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*Molecular mechanisms responsible for
radiation resistance in colorectal tumours*

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Indice

Abstract.....	3
Introduction.....	9
1. Rectal cancer and chemoradiation.....	12
1.1 Pretreatment evaluation.....	14
1.2 Preoperative versus postoperative radiation.....	16
1.3 Long-course or short-course radiation.....	18
1.4 Concurrent chemoradiation.....	20
1.5 Addition of capecitabine.....	23
1.6 Addition of oxaliplatin.....	24
1.7 Addition of targeted agents.....	25
1.8 Technique of radiation therapy.....	26
1.9 Preoperative restaging after neoadjuvant therapy.....	27
1.10 Timing of surgery after preoperative radiation.....	28
1.11 Surgery.....	29
1.12 Pathological evaluation of tumour response and perspectives....	30
2. Molecular basis of chemoradiosensitivity in rectal cancer..	34
2.1 Whole-genome analyses.....	35
2.1.1 Gene expression profiling.....	35
2.1.1.1 Microarrays in tumour tissue.....	35
2.1.1.2 Microarrays in peripheral blood.....	38
2.1.1.3 Microarrays of microRNA.....	39
2.1.2 Chromosomal aberrations.....	40
2.2 Single-biomarker and multibiomarker analyses.....	41
2.2.1 Single-nucleotide polymorphisms.....	41
2.2.2 Immunohistochemistry.....	43
2.2.2.1 Cell cycle.....	44
2.2.2.2 Proliferation markers.....	48
2.2.2.3 Apoptosis.....	49
2.2.2.4 Angiogenesis.....	52
3. The MAPK network: KRAS and BRAF.....	55
4. Materials and methods.....	65
4.1 Patients and chemoradiotherapy.....	65
4.2 Cell cultures, siRNAs and chemicals.....	67
4.3 Cell culture irradiation.....	68

4.4 Colorimetric MTT assays.....	70
4.5 Apoptosis assays.....	71
4.6 Colony forming assays.....	71
4.7 Immunoblot analysis.....	72
4.8 Statistical analysis.....	73
5. Results.....	74
5.1 Pathological and clinical outcomes of chemoradiation.....	74
5.2 Dose finding for in-vitro experiments.....	76
5.3 Radioresistance after exclusive radiation treatment.....	77
5.4 The effects of chemotherapy on radiation resistance.....	83
5.5 BRAF silencing/inhibition.....	86
5.6 TRAP1 silencing/inhibition.....	88
6. Discussion.....	97
7. Conclusion.....	113
Bibliography.....	115

Abstract

Keywords: rectal cancer, radiation resistance, BRAF mutations, TRAP1.

Introduction

Radiotherapy is a well-established therapeutic modality for cancer. It is considered a crucial treatment for most common types of cancer and is usually used in conjunction with chemotherapy, hormone therapy or surgery. However, the presence of radioresistant cells is one of the major obstacles to successful treatment with radiotherapy.

Ionizing radiation exerts its cytotoxic effect by the induction of double strand breaks (DSBs) and non-DSB highly clustered DNA lesions consisting in a combination of single strand breaks (SSBs), abasic sites and oxidized bases within 5–10 base pairs.

Radiation is known to activate multiple signaling pathways, causing cancer cells to become inactivated and resulting in diverse types of stress responses, including apoptosis, cell cycle arrest, senescence and gene induction. However, a large number of tumours fail to respond to radiotherapy as they are less sensitive or more resistant to radiation.

Various studies on the molecular mechanisms of resistance to radiotherapy have been carried out. However, obstacles related to overcoming this resistance remain to be solved. Therefore, identification of the radiation-responsive genes may aid to better understand the molecular mechanisms involved in the response of tumours to radiation and, ultimately, improve radiotherapy.

We focused our attention on colorectal cancer (CRC), which is one of the three leading causes of cancer deaths worldwide. Most colorectal cancers are sporadic, with dietary risk factors implicated in their development. Despite curative surgery, patients still have a significant probability of disease relapse and poor survival. Much interest has been generated in the last years in neoadjuvant treatment that would improve operability and prevent recurrent diseases.

