ability to initiate and sustain tumor growth and to generate the heterogeneous cell population forming the entire tumor. The biology of CSCs is closely associated with

tumorigenesis and therapeutic resistance. Indeed, conventional anti-cancer therapies, which are able to kill the majority of differentiated tumor cells, may spare CSCs which remain unaffected and may be responsible for tumor recurrence and progression. Hence, CSCs represent the primary therapeutic target for complete tumor eradication. In this study we will propose a model for the prediction of response to radiotherapy in rectal cancer using *in vitro* irradiation of patient-derived CSCs.

1.3 Colonrecta Cancer Resistance

Conventional treatments for colon rectal cancer are surgery, radiotherapy and chemotherapy. Resistance of CRC to radiotherapy and chemotherapy is a major relevant cause of treatment failure and death. Radiotherapy and chemotherapy can efficiently kill more differentiated cell in a mass, but have partial effects on CSCs. In fact, colorectal CSCs are generally resistant to radio- and chemotherapy [51–55]]. CSCs might enter in the proliferating cell cycle, but be quiescent in the G_0 phase and consequently these cells are resistant to radio- and chemotherapy [56]. CSCs have capacity of DNA damage repair and are resistant to DNA-damaging radiation [57]. Therefore, CSCs arise high concentration of anti-apoptotic proteins, as Bcl-2 family and apoptotic inhibitors [58].

Radiotherapy is the most important treatment for CRC, causing cancer cells death through DNA damage by ionizing radiation. Colorectal CSCs shown a properties, such as upregulated anti-apoptotic proteins and thus are radioresistant [59, 60]. This mechanism is due to DNA damage repair, cell cycle activity reduction, elevated ROS inhibitors, stimulation of survival pathways, protein kinase C δ signaling pathways, e.g., c-Jun N-terminal kinase and Notch [51, 61–63]. The radiation can induce cancer cells to gain the phenotypes and role of CSCs [64]. Colorectal CSCs may be a significant aspect that promotes CRC relapse.

The CSCs endure the chemotherapeutic agent and radiotherapy and survive for their high tumorigenicity, a small portion of CSCs in the quiescent status return into cell cycle for proliferation [65].

The aim of Radiotherapy Treatment in CRC is to reduce the number of relapse. Many RT regimens with different plans of fractionation have been used but two different courses are used. Moreover, a conventionally doses of 2 Gray x 25days with delayed surgery most frequently combined with chemotherapy. The other view is a short doses 5 Gray for 5 days.

1.3 Colon Cancer and Liquid Biopsy

The genetic molecular analysis of colon cancer has been addressed to the identification of different genes involved in the progression of tumor. The dysregulation of some pathways involved in the cancer progression have an important prognostic and predictive role.

Tissue biopsy is the gold standard for tumor analysis and the histological analysis is the source for pathological diagnosis of cancer; these valuations are not sufficient to predict the treatment response and the development of the disease.

For patient management and therapies decisions, the valuation of cancer genetic alterations is decisive in the progresses of precision medicine [66]. The evaluation of the mutational status of cancer is strongly needed for targeted therapies.

There are important limitations to use of tissue specimen in clinical setting, tumor heterogeneity, the patient compliance, clinical risks and surgical complications, due to multiple or serial biopsies, are often impractical [67].

A limitation of the tissue biopsy is the impossibility of obtaining tissue samples repeated over time. Furthermore, the presence of drug resistance is also impossible to manage with the only tissue biopsy [68-69]

Despite tissue biopsy represents the gold standard, for the valuation of clinical biomarkers mutational status there are valid alternative approaches. Numerous studies have recently shown how the liquid biopsy in oncology field can be a potential new alternative to traditional tissue biopsy.

Liquid biopsy is considered a minimally invasive and repeatable test that represents an appropriate method to shown the tumor mutation over time. Serial blood extraction can be simply obtained for analysis mutation pattern at different time points [70,71]. The liquid biopsy provides the prospect of earlier therapeutic intervention.

Another application of liquid biopsy could be tumor detection using a simple blood test for screening a healthy population [69]. Liquid biopsy is considered a non-invasive approach that offers more precise representations of diseases biology [71].

The Next Generation Sequencing (NGS) is the most innovative technique that makes possible to use liquid biopsy and could be useful to achieve the goal of precision cancer medicine [72].

The liquid biopsy is based on isolation and analysis of material derived from the tumor. This represents a minimally invasive instrument that offers the possibility of capturing the cancer molecular properties and its evolution [73].

The tumoral components include circulating tumor cells (CTCs), the exosomes, subcellular particles, extracellular membrane-encased vesicles that contain tumor-specific proteins and nucleic acids. Circulating cell-free nucleic acid derived from tumor cells containing microRNAs (miRNAs), non-coding RNA, cfRNA (less stable), and cfDNA [69].

CfDNA is present in the bloodstream as double-stranded fragments of about 150 to 200 base pairs. In cancer patients, the cfDNA is released from tumor cells as circulating tumor DNA (ctDNA); it represents the only 0.1% of the overall cfDNA [70]. The ctDNA present different characteristics from cfDNA, such as methylation, copy number variations, variants and amplifications or deletions associated with tumor [74].

2. AIM OF STUDY

The purpose of my PhD project is to find a strategy for clinical management of colon cancer patients.

The first aim of this study is to develop an *in vitro* and *in vivo* model of radiotherapy, based on CRC patient-derived CSCs, for the prediction of treatment efficacy to support clinical decisions. (Extracted from: "***Radiosensitivity of cancer stem cells has a potential predictive value for individual responses to radiotherapy in locally advanced rectal cancer". Caterina Puglisi et all.*****Cancers (Basel). 2020 Dec 7;12(12):3672. doi: 10.3390/cancers12123672.)**

The second purpose of this study is to identify prognostic factors related to ~~adjuvant~~ therapy in colon stage III tumors, in order to guide the therapeutic decision after surgery.

The proposed strategy is to identify variations in molecular profiles detected in circulating cfDNA before and after surgery.

3.MATERIALS AND METHODS (first object)

3.1 Patient enrolment and primary human tumor collection

Newly diagnosed T3 or T4 colorectal cancer patients who were eligible for neo-adjuvant radiotherapy and subsequent surgical resection were enrolled at the Mediterranean Institute of Oncology (IOM).

Rectal cancer biopsy samples were obtained before the administration of neo-adjuvant radiotherapy. Eight weeks after neo-adjuvant radiotherapy, the individual responses has been evaluated via CT-scan and Magnetic Resonance, and the patients were classified.

Table 1 reports the clinical characteristics of the involved patients. No evident correlation between the clinical characteristics and the ability to generate growing cultures was observed.

CRC line	AGE	SEX	DIAGNOSIS	T	N	M	STAGE	TREATMENT	TRG	MUTATION STATUS		
										KRAS	NRAS	BRAF
1	60	M	Adenocarcinoma	3	2	X	2	5FU+ 50GY RADIO NEOADJ	2	WT	WT	WT
2	50	M	Adenocarcinoma	3	0	0	2A	5FU+ 50GY RADIO NEOADJ	1	WT	WT	WT
3	46	F	Adenocarcinoma	3	1	0	3B	5FU+ 50GY RADIO NEOADJ	1	MUT	WT	WT
4	78	F	Adenocarcinoma	2	0	0	2	5FU+ 50GY RADIO NEOADJ	2	WT	WT	WT
NE1	*68*	*F*	*Adenocarcinoma*	*3*	*1*	*0*	*3B*	*5FU+ 50GY RADIO NEOADJ*	*0*	*WT*	*WT*	*WT*
NE2	*67*	*F*	*Adenocarcinoma*	*3*	*1*	*0*	*3B*	*5FU+ 50GY RADIO NEOADJ*	*0*	*WT*	*WT*	*WT*
NE3	*61*	*M*	*Adenocarcinoma*	*3*	*1*	*0*	*3B*	*5FU+ 50GY RADIO NEOADJ*	*3*	*WT*	*MUT*	*WT*

Table 1: Clinical characteristics of the patients enrolled. Patients for whom cell lines were not obtained are reported in Italic.

The study is compliant with all relevant ethical regulations involving human participants and was approved by the Institutional Review Board protocol (project ID code: 157_1 of 20 February 2017, IOM Institutional Review Board). Informed consent was obtained from all subjects.

3.2 CSC isolation and culture establishment

Seven fresh human colorectal cancer biopsies were obtained in accordance with the standards of the ethics committee on human experimentation of the IOM. Biopsy samples were collected before the administration of neo-adjuvant radiotherapy. CSC isolation from the biopsies was performed as previously described (22, 37). Briefly, for the dissociation, biopsies were first extensively washed in PBS (GIBCO, Thermo Fisher Scientific, Carlsbad, CA) and then subjected to mechanical and enzymatic digestion with Collagenase type II (Thermo Fisher Scientific) and DNAse I (Roche Diagnostics, Indianapolis, IN) at 37 °C for 1 hr. The cell suspension was then filtered through a 100 μm nylon filter and the cell pellet was resuspended in a CSC medium for spheroid growth (Tumorsphere Medium XF, PromoCell, Heidelberg, Germany), plated in ultra-low attachment tissue

culture flasks (Corning Costar, Cambridge, MA), and incubated at 37 °C under a humidified atmosphere of 5% CO_2. Every 2/3 days, half of the culture medium was refreshed. In these severe culture conditions, immature cells grew slowly and formed non-adherent clusters called tumor spheres, while non-malignant cells or differentiated cells died. Tumor spheres became evident after a variable length of time, ranging from 5 to 7 days to 3 weeks. Regular culture splitting (1:2) was usually needed after 3–6 weeks from isolation. Spheroids were weekly subjected to mechanical or enzymatic dissociation via incubation for 10 minutes at RT with the Accutase enzyme (GIBCO).

From a total of 7 surgical samples, 4 CSC lines were established and validated to determine their ability to generate tumors in mice, and 3 samples did not generate growing in vitro cultures (1 culture was affected by bacterial contamination, while 2 did not expand in the culture medium, remaining in a quiescent state for several months).

3.3 Evaluation of stem cell marker expression by flow cytometry

The expression of stem cell markers was evaluated by flow cytometry analysis using FACSAria II (Becton Dickinson (BD), Franklin Lakes, NJ, USA). Single cells dissociated from spheroids were incubated with the appropriate dilution of a specific antibody: anti CD133-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti CD44-FITC (BD Bioscences). Unstained cells were used as the negative control.

Cell stemness was also evaluated by using an ADEFLUOR KIT (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. The ALDEFLUOR™ reagent system is a non-immunological method to identify stem/progenitor cells by their aldehyde dehydrogenase (ALDH) activity.

3.4 In vitro cell irradiation

CSCs were seeded into 35x10 mm dishes (Corning, NY, USA) and reached approximately 80% confluency at the time of irradiation.

A system for the *in vitro* irradiation of CSCs and custom-designed irradiation geometry were developed. This system utilizes the same equipment used for patient treatments. To simulate the flow of radiation beams through human tissues before reaching the target tumor area, a single dish containing cultured CSCs was inserted into a custom-built phantom made of plexiglass, a material similar to water, which is the main component of human tissue. The dish was housed within a niche created inside the phantom to place the cells at the radiation isocenter.

The phantom containing the colorectal CSCs was irradiated through a Varian Novalis-TrueBeam STx linear accelerator, which is able to perform stereotactic treatments with very high precision. This

radiotherapy equipment uses the high dose rate Flattening Filter Free (FFF) technique and a High Definition Multilamellar Collimator (MLC); with a minimum leaf size at the isocenter of 2.5 mm, this device is specifically designed to treat small lesions. A fractionated dose of 25 Gy (5 daily fractions of 5 Gy) was administered [38] in combination with different dose rates (600 MU/min, 1400 MU/min, and 2400 MU/min) to assess the eventual impact of dose rates on apoptosis and cell viability. The plan consisted of two opposed photon beams of 8x8 cm^2 defined at the machine isocenter located at the center of the niche containing the plate.

After irradiation, cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, 48 h, 72 h, 7 d, and 14 d, and then analyzed for cell viability, apoptosis, and the ability to give rise to new clones.

3.5 Evaluation of cell viability

The cell viability assay was performed using a CellTiter96® Aqueous One Solution Cell Proliferation Assay Kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The fluorescence signal was detected with Synergy HT (Biotek Instruments Inc., Vermont, USA) at 24 h, 48 h, 72 h, 7 d, and 14 d.

3.6 Evaluation of apoptosis

Annexin V staining of the phosphatidylserine (PS) in the outer surface of the cellular membrane is a widely used assay for studying cellular apoptosis, as an increase in PS staining is directly connected with early apoptosis. Here, $1x10^5$ cells for each sample were stained at 24 h, 48 h, 72 h, 7 d, and 14 d, with Annexin V fluorescein isothiocyanate (FITC) at a final concentration of 0.375 µg/ml (BD Biosciences), according to the manufacturer's instructions. To distinguish between early apoptotic cells with intact cellular membranes and necrotic or late-apoptotic cells, 1 µg of propidium iodide (PI) was added to each sample. Cytometric analysis was performed with a FACS-Aria II flow cytometer (BD Biosciences). For each measurement, $1x10^4$ cells were counted, and the results were analyzed. Three replicates were analyzed for each CSC line in each condition assessed.

3.7 Single-cell cloning

CSCs were dissociated with Accutase (Gibco) and then resuspended in a fresh medium to generate a single-cell suspension with a density of 10 cells/ml. Then, 200 µl of the single-cell suspension was dispensed into each well of a 96-well non-treated plate. The day after plating, only wells that contained 1 viable cell were selected, excluding wells with no cells or more than one cell.

Single-cell cultures were maintained in a medium and checked after 14 days to evaluate their clonogenic potential.

3.8 In vivo procedures

All animal procedures were performed according to the Italian national animal experimentation guidelines (D.L.116/92) upon approval from the experimental protocol by the Italian Ministry of Health's Animal Experimentation Committee. In this study 4-to-6-week-old female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME) were used.

3.9 In vivo evaluation of CSCs' tumor initiating capabilities

The main feature of stem cells is their ability, once implanted in a hosting mouse, to reproduce a tumor with the same phenotype of the original one. To this end, 5×10^5 cells were resuspended in 100 μl of a 1:1 growth medium/Matrigel (BD Biosciences) solution, and the cell suspension was injected subcutaneously into the flank of the animal. For each CRC cell line, 5 replicates of xenotransplants were performed. For all 4 lines, a tumor mass was detectable within 3–5 weeks in at least 3 out of 5 mice. As soon as the tumor mass reached a diameter of 10 mm, xenografts were explanted, and one-half of the mass was formalin-fixed, paraffin-embedded, and processed for histology to evaluate the tumor phenotype in comparison with the parental human tumor. The other portion of tumors was dissociated into single cells that were seeded in a tumor sphere medium and expanded to be assayed once again for stemness markers (CD44 and CD133 expression, ALDH activity, self-renewal, and high proliferative capacity).

3.10 Histology and Immunohistochemistry

Tumors were fixed with 10% formalin and paraffin-embedded for histological analysis. Three-millimicron-thick sections were cut with a microtome and automatically stained with hematoxylin-eosin (Ventana Symphony Stainer, JMD Histology and Histologistics Inc., Dudley, MA, USA).

The presence of colon adenocarcinoma was also evaluated via immunohistochemical analysis. Three-millimeter-thick sections were cut from the FFPE blocks. Anti-human CK20 (clone SP33) and CDX2 (clone EPR2764Y) rabbit monoclonal primary antibodies (Ventana, Roche Diagnostic, Basel, Switzerland) were used for the analysis. Slides were incubated using the BenchMark ULTRA platform, and an OptiView DAB IHC Detection Kit (Roche Diagnostic) was used to detect protein expression. Tissues were counterstained with Hematoxylin II (Roche Diagnostic) for 4 min. Control of immunostaining specificity was performed by omitting the primary antibody.