Neoadjuvant chemoradiotherapy is an area of active research in rectal cancer. Indeed, outcomes of patients with rectal cancer have improved over the last decade, but this benefit has not extended to all subtypes of this disease. Many trials have been conducted to improve the outcome and decrease recurrence possibility. It is without doubt that tracing the underlying molecular mechanisms within the adopted strategy for cell death is a cornerstone for the

success of such trials. The purpose of our study was to evaluate if inhibition of determinate key points could enhance radiosensitivity in colorectal cancer cell lines.

Materials and Methods

Patients (pts) and neoadjuvant therapy. Between October 2006 and December 2013, 116 pts with locally advanced or distal T2 rectal tumours were treated by chemoradiation followed by surgery. Median follow-up was 66 months (IQR 56–73) for all pts.

Cell lines. We used cells of human colorectal adenocarcinoma: COLO320 (RAS and BRAF wild-type), HCT116 (RAS-mutated), HT29 (V600E BRAF mutated). Cell lines were supplied by the American Type Culture Collection (ATCC).

Chemotherapeutic. 5-fluorouracil (5-FU), commonly used to treat colorectal malignancies in association with radiotherapy. 5-FU was used at concentration of 5-500 nM.

Inhibitors. PLX4720, a potent and selective inhibitor of the V600E mutant form of the B-Raf protein, and HSP990, a dual HSP90/TRAP1 inhibitor.

Gene silencing by siRNA. Cells were transfected with BRAF-targeting siRNA, TRAP-targeting siRNA or nontargeting siRNA.

Cell culture. Cells were seeded in a 6-well plate at a density of 300 and grown in a medium containing 10% heat-inactivated fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Irradiation. A preliminary dosimetric study was necessary. Plates with cell lines were CT scanned. The CT data were imported into a treatment planning system and elaborated allowing isodose coverage of 95–107%: 1 cm plexiglas layer was used for radiation dose build-up, due to its tissue equivalent characteristics. Radiation was carried out at room temperature and delivered with 1.8-24 Gy, using a therapeutic linear accelerator (Elekta Sinergy) and 6-MV X-rays.

Cytofluorimetric Assay. After irradiation, cell lines were labeled using the Annexin V-FITC (fluorescein isothiocyanate) / 7-AAD (7-amino-actinomycin D) kit. Apoptosis was assessed by flow cytometric analysis of Annexin V and 7-AAD positive cells.

Clonogenic Survival Assay. Cell were grown in standard medium for about 2 weeks after irradiation to assess the capacity to form colonies. Cells were fixed with cold methanol for 25 minutes and stained with 1% crystal violet. The

number of colonies containing at least 50 cells was determined. Surviving fractions for each treatment were determined by normalizing the average plating efficiency for each dose to the plating efficiency at 0 Gy.

Western Blot. Cell lysates were prepared using buffer containing phosphatase and protease inhibitors and protein concentration was determined. Equal amounts of protein were subjected to electrophoresis performed under reducing conditions and gels were blotted to nitrocellulose membranes. All western blotting experiments were conducted in duplicate.

Statistical analyses. Comparison of the relative sensitivity of the cell lines was conducted using Analysis of Variance for three or more groups. Unpaired t-tests were used for comparisons of cytotoxicity between two conditions or cell lines.

Results and Discussion

Although current total mesorectal excision is curative for small tumours, the risk of locoregional recurrence, distant metastasis and death increases with tumours extending through the muscularis propria (T3 or T4) or nodal involvement (N1 or N2; stage II and III tumors).

One strategy to minimize recurrence in patients with rectal cancer is neoadjuvant chemoradiotherapy. Preoperative ionizing radiation downstage tumours and is well tolerated. Ionizing radiation, however, results in a wide spectrum of clinical response and the magnitude of benefit is heterogeneous. We analyzed our cohort of pts with locally advanced or distal T2 rectal tumours and we observed 21% pathological complete responses, 39% partial responses and 40% stable diseases. The estimated 2-year disease-free survival was 91.0% (95% CI 84.8-97.6). The estimated 2-year overall survival 96.2% (95% CI: 92.0-100).