3.11 In vivo tumor irradiation and evaluation of therapeutic response

4- to 6-week-old female NSG mice (The Jackson Laboratory, Bar Harbor, ME) were randomly assigned into 4 groups, one group for each CSC line. Each group was formed from 11 mice. For each line, the CRC-SCs were resuspended in 100 µl of a 1:1 growth medium/Matrigel, and 5×10^5 cells were injected subcutaneously into the flank of the animal (39). Tumor growth was measured twice weekly wih a digital caliper, and volumes were calculated using the following formula: $\pi/6 \times d^2 \times D$, where d and D represent shorter and longer tumor measurements, respectively. When tumors reached a dimension of 100–150 mm^3, the mice were randomly assigned to the control (3 mice/group) and treatment groups (8 mice/group). On the day of the radio treatment, mice belonging to the treatment group were moved, one by one, from the cage into a plexiglass box where an anesthetic gas containing Vetflurane (Virbac, Barcelona, Spain) was insufflated. All the following procedures, including image acquisition, contouring, elaboration of the treatment plan, and the same radio-treatment, were performed with the mice inserted and immobilized inside the plexiglass cage. Here, mice were first subjected to a computed tomography (CT) scan, and the CT images were the sent to the treatment planning system (TPS) dedicated to stereotactic radiotherapy treatments. Here, contouring of the volumes of interest was performed, including the target volume, spinal cord, heart, lungs, and bowels. At this point, the treatment plan was elaborated. This plan consisted of two non-coplanar dynamic conformal arcs with the optimized opening of MLC leaves based on dose constraints established during planning. Plan evaluation was performed carefully while observing the dose distributions on each CT image and the dose–volume histograms to assess the radiation dose that reached the target and the neighboring organs. To set-up the verification, we used the image-guided radiotherapy system "ExacTrac X-Ray 6D", using which it was possible to carry out pre-positioning through the infrared system and positioning using the X-Ray imaging system. Irradiation of the mice was performed by delivering 5 Gy for 5 consecutive days, for a total dose of 25 Gy at a maximum dose rate of 2400 MU/min using the 10 MV FFF photon beam produced by the Varian Novalis-TrueBeam STx linear accelerator.

Control animals were inoculated but not treated. Thirty days after treatment, a CT scan was performed to verify the tumor dimensions.

This study was performed in accordance with the ethical statement established by Italian law (Decreto legislativo 4 marzo 2014, n. 26) and authorized by the Italian Ministry of Health with the code 0D183.2.

3.12 Patient study

The patients enrolled in this observational study, whose isolated cells were used for *in vitro* and *in vivo* studies, were treated according to the actual therapeutic protocol. At the end of the treatment, the individual responses to therapy were evaluated via CT-scan, Magnetic Resonance, and colonoscopy, and the data were compared with the *in vitro* results.

3.13 Statistical analysis

Quantitative endpoints (MTS, Annexin V and subcutaneous tumor masses volume) measured at different timepoints were evaluated between groups of the treatments and controls using a repeated measures ANOVA (RMAN). Statistical analysis was performed using the R statistical environment (R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

4. MATERIALS AND METHODS (second object)

4.1 Patient enrolment

18 stage III patients with high probability of recurrence were enrolled for the study at Mediterranean Institute of Oncology (IOM) of Valverde (Catania, Italy). All patients were subjected to curative surgery and subsequent adjuvant chemotherapy with FOLFOX (5-fluorouracil [5-FU], folinic acid and oxaliplatin) o XELOX/CAPOX (capecitabine, oxaliplatin) for at least 6 months. Tumor staging were confirmed for all patients by histological analysis on surgical specimen. Patients were subjected to blood withdrawal before surgery (T0), one month after surgery before the start of adjuvant chemotherapy (T1) and 3 months after the start of chemotherapy (T2). Blood samples were collected on EDTA tubes and processed within 2 hours to avoid cfDNA contamination by DNA from blood-cells lysis. Primary tumor tissues were also sampled, and nucleic acids extracted from formalin-fixed, paraffin embedded (FFPE) sections). The study is compliant with all relevant ethical regulations involving human participants and was approved by the Ethical Committee (Catania 2). Patients involved in this study were recruited on a voluntary basis and informed consent was obtained from all subjects.

4.2 Cell-free Plasma Isolation

The plasma was obtained by double centrifugation at RT (room temperature): the first centrifugation at 1600 rcf for 10 minutes, and the second one at 3000rcf for 10 minutes to remove eventual cells and

cellular debris from the plasma. After that, the plasma was stored at -80 °C or directly processed for cf-DNA extraction.

4.3 Extraction of the circulating DNA

The extraction of the cfDNA was obtained from 3mL of plasma through the use of the commercial QIAamp® Circulating Nucleic Acid (Qiagen) kit which uses a vacuum pump to increase the extraction speed and efficiency. The extraction involves: a first phase of lysis, a purification phase through various washes, during which the DNA is retained by a membrane present in the column provided by the kit, and a final phase of elution in which the purified DNA is removed from the membrane by centrifugation. after the cfDNA was recovered to proceed to the Quantity, and Quality control step using the QuantiFluor Assay Kits and Agilent2100 Bioanalyzer respectively.

4.4 *Extraction of tissue DNA and RNA*

DNA was extracted from the tissues using the QIAamp® DNA Mini Kit (Qiagen). Nucleic acid quality and quantity were assessed using the QuantiFluor Assay Kits and Agilent2100 Bioanalyzer respectively. The highly intact and non-degraded RNA-free genomic DNA was subjected to library preparation prior to sequencing.

4.5 *Next Generation Sequencing on DNA and RNA*

The NGS analysis was conducted on both DNA and RNA from (FFPE) and cfDNA. The AmpliSeq Comprehensive Panel v3 (Illumina) was used to perform the library preparation procedure for DNA and RNA from FFPE. The sequencing libraries were then analyzed on MiSeq platform (Illumina). RNA sequencing is used to efficiently identify fusion variants.

The AmpliSeq for Illumina Comprehensive Panel v3 enables the use of targeted resequencing to study somatic mutations across 161 genes with known associations to cancer.

The Comprehensive panel v3 is part of a streamlined workflow that includes PCR-based library preparation, Illumina sequencing by synthesis (SBS) chemistry and next-generation sequencing (NGS) technology, and automated analysis. The AmpliSeq for Illumina Comprehensive Panel v3 requires as little as 1 ng high-quality DNA or RNA and is compatible with various sample types, including formalin-fixed, paraffin-embedded (FFPE) tissues. The high gene content and low DNA/RNA input requirement enable a single, streamlined workflow for the quick assessment of cancer-related genetic variations, affording researchers the potential to unlock a wealth of genomic information from many tumor types. (support.illumina.com)

Sequencing of cfDNA has been performed using Cell3™ Target sequencing panel by Nonacus on the same Illumina MiSeq platform which is able to sequence all the exonic regions of 50 cancer related genes that has been characterized in primary tumor tissues. This panel allow the accurate detection of ultra-low frequency mutations (below 5% VAF) reducing false positives or artefactual variant calls. This technical solution was required since the need to accurately call variants below 5% VAF is important for the achievement of non-invasive techniques like liquid biopsy testing where the sample used, circulating tumor DNA (ctDNA), is in low abundance and requires ultra-sensitive sequencing methods to detect it. The target enrichment protocol uses error suppression technology to ensure confident calling of all mutations down to 0.1% VAF.

Variants identified in tumor tissues has been matched with sequencing results obtained from cfDNA sequencing to identify eventual clonal reappearance that may suggest disease recurrence.

5. **RESULTS (first object)**

5.1 Molecular phenotype of tumor sphere cultures

CD44 and CD133 are two common surface markers used for the identification and isolation of colorectal CSCs (77, 22). Flow cytometry analysis revealed a modest expression of CD133 (around 20–25%) for all four lines analyzed. Instead, CD44 showed a much higher expression for lines 2 and line 3 (70.7% and 76%, respectively), while lines 1 and 4 revealed expression of CD44 comparable to that of CD133 (around 21%) (Table 2).

Table 2. *Typical stem-cell markers evaluated on tumor sphere cultures*

	CSC line 1	***CSC line 2***	***CSC line 3***	***CSC line 4***
CD44	*21%*	*70.70%*	*76%*	*21.40%*
CD133	*21.50%*	*25.50%*	*25.70%*	*21.75%*
ALDH	*MEDIUM*	*MEDIUM*	*MEDIUM*	*MEDIUM*

Increased aldehyde dehydrogenase (ALDH) activity has been described in cancer stem cells from different carcinomas (78-82). Moreover, several ALDH isoforms have been identified as CSC markers in different tumor types, including colon cancer (83). Medium ALDH activity was found in all four lines analyzed (Table 2).

5.2 Animal model-derived tumors present the same phenotype as human parental cancer

A pre-requisite of putative CSCs is their capacity to develop tumors that have the same phenotype as human parental cancer when transplanted in hosting mice.

CSCs derived from CRC biopsies were injected subcutaneously into the flanks of thymic immunocompromised mice. This procedure resulted in 100% tumor formation efficiency within a few weeks (2-4 weeks) from injection for all four cell lines (Table 2). The tumor mass explanted from the animal was processed for histologic and immunohistochemical analysis to verify that its phenotypical features reproduced those of the original human tumor.

As shown in Figure2, the xenografts presented the histological (A) and immunohistochemical (B, C) features of human colorectal cancer (D-F), including the formation of glands (arrows) and stromal components. Like their human counterparts, the adenocarcinomas here showed variability in the size and configuration of their glandular structures. In well and moderately differentiated adenocarcinomas, the epithelial cells were usually large and tall and often contained cellular debris in the gland lumen. In the mouse, the xenograft glands maintained the expression of anti-human CK20 (Fig. 2B) and CDX2 (Fig. 2C), two typical CRC markers.

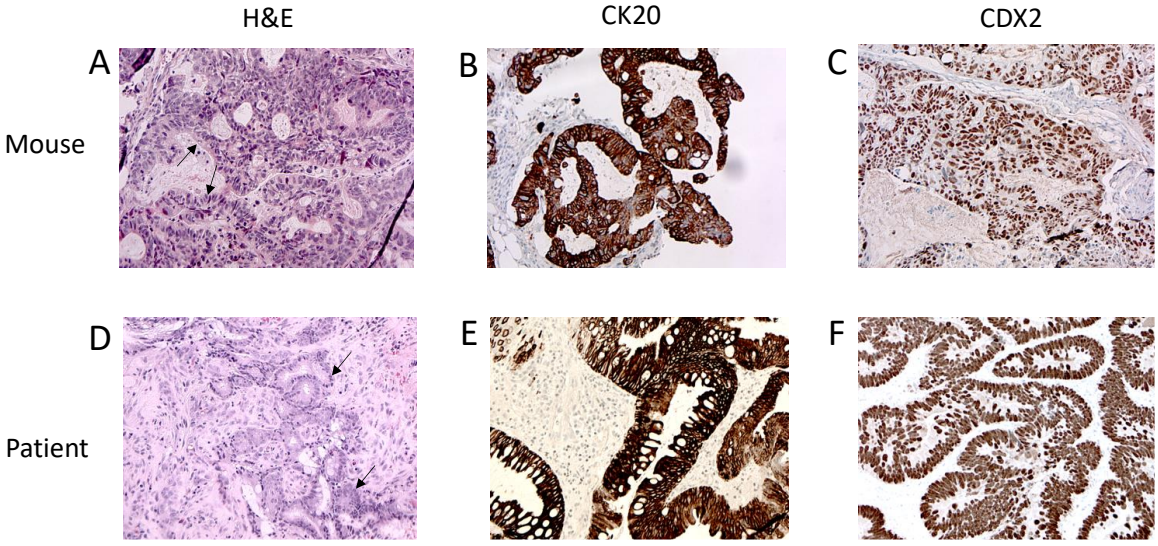

Figure 2. Animal model-derived tumors presented the same phenotype as human parental cancer.

(A) Hematoxylin and Eosin staining, **(B)** CK20 and **(C)** CDX2 immunohistochemistry of the mouse xenograft. **(D)** Hematoxylin and Eosin staining, **(E)** CK20 and **(F)** CDX2 immunohistochemistry of human colorectal cancer biopsy. Arrows indicate the gland structures. Magnification 20X.

5.3 CSCs from different rectal biopsies showed different in vitro sensitivity levels to radiotherapy

The establishment of CRC stem cell cultures may facilitate the direct evaluation of radiation cytotoxic activity on the putative cells responsible for tumor growth and spread, which represent optimal cellular targets for successful therapy. As a fractioned 25 Gy dose administered daily (5 Gy/Day) is the most commonly updated protocol used before proceeding with curative surgery for rectal cancer (75), the same schedule was used to treat the CSC *in vitro*.

To evaluate the effects of the radiotherapy treatment *in vitro*, different assays, including proliferation, apoptosis, and single cell cloning assays, were performed on irradiated cells at different time points (24, 48, and 72 hours and 7 and 14 days). Since radiation can be administered using different dose rates during the daily sessions, the effects of the dose rate in terms of cell growth arrest and apoptosis induction were investigated. *In vitro* cultures were treated using a 5 Gy daily dose at 600, 1400, and 2400 MU/min, and the MTS and Annexin V results were compared. No statistically significant difference was observed among the different dose rate treatments in all the lines assessed (Fig. 3 A-H). On the other hand, different cell lines showed significantly different overall sensitivity to the treatment. In particular, Line1 and Line 4 CSCs displayed similar behavior, both becoming rather resistant to radiation (Fig 3 A-B and G-H). Indeed, cell proliferation was not significantly reduced at any dose rate or at any time point considered for either Line 1 or Line 4, compared to the control sample (Figure 3 A and G; RMAN p values, respectively, of 0.113 and 0.233). Accordingly, the apoptosis assay did not show any significant difference in the percentage of cell vitality between the treated and NT samples at any dose rate or at any time point considered (Fig 3 B and H; RMAN p value respectively of 0.467 and 0.619). Finally, the single cell cloning assay exhibited only a slight reduction for both lines (25% and 30%, respectively) in their ability to give rise to new cell clusters 7 days after treatment.

On the contrary, the other two CSC lines (Lines 2 and 3) were shown to be highly sensitive to radiotherapy treatment. The proliferation assay revealed a significant reduction in cell proliferation for both lines at all dose rates tested (600 MU/min, 1400 MU/min, and 2400 MU/min) compared to the NT samples. In particular, after 14 days, almost all the cells were shown to have lost their proliferative potential in all treated samples (Fig. 3 C and E; RMAN p values both below 0.001 with no significant differences between dose rates). The Annexin V assay confirmed these data, already showing a reduction in cell vitality of about 80% at 14 days after treatment (Fig. 3 D and F; RMAN p values both below 0.001 with no significant differences between dose rates). Although all assays

were performed on irradiated cells at 5 different time points (24, 48, and 72 hours and 7 and 14 days), data are reported only for the most informative time points (24 h, which is the early timepoint and 14 days, which is the late timepoint). In accordance with the radiobiology of the treatment, the cells start to die only a few days after the treatment because they were unable to repair DNA damage and accumulated mutations. At the earlier time points, the induction of cell death was not noticeable, even when already triggered.

Seven days after radiation, both cell lines (Lines 2 and lines 3) showed a 100% reduction of their clonogenic potential.

These *in vitro* results demonstrated that the CSCs derived from different patients showed diverse sensitivity levels to radiation treatment, although no differences were found among the different dose–rate protocols tested.