One of the hallmarks of cancer cells is the up-regulation of cellular pathways that provide survival advantages by promoting proliferation and/or decreasing cell death. We evaluated the hypothesis that the RAS/RAF mutational status may influence cell response/resistance to radiation/chemoradiation. Upon analysis of clonogenic ability and apoptotic response to radiation in HCT116, COLO320 and HT29 cell lines, COLO320 cells showed sensitivity to radiation, while HT29 cells (BRAF-mutated) revealed to be the more radioresistant and HCT116 cells (KRAS-mutated) an intermediate phenotype. Our data suggest that resistance to ionizing radiation is mediated by activation of the Ras/MAPK pathway. Such pathway is the

Ras/Raf/mitogen activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) pathway, which is involved in cell proliferation, differentiation, apoptosis, and survival.

CRCs frequently exhibit activation of the Ras/MAPK pathway via activating mutations in Ras and/or Raf. The presence of either a Ras or Raf mutation is associated with an inferior prognosis compared to non-mutated tumours. Less than 10% of patients with metastatic CRCs have tumours with a point mutation in BRAF, a component of the RAF/MEK/ERK signaling pathway. Similar to other cancers, more than 95% of the BRAF mutations in CRC affect the V600 position of the protein, resulting in constitutive RAF/MEK/ERK pathway activation.

Inhibition of the Ras/MAPK pathway has also been exploited as a means to sensitize tumour cells to cytotoxic chemotherapy. As some studies hypothesized, in our experience inhibition of signaling via the Ras/MAPK pathway enhances sensitivity also to radiation.

To assess if specific inhibitors could enhance the radiation sensitization observed with 5-FU, we performed clonogenic survival assays with three tumour cell lines. Doses of X-rays and inhibitors and timing of 5-FU were chosen based on published data and preliminary work performed in our laboratory confirming radiation sensitization.

In all cell lines the combination of 5-FU and radiation showed to arrest cell growth to a greater extent compared to radiotherapeutic treatment alone. However, HT29 cells were confirmed to be more resistant than COLO320 and HCT116 cells also to the combination of 5-FU and radiation.

Inhibition of RAS/RAF/ERK pathway was evaluated as a strategy to sensitize rectal cancer cell lines to radiation. This was achieved by specific BRAF inhibitors, BRAF silencing and HSP990, a dual inhibitor of HSP90 and TRAP1. The inhibition of B-Raf protein with PLX4720 showed a moderate but not significant sensitizing effect, according to literature results. Conversely, BRAF siRNA silenced cells resulted significantly more sensitive than control and negative transfected cells. HSP990 significantly increases the radiosensitivity of BRAF-mutated cells, consistently with the chaperoning activity of HSP90 chaperones toward BRAF.

TRAP1 is a HSP90 molecular chaperone deregulated in human tumours and responsible for specific features of cancer cells, i.e., protection from apoptosis, drug resistance, metabolic regulation, and protein quality control/ubiquitination. Because HSP90 is the main molecular chaperone

responsible for BRAF folding, with specific affinity greater for its mutated form, HSP90 targeting is presently evaluated as an antitumour strategy in human BRAF-mutated neoplasms. In this scenario, we found that TRAP1 could play a role in radiation resistance. Indeed, transfection of resistant HT29 cells with TRAP1 siRNAs increased cancer cell killing. The role of TRAP1 in radioresistance is confirmed in stable clones of HCT116 cells in which TRAP1 is silenced. Lastly, with daily irradiation the expression of TRAP1 increased, suggesting a role also in the adaptive response to radiation.

Conclusions

Concurrent radiotherapy and radiation sensitizing 5-FU based chemotherapy is a common treatment strategy for colorectal malignancies. Despite aggressive chemoradiotherapy, a subgroup of tumours which undergo to chemoradiation do not achieve a clinically valuable response and local failure remains a troubling clinical problem that requires development of more effective regimens. In such a context, CRCs are known to frequently have activation of the Ras-Raf-MEK-ERK pathway via activating Ras/Braf mutations or EGFR pathway activation, and this likely represents a mechanism responsible for resistance to chemoradiation. Specifically, our study highlight the relevance of BRAF mutations as determinant of radioresistance.

To improve outcomes in patients with CRCs with a BRAF mutation, there is a critical need to better understand the mechanisms of resistance. We describe our attempts to enhance the radiation response with 5-FU. Our data suggest that concurrent treatment with 5-FU may be used in patients with colorectal malignancies to augment radiation response.