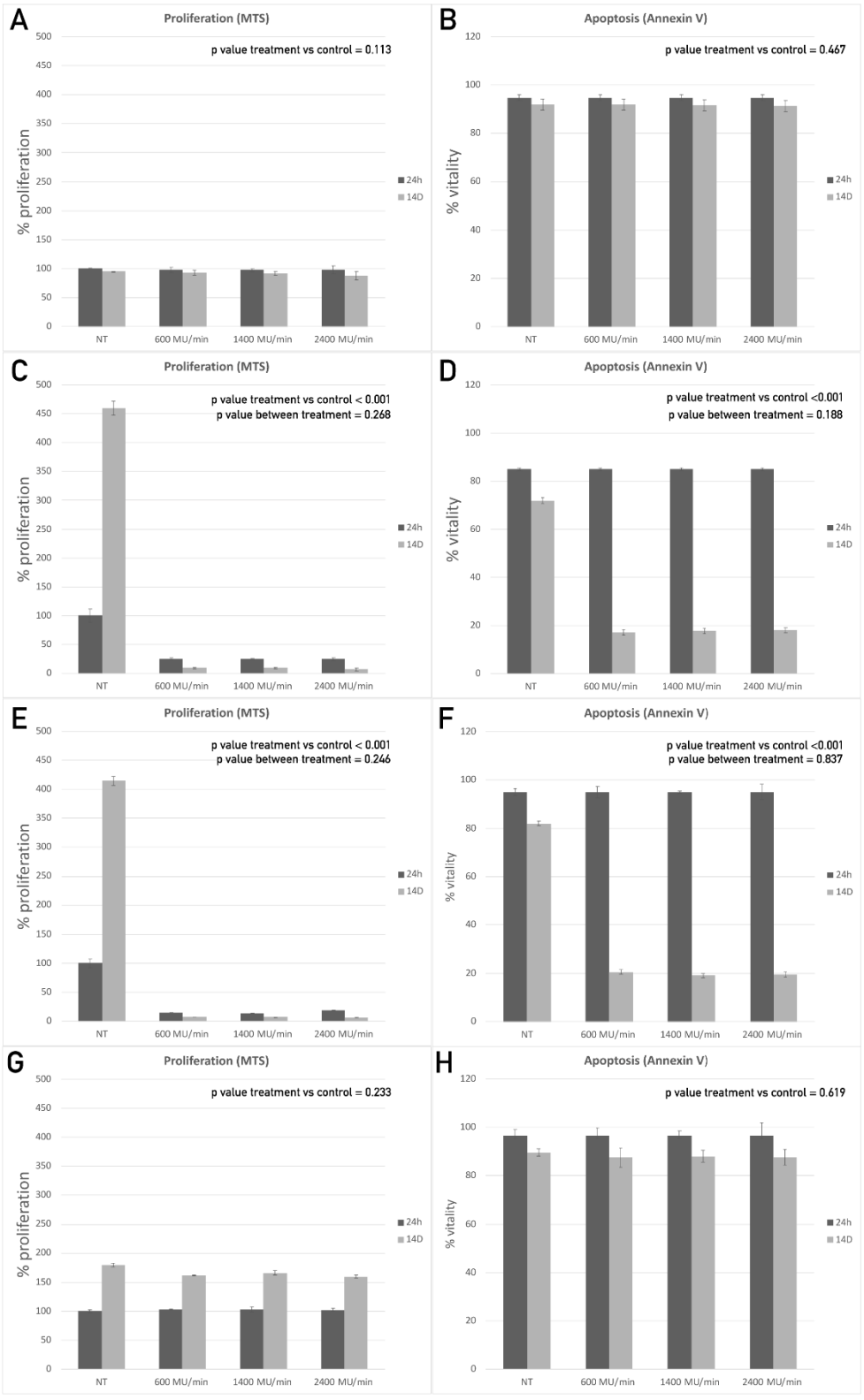

Fig. 3: CSCs from different rectal biopsies show different *in vitro* sensitivity levels to radiotherapy.

Graphical representations of the proliferation **(A, C, E, G)** and apoptosis assay **(B,D, F, H)** 24 h and 14 days (14D) after treatment with a 5 Gy dose radiation supplied for 5 consecutive days in combination with different dose rates (600 MU/min, 1400FFF MU/min and 2400

MU/min) in comparison with the non-treated sample (NT) for CSC Line 1 **(A-B)**, Line 2 **(C-D)**, Line 3 **(E-F),** and Line 4 **(G-H)**.

5.4 CSCs from different rectal biopsies showed in vivo sensitivity to radiotherapy comparable to that found under in vitro treatments

The individual responses of CRC patients to neoadjuvant radiation may vary in relation to the unique characteristics of each individual tumor, differently affecting the response to the radiotherapy in terms of mass reduction and risk of recurrence.

In vitro experiments already demonstrated that the cells isolated from several colorectal cancer patients present different sensitivity levels to radiotherapeutic treatments. To evaluate the *in vivo* responses to radiotherapy, animal models were exposed to the same radiotherapy protocols used in *in vitro* experiments.

To this end, cancer stem cells derived from patients with colorectal tumors were first expanded *in vitro* and, after reaching a sufficient amount, were injected into the flank of female athymic immunodeficient mice. Tumor growth was evaluated every 4 days using an digital caliper. Within 2-4 weeks, all the animals showed the formation of a tumor mass for all four cell lines tested. Mice were divided into two groups: a control group (NT: not treated) composed of three animals and a treated group containing eight animals. Once the tumors reached a diameter of about 100–150 mm^3, the animals were subjected to a TC scan to better estimate the tumor dimensions and exactly define the zone to expose to the radiation. Mice were treated with 5 Gy radiation for 5 consecutive days during the same hour and under the same experimental conditions. Tumor growth was evaluated by caliper measurements every 4 days for 4 weeks. At the end of this period, the mice were subjected to a final TC scan to exactly evaluate their tumor mass reduction in comparison with the initial TC scan. The *in vivo* results were fully consistent with those obtained *in vitro*. In particular, the radiation treatment was ineffective for Line 1- and 4-derived tumor grafts, as shown in Fig. 5A and F, where the trend of tumor growth for the treated group was fully comparable to that of the NT group. The CT-scan images of the tumor at the beginning (0D) and end (30D) of the treatment confirmed the data obtained with the caliper (Fig 4C) for line 1. Line 4 produced fat-growing refractory tumors that rapidly reached the maximum volume allowed by the in vivo protocol in all the animals implanted, both treated and untreated. The animals were sacrificed 20 days after the start of the treatment. For this reason, D30 CT scans are not available for these animals. Figure 4 H reports some representative

ex vivo measures of the tumor masses. Conversely, Line 2 and 3 tumor grafts displayed a high sensitivity to the treatment, revealing a steady decrease in tumor growth over time, down to zero (Fig. 4 B and E; RMAN p values below 0.001). The same trend was observed in the CT-scan images at the beginning (0D) and end (30D) of the treatment (figure 4 D and G).

Ex vivo histological analysis confirmed tumor regression in the treated explants derived from lines 2 and 3. In these tissues, no viable tumor cells were observed. On the other hand, explanted tissues derived from treated tumors derived from line 1 and 4 presented residual cancer outgrowth in the fibrotic areas.

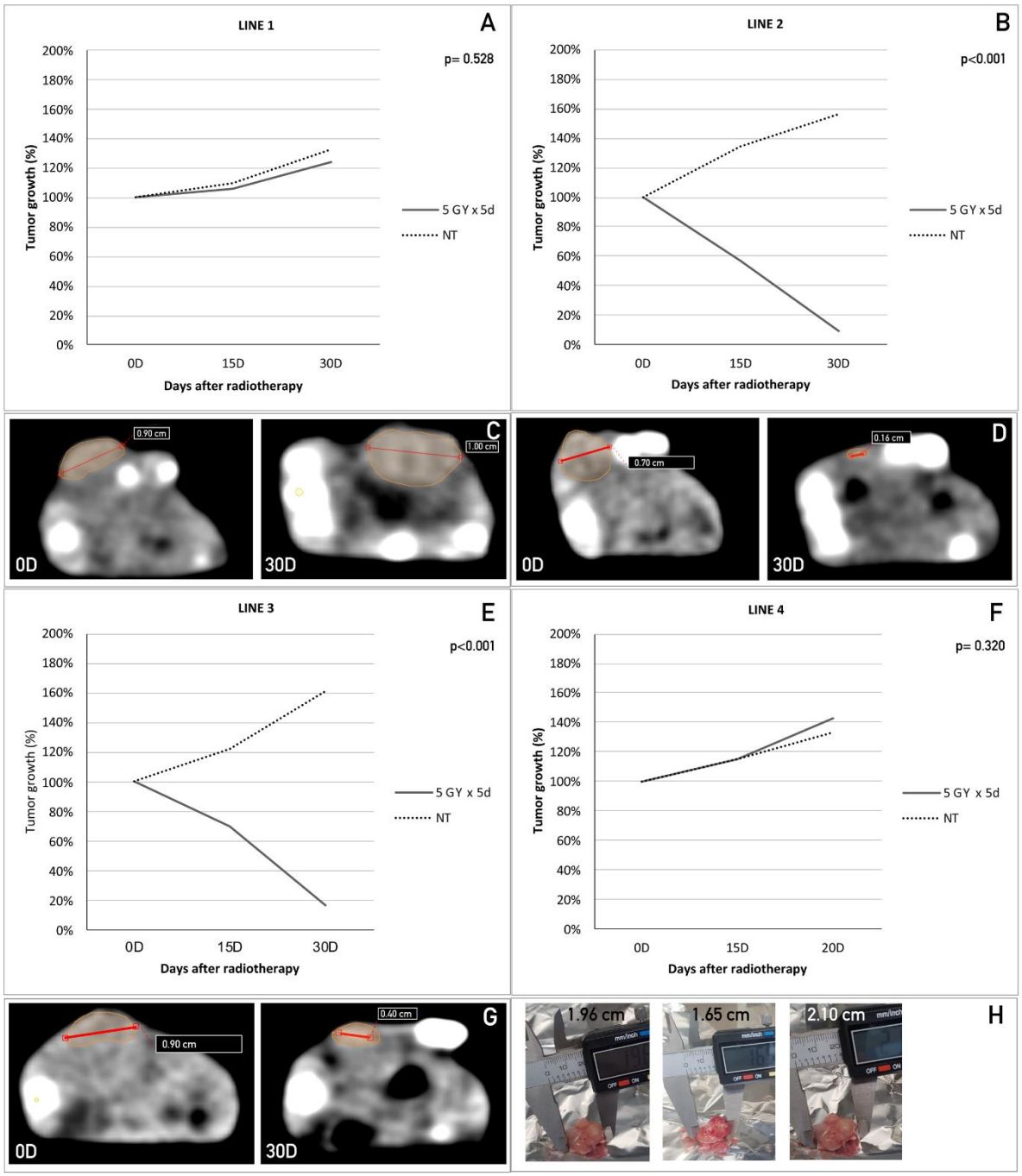

Figure 4. CSCs from different rectal biopsies showed *in vivo* sensitivity to radiotherapy comparable to that found under *in vitro* treatments.

Graphical representations of the *in vivo* tumor growth (%) measured at three different time points (0D, 15D, and 30D) after treatment with a 5 Gy dose of radiation supplied over 5 consecutive days in combination with a dose rate of 2400MU/min compared to the non-treated sample (NT), for CSC Line 1 **(A)**, Line 2 **(B)**, Line 3 **(E),** and Line 4 **(F)**. (**C, D, G**) Representative CT scan images taken on the day of the last irradiation (D0) and 30 days after treatment for lines 1, 2, and 3. Line 4 produced fast growing refractory tumors with a consequent risk of ulceration. All mice implanted with line 4

had to be sacrificed on day 20 because they reached the tumor mass limit. For this reason, CT scan images are not available. Representative images of *ex vivo* measure for line 4 **(H**).

5.5 In vitro treatment predicted the clinical outcomes of rectal cancer patients treated with neo-adjuvant radiation

In the clinic, all four CRC patients whose CSCs were isolated and used for the *in vitro* experiments were subjected to neo-adjuvant radiotherapy. In this way, we compared the *in vitro* responses with the clinical outcomes.

Our data show that the patients achieved a good clinical response when their isolated CSCs were sensitive to *in vitro* treatments, as shown in Fig. 5 (A-B), where a significant reduction in tumor mass was obtained after radiotherapy in patients whose cells became sensitive when treated *in vitro*. On the other hand, the patients whose CSCs became resistant to *in vitro* radiation presented a poor clinical response and could not be subjected to post neoadjuvant surgical treatment because of their unaltered tumor dimensions (Fig. 5C-D).

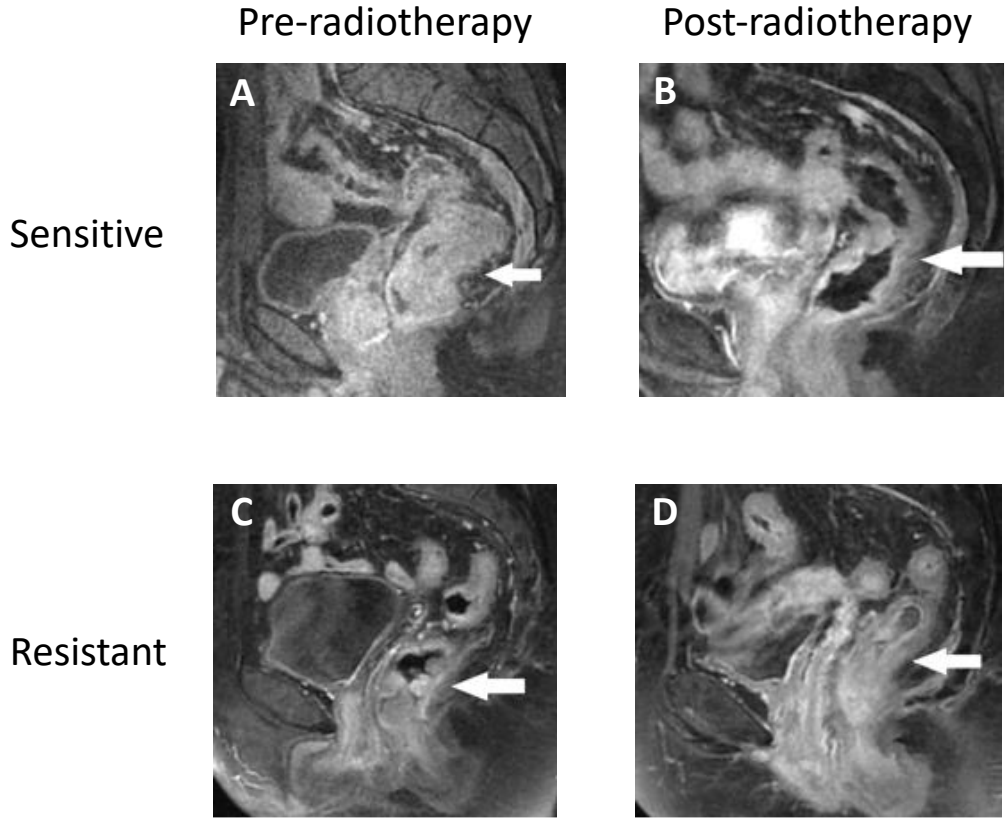

Figure 5. *In vitro* treatment predicted the clinical outcomes of rectal cancer patients treated with neo-adjuvant radiation. Representative MRI images before **(A)** and after **(B)** the radiotherapy of a patient whose CSCs became sensitive

to in vitro treatment. Representative MRI images before **(C)** and after **(D)** radiotherapy of a patient whose CSCs became resistant to in vitro treatment.

6. RESULTS (second object)

6.1 *NGS profiling of tumor tissues*

Primary tumor tissue DNA and RNA were extracted from FFPE sections of tumors obtained from patients affected by stage III colon cancer. All section has been evaluated by a pathologist in order to select samples in which tumor cells represented at least the 80% of the total area.