Our study shows that in CRC cell lines there is a relationship between V600E BRAF mutation and response to radiation with or without 5-FU, suggesting that BRAF mutation might be used as a predictive biomarker of response to neoadjuvant therapy in CRCs. However, the low frequency of V600E BRAF mutation must be considered (3-15%), as well as the need to confirm in vivo the results we have obtained.

Our study also suggests that the inhibition of molecules as TRAP1, involved in the regulation of B-Raf, may represent a treatment strategy for V600E-mutated CRCs, a subgroup characterized by a more aggressive biological behavior and a reduced responsiveness to conventional treatments.

In conclusion, our study may aid in better understanding the molecular mechanism that control response to radiation in cancer cells. Additionally, our

findings may contribute to the development of more effective strategies of combining radiation therapy with other systemic therapies. Our results provide rational therapeutic strategies for clinical studies in this poor prognosis subtype of CRC.

Introduction

Colon and rectal cancers are typically grouped and staged similarly. However, their management is different. Owing to the pelvic location of the rectum and its proximity to the anal sphincter and bladder, as well as to sympathetic and parasympathetic nerves, patients with rectal cancers present a substantial surgical challenge compared with individuals affected by colon cancer. Although current sharp radial resection of the tumour (total mesorectal excision) is curative for small tumours, the risk of locoregional recurrence, distant metastasis and death increases with tumours extending through the muscularis propria (T3 or T4) or nodal involvement (N1 or N2; stage II and III tumours).

One strategy to minimize recurrence in patients with rectal cancer is neoadjuvant chemoradiotherapy. Preoperative ionizing radiation might downstage tumours, facilitate surgical intervention, minimize surgical complications and decrease locoregional recurrence. Individual, randomized, controlled studies using low doses of neoadjuvant radiation therapy have failed to demonstrate a survival advantage in patients with rectal cancer. Yet, recent studies employing higher doses of preoperative radiation and chemotherapy revealed a distinct survival advantage utilizing this preoperative modality.

Regardless of the benefits in locoregional recurrence or survival in patients subjected to ionizing radiation, a major objective of this preoperative treatment is to reduce the tumour. Complete pathological response has been observed in most studies evaluating neoadjuvant chemoradiation, and it is a desirable outcome prior to surgical intervention. The rate of complete response in a given patient population is an objective and measurable outcome to assess the effectiveness of a neoadjuvant chemoradiotherapeutic regimen. Pre-sensitizing agents have classically been 5-fluorouracil-based given in combination with ionizing radiation. However, regardless of the preoperative agent used (i.e., 5-fluorouracil, irinotecan, oxaliplatin, capecitabine, bevacizumab and cetuximab), the rate of complete response remains substantially wide.

Preoperative radiation, alone or in combination with chemotherapy, results in a tremendously wide range of response. From one side of the spectrum, 10-25% of patients are able to achieve a complete pathological response following neoadjuvant chemoradiation. Yet, on the other side of this wide range, a considerable number of patients do not respond to ionizing radiation. In fact, in some cases, the tumour continues to grow in spite of neoadjuvant treatment.

Thus, the magnitude of benefit is heterogeneous across all trials and the ability of neoadjuvant therapy to minimize tumour burden is extraordinarily unpredictable.

While patients with breast cancers are not subjected to the adverse side effects of tamoxifen or trastuzumab if their tumours are negative for estrogen, progesterone or Her-2/Neu, neoadjuvant ionizing radiation with concurrent chemotherapeutic agents is administered almost universally to patients with stage II/III rectal cancers, despite the tremendously wide range of response to this preoperative modality in patients receiving the same form of treatment. The specific phenotype of the tumour plays a major role in rendering tumour survival advantage to the cytotoxic effects of chemoradiation. Pathways such as proliferation, cell cycle, apoptosis and hypoxia have been investigated under a variety of conditions in pre-irradiated tissues and post-irradiated tumours.

Ionizing radiation results in intracellular free radical formation, which leads to DNA damage by causing DNA base damage, DNA single-strand breaks (SSBs), DNA double-strand breaks (DSBs), DNA protein cross-links and installed replication forks. Alternatively, membrane effects cause signal transduction, which might result in gene expression of cell cycle regulators, growth factor production, or oxidative-stress pathway activation. Another pathway leading to cell death by ionizing radiation results from the induction of DNA damage, which interferes with DNA replication leading to premature segregation into mitosis and defects in maintaining cell cycle arrest. This leads to mitotic catastrophe.