DNA and RNA have been used to construct sequencing libraries. DNA libraries have been sequenced to identify single nucleotide variants and small indels, while RNA libraries have been used to identify gene fusions.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AKT1	2	3	1	2	2	3	0	2	0	0	1	1	1	2	0	0	0	0
AKT2	1	1	1	2	0	1	1	1	1	0	0	0	0	0	0	0	0	0
ALK	1	1	1	2	0	1	1	1	0	0	1	0	0	0	0	2	0	0
ARID1A	4	4	3	7	5	3	3	5	0	0	0	0	0	4	0	0	0	0
ATM	21	26	23	31	18	37	27	23	23	22	22	21	21	22	20	22	21	22
ATR	15	17	30	30	12	29	27	24	19	19	17	17	18	17	17	19	17	17
ATRX	2	5	4	7	3	5	8	6	1	1	0	0	2	0	2	2	2	2
AXL	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0
BAP1	0	1	1	1	1	2	4	3	0	0	0	0	0	0	0	0	0	0
BRAF	9	4	7	9	9	8	12	12	4	4	3	6	5	6	4	4	3	4
BRCA1	18	7	19	23	4	18	19	8	11	10	9	12	9	12	10	9	11	9
BRCA2	16	13	17	17	15	20	17	15	11	10	10	12	11	10	13	10	12	11
BYSL	2	0	2	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0
CASC11	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	2	0	2
CCND1	1	1	2	3	1	2	2	4	1	0	0	0	0	1	0	0	0	1
CCND2-AS1	2	2	2	2	1	2	1	2	0	0	0	0	1	0	0	0	1	0
CCNE1	2	1	1	2	2	3	2	0	0	1	1	0	1	0	0	1	1	0
CDK12	2	2	3	1	4	3	2	2	0	1	1	0	0	0	0	1	0	0
CDK6	2	0	2	3	0	1	3	0	0	1	1	0	0	0	1	0	0	0
CDKN1B	2	0	0	1	1	0	1	1	0	1	1	1	1	11	0	0	0	0
CDKN2A-AS1	1	2	1	1	1	1	1	1	1	0	0	0	0	0	1	1	1	0
CDKN2B-AS1	0	0	0	2	0	0	0	0	0	1	1	0	1	0	0	0	0	0
CHEK1	4	2	2	2	4	3	3	2	0	0	0	1	0	1	0	0	0	1
CREBBP	8	7	6	8	8	12	9	16	3	4	5	3	5	3	3	3	3	3
CSF1R	2	1	0	1	1	0	3	3	0	0	0	0	0	0	0	0	0	1
DDR2	1	3	1	3	1	1	2	1	0	0	0	0	0	0	0	0	0	1
DNAH1	0	0	0	1	0	0	0	0	0	0	0	2	0	0	0	0	0	1
EGFR	4	2	3	2	2	2	3	2	0	0	0	0	0	0	0	0	0	1
ERBB2	1	2	2	1	7	2	1	1	0	0	0	0	1	0	0	0	0	0
ERBB3	1	2	1	1	1	2	1	0	0	0	1	0	0	0	0	0	0	0
ERBB4	2	2	2	3	1	3	3	2	0	0	0	0	0	0	0	0	0	0
ERCC2	0	0	0	1	0	0	0	0	0	0	2	0	1	0	1	1	0	0
ESR1	3	2	3	2	3	4	3	2	0	0	0	0	0	0	0	0	0	0
ETFRF1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FANCA	2	4	25	4	3	8	10	32	8	6	6	8	7	7	6	6	7	6
FANCD2	13	12	13	28	10	30	26	27	14	16	14	16	14	16	17	17	16	16
FANCI	4	10	2	4	8	8	2	2	1	2	2	0	0	0	0	0	0	0
FBXW7	5	2	4	5	5	7	5	4	0	0	2	0	0	0	0	2	1	0
FGF3	1	1	1	2	1	3	3	2	0	0	0	0	0	0	0	0	0	0
FGFR2	1	1	1	2	1	0	1	1	0	0	0	0	1	0	0	0	0	1
FGFR3	2	5	1	3	5	1	1	3	1	1	0	0	0	0	0	2	1	1
FGFR4	1	1	1	1	1	1	2	0	0	0	1	1	0	0	0	1	1	0
FLT3	1	2	1	1	2	1	1	1	0	0	0	1	0	0	0	1	0	0
GNAS	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
HMGXB3	1	1	1	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0
HNF1A	1	2	0	1	2	2	2	2	0	1	0	0	1	1	0	0	0	0
HRAS	0	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0
IGF1R	2	2	2	2	2	2	2	2	0	1	1	0	1	0	1	1	1	0
JAK1	3	1	1	1	1	1	1	0	0	1	1	0	2	0	1	1	1	0
KIT	0	1	3	2	2	2	0	0	0	1	1	0	3	0	1	1	1	0
KRAS	2	2	2	2	1	2	2	3	0	0	0	0	0	1	0	0	0	0
LETM2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LINC00441	1	0	1	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
LOC100130075	0	0	2	1	1	0	2	1	0	0	0	1	0	0	0	0	0	0
LOC101928728	0	1	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0
MDM2	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
MDM4	2	1	2	2	2	3	2	0	1	1	1	0	0	0	0	0	0	0
MET	2	3	2	0	2	0	2	2	0	0	0	0	0	0	0	0	0	0
MLH1	4	8	2	4	4	10	10	7	1	1	1	0	0	1	0	0	3	1
MRE11	4	6	6	7	4	4	2	2	0	0	0	0	0	0	0	1	0	1
MSH2	15	15	12	11	11	15	9	13	10	10	10	9	10	10	7	9	8	8
MSH6	21	13	10	28	16	24	20	12	13	13	12	13	13	15	15	15	15	14
MTOR	1	1	1	2	1	1	1	1	0	0	0	0	0	0	0	0	0	0
MYCL	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
MYCNOS	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
NBN	1	10	5	13	2	14	7	12	3	4	4	5	4	2	2	5	5	4
NF1	23	17	24	28	15	34	23	24	19	18	19	18	19	20	20	21	19	21
NF2	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
NOTCH1	21	20	8	15	18	22	21	13	11	14	14	12	14	14	13	11	12	12
NOTCH2	10	9	11	10	9	11	11	10	7	7	5	5	7	5	7	4	7	7
NOTCH3	18	20	18	14	18	17	17	18	14	13	13	15	13	13	13	14	14	15
NTRK1	1	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
NTRK2	0	1	0	1	0	0	0	1	1	1	0	0	0	1	0	0	0	1
NTRK3	1	2	2	0	2	0	2	1	0	0	0	0	0	0	0	0	1	0
P2RX2	2	1	1	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1
PALB2	3	1	0	6	1	3	7	1	1	0	0	0	0	0	0	0	0	0
PDGFRA	1	1	1	1	3	1	2	3	0	0	0	0	0	1	0	1	1	0
PDGFRB	1	0	3	2	0	1	2	1	1	1	0	0	0	0	0	0	0	0
PIK3CA	0	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	1	0
PIK3R1	2	1	4	5	4	5	4	7	0	0	0	0	0	0	0	0	0	0
PMS2	8	9	6	13	6	8	7	9	4	2	4	4	5	4	5	4	4	4
POLE	17	22	20	19	22	18	23	8	13	15	13	16	13	16	13	13	14	14
PPARG	1	0	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0
PTCH1	7	3	2	5	1	5	3	2	0	0	0	0	0	0	0	1	0	0
PTEN	2	2	6	9	1	8	4	6	2	0	0	2	0	1	2	0	0	2
RAC1	2	2	2	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
RAD50	2	4	13	10	3	11	11	10	2	5	4	4	5	2	2	2	5	5
RAD51	1	2	2	3	1	9	3	5	0	0	0	0	0	0	0	0	0	0
RAD51-AS1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RAD51B	8	11	9	18	7	18	11	9	8	8	8	6	6	5	6	7	7	7
RAD51C	1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
RAD51L3-RFFL	0	0	2	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0
RB1	13	16	15	14	11	15	10	9	8	10	7	7	7	9	9	10	9	10
RET	3	2	1	3	2	2	1	4	0	0	0	0	0	0	0	0	0	0
RICTOR	1	1	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
RNF43	2	1	2	1	1	2	1	2	1	1	1	0	1	0	0	0	0	0
ROS1	5	4	1	7	4	5	0	1	0	0	0	0	0	0	0	0	0	0
RSPH10B	1	1	0	1	1	0	1	0	0	0	0	0	0	0	0	1	0	0
SETD2	13	10	12	13	7	16	13	11	8	6	6	7	8	9	6	7	6	7
SH2D2A	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
SLX4	1	0	5	4	4	3	4	24	3	0	3	0	0	0	2	3	3	0
SMARCA4	7	5	6	5	10	5	4	4	0	1	0	3	2	3	0	0	0	2
SMARCB1	3	1	4	0	2	0	0	1	1	0	0	0	0	0	0	0	0	0
SMO	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SPOP	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0
SRC	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	0
STAT3	1	0	1	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0
STK11	5	5	0	5	0	2	6	0	0	0	0	0	1	1	0	0	0	0
STT3A	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	0	0	0
TERT	0	1	0	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0
TEX14	0	0	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0
TOP1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TP53	5	1	3	1	2	4	3	2	0	0	0	0	0	0	0	0	0	0
TSC1	4	10	7	8	5	11	5	5	2	4	4	2	4	1	3	3	4	1
TSC2	3	8	5	1	2	1	3	5	1	0	1	0	0	0	1	0	0	0
TSPAN31	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TSPOAP1-AS1	1	1	1	2	2	3	0	1	1	0	0	0	0	0	0	0	0	0
XRN1	0	1	1	1	1	1	1	1	0	0	2	0	0	1	0	1	0	0
ZAR1L	1	1	1	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0
ZNF276	0	0	6	0	0	1	1	7	0	0	0	1	0	1	0	0	0	0

Figure 6. *Variants identified in tumor tissues*

These variants comprise both de-novo somatic mutations and inherited germline mutations. Tissue mutations have been matched with the corresponding cfDNA mutation profiles. T1 profiles have been used as baseline to select for variants that are likely to be inherited (germline) that will be ignored for monitoring purposes.

6.2 NGS profiling of cfDNA

The table 3 illustrates the number variant identified in the cfDNA of T1 samples. Cells in green report variants that are not present in the T1 cfDNA but are present in tissue (see table below). These mutations are almost often present also in cfDNA of T0 samples suggesting that circulating tumor DNA can be detected before surgery. The lack of a variant which is present in tumor tissues but absent if T1 cfDNA samples, ma be explained by the corresponding absence on tumor cells of by concentration belowe sensitivity threshold. The presence of a variant in T0, T1 cfDNA and tissue DNA can be explained by cfDNA contamination from germline circulating DNA.

Table 3.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AKT1	2	3	1	2	2	3	0	2	0	0	1	1	1	2	0	1	1	0
ALK	1	0	1	2	0	1	1	1	0	0	1	1	1	0	0	2	0	0
ATM	21	26	23	31	18	37	27	23	22	22	22	21	21	21	20	22	21	22
BRAF	9	4	7	9	7	8	12	12	4	3	3	6	5	6	4	4	2	4
CSF1R	2	1	0	1	1	0	3	3	2	0	2	0	0	0	0	0	0	1
EGFR	4	2	3	2	2	2	3	2	0	0	0	0	0	0	0	0	0	1
ERBB2	1	2	2	1	7	2	1	1	0	0	0	0	0	0	0	0	0	0
ERBB4	2	2	2	3	1	3	3	1	0	0	0	0	0	0	0	0	0	0
FBXW7	5	2	4	5	5	7	5	4	0	0	1	0	0	0	0	2	1	0
FGFR2	1	1	1	2	1	0	1	1	0	0	0	0	1	0	0	0	0	1
FGFR3	2	5	1	3	5	1	1	3	1	1	0	0	0	0	0	2	1	1
FLT3	1	2	1	1	2	1	1	1	0	0	0	1	0	0	0	1	0	0
GNAS	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
HNF1A	1	2	0	1	2	2	2	2	0	1	0	0	0	0	0	0	0	0
HRAS	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
KIT	0	1	3	2	2	1	0	0	0	1	1	0	3	0	1	1	1	0
KRAS	2	2	2	2	1	2	2	3	0	0	0	0	0	0	0	0	0	0
MET	2	3	2	0	2	0	2	2	0	0	0	0	0	0	0	0	0	0
MLH1	4	8	2	4	4	9	10	7	0	1	1	0	0	1	0	0	2	1
NOTCH1	21	20	8	15	18	20	21	13	11	14	14	12	14	14	13	11	12	12
PDGFRA	1	1	1	1	3	1	2	3	0	0	0	0	0	1	0	1	1	0
PIK3CA	0	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	1	0
PTEN	2	2	6	9	1	8	4	6	2	0	0	1	0	1	2	0	0	0
RB1	13	16	15	14	11	13	10	9	8	10	7	7	7	9	9	10	9	10
RET	3	2	1	3	2	2	1	4	0	0	0	0	0	0	0	0	0	0
SMARCB1	3	1	4	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0
SMO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SRC	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	0
STK11	5	5	0	5	0	1	6	0	0	0	0	0	0	1	0	0	0	0
TP53	3	1	3	0	0	4	3	2	0	0	0	0	0	0	0	0	0	0

6.3 *NGS profiling of T2 cfDNA samples and patients followup*

Blood samples collected 3 months after the start of chemotherapy have been used for the identification of cfDNA. Recurring variants may be a symptom of a secondary resistance during treatment.

The scheme below reports the sequencing results.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AKT1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ALK	1	0	1	2	0	1	1	1	0	0	1	1	1	0	0	2	0	0
ATM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BRAF	9	4	7	9	7	8	5	12	4	0	3	0	5	6	4	4	0	4
CSF1R	2	1	0	1	1	0	3	3	2	0	2	0	0	0	1	0	0	1
EGFR	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ERBB2	1	2	2	2	7	2	1	1	0	0	0	0	0	0	0	0	0	0
ERBB4	2	2	2	3	1	3	3	1	0	0	0	0	0	0	0	0	0	0
FBXW7	0	0	0	5	0	0	4	3	0	0	1	0	0	0	0	2	1	0
FGFR2	1	1	1	2	1	0	1	1	0	0	0	0	1	0	0	0	0	1
FGFR3	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1
FLT3	1	2	1	1	2	1	1	1	0	0	0	1	0	0	0	1	0	0
GNAS	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0
HNF1A	0	2	0	1	2	2	2	2	0	1	0	0	0	0	0	0	0	0
HRAS	0	1	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0
KIT	0	1	3	2	2	1	0	0	0	1	1	0	0	0	1	0	0	0
KRAS	2	0	0	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0
MET	2	3	2	0	2	0	2	2	0	0	0	0	0	0	0	0	0	0
MLH1	4	8	2	4	4	9	10	7	0	1	1	0	0	1	0	0	2	1
NOTCH1	21	20	8	15	18	20	10	13	11	10	14	12	14	14	13	11	12	12
PDGFRA	0	0	0	1	0	0	2	3	0	0	0	0	0	1	0	1	1	0
PIK3CA	0	0	0	1	0	1	1	1	0	1	1	0	0	0	0	0	1	0
PTEN	2	2	6	9	1	8	4	6	2	0	0	1	0	1	2	0	0	0
RB1	13	16	15	14	11	13	10	9	8	10	7	0	7	9	9	10	9	10
RET	3	2	1	3	2	2	1	4	0	0	0	0	0	0	0	0	0	0
SMARCB1	3	1	4	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0
SMO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SRC	1	1	1	1	1	1	1	1	0	0	0	0	0	0	1	1	0	0
STK11	5	5	0	5	0	1	6	0	0	0	0	0	0	1	0	0	0	0
TP53	3	1	3	1	0	4	3	2	0	0	0	0	0	0	0	0	0	0

relapse of a variant present in tissue but absent in T1

newly identified variant

variants potentially identified as somatic cancer cfDNA variant

missing previously identified variant(s)

After 3 months from the start of adjuvant therapy, in 17 out of 18 patients' variants previously identified as candidate somatic tumor biomarkers were not detected. The absence of such variants may suggest a tumor burden, at the time of sampling, which is null or very low. In one patient different variants, related to 6 different genes, which were previously detected in tumor tissue but absent in T1 have been detected in T2 cfDNA. This recurrence may be suggestive of a disease recurrence during treatment. Patient will be monitored carefully during follow up. No symptoms of disease are present at this moment. A new variant in ERBB2 gene has been detected in this sample. This newly identified variant may be previously missed for technical reason of may constitute a de-novo mutation which is symptomatic of a clonal evolution.

7. DISCUSSION AND CONCLUISION

The aim of this study is to improve the management of cancer through the development of new and better technological solutions that address two main issues: early diagnosis and precision medicine. In this study, we demonstrated the intrinsic individual sensitivity of CSC to the radiotherapy. This sensitivity profile was conserved in both *in vitro* and *in vivo* treatments of all the assessed samples, thus indicating that the subcutaneous tumors inherited this feature from their parental cells. Sensitive CSC were observed to be obtained by patients showing an optimal response to the therapeutic protocol. On the other hand, cells isolated from the biopsies of radioresistant tumors give rise to radioresistant CSC cultures. This suggest that the effect exerted by the administration of radiotherapy *in vitro* may be useful to predict the outcome of the treatment in donor patients. The limited size of the patient cohort also highlights the preliminary nature of the presented study. Nevertheless, the novelty of the approach may help deepen the suitability of the model for translation into a clinical setting through a broader study. The proposed approach may be suitable thanks to its time frame, which may fit the current therapeutic settings for LARC. In conclusion, assessment of the *in vitro* based model predicted CRC patient responses to radiotherapy treatment, so this model could be developed as a powerful diagnostic tool for CRC treatment.

On the other hand, this study proposes an innovative approach, the so called liquid biopsy which is promising to be a new and efficient instrument for the clinical management of cancer. Liquid biopsy is a new approach for the development of techniques targeted for personal and precision medicine [84]. The molecular characterization of the tumor, also at time of relapse, will allow a personalized and timely approach to therapy, improving quality of life and hopefully prolonging overall survival of patients.

The therapies based on molecular profiles of the primary tumor are not always efficacy because they are not representative of the evolving disease [85]. The proposed strategy is to identify variations in molecular profiles detected in circulating DNA before and after surgery.

In this study we have shown that Cf DNA is really a biomarker capable to monitor the clones' evolutions and to follow the progression of the disease.

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