The classical pathway of radio-induced cell death begins with signal transduction mechanisms that cause cell cycle arrest and initiate DNA repair mechanisms. If the cell is unable to successfully repair the DNA damage induced by ionizing radiation, it undergoes apoptosis. Radiation injury causes increased levels of p53, and cell cycle arrest ensues via the up-regulation of cyclin-dependent kinase inhibitors p21 and p27. DNA damage leads to an increase in p53, which then causes cell cycle arrest via an increase in cyclin-dependent kinase inhibitor (p21). If the cell is unable to repair itself, the sustained levels of p53 lead to a release of BAX, which then leads to apoptosis. Bcl-2 keeps a baseline suppression on p53. Similarly, p53 down-regulates survivin, which in turn stimulates apoptosis by removing the inhibitory effects of survivin on the caspases.

Resistance to drugs and radiation is the major cause of anticancer treatment failure in rectal cancer. Indeed, resistance is a multifactorial phenomenon

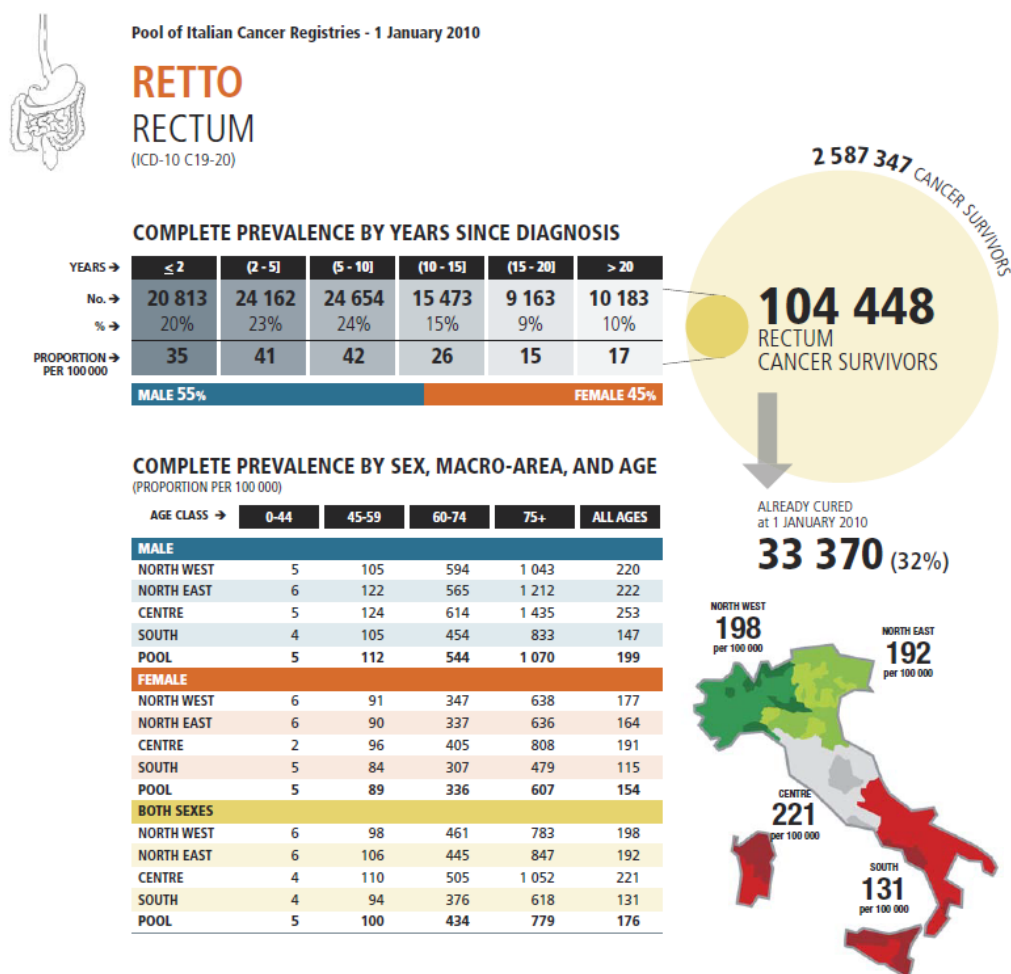
involving multiple pathways, including changes in cellular responses, increased ability to repair DNA damage or tolerate stress conditions, acquired mechanisms foreshadowing apoptosis. Adaptive responses to stress conditions, such as increases in DNA repair activities or antioxidant defenses, may contribute to resistance and escape from apoptosis in tumour cells. Cancer cells produce increased amounts of reactive oxygen species (ROS), especially when they are irradiated. Adaptive responses to oxidative stress can cause the activation of pro-survival mechanisms.

A wide variety of pathways have been identified as responsible for drug resistance; results about radiation resistance are limited and sometimes conflicting. In such a perspective, the aims of this research are: to review literature investigations that have been performed to identify molecular biomarkers differentiating responsive and resistant tumours; to summarize our experience of neoadjuvant chemoradiotherapy in rectal cancer patients; to find a range of radiation dose to which colorectal cancer cell lines are sensitive; to establish whether BRAF-mutated rectal carcinoma cells are resistant to radiation; to assess whether TRAP1 is responsible for this radiation-resistant phenotype in human colorectal carcinoma cells; to hypothesize therapeutic approaches to overcome radiation resistance and restore sensitivity to neoadjuvant treatments.

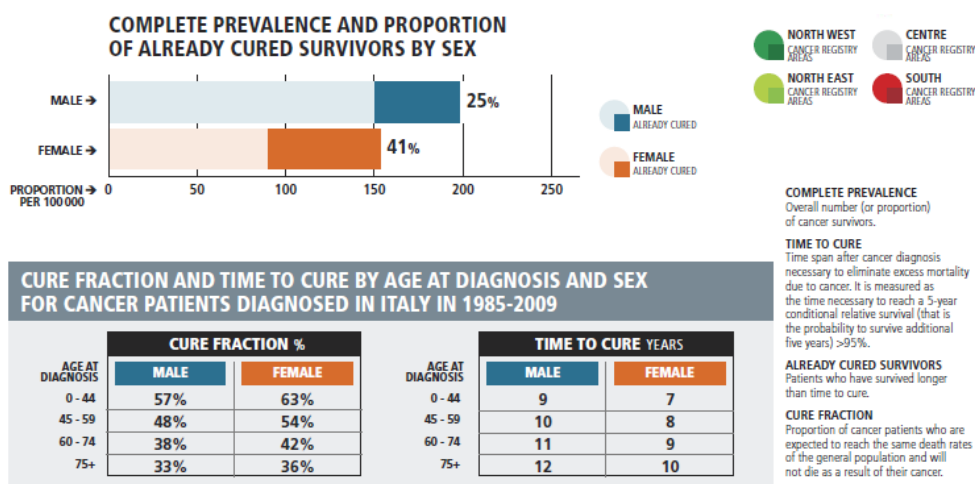
An understanding of the mechanisms leading to tumour cell radiation resistance might result in optimal operative intervention. Tailoring treatment to a specific molecular phenotype should be the cornerstone of chemoradiotherapeutic interventions. If radiosensitive tumours could be identified, a selective and individualized form of chemoradiation might be instituted and radioresistant tumours could be sensitized.

1. Rectal cancer and chemoradiation

Colorectal carcinoma is among the top five causes of cancer death in developed countries. Epidemiological information about rectal tumours in Italy at 1st January 2010 are illustrated in tabs. 1-2.



Tab. 1- Epidemiological data on rectal cancer in Italy (part A).



Tab 2- Epidemiological data on rectal cancer in Italy (part B)¹.

More than a quarter of these deaths are from tumours that arise from the rectum². In the setting of advanced disease, tumours originating in the colon and rectum are treated identically and clinical trials of systemic therapy routinely include tumours arising from all sites in the large bowel, including the rectum. However, in the setting of early-stage disease, there are distinct natural histories and approaches to treatment that stem from the vascular supply of the rectum, which drains to inferior vena cava rather than the portal vein. As a result, rectal cancers are somewhat less likely to metastasize to the liver and more likely to spread to the lung. However, the most notable feature of rectal cancer is that it has propensity to recur locally in the pelvis. As a consequence, the approach to treatment necessitates emphasis on both local control and distant spread.

The mainstay of treatment for rectal cancer is surgery. During the 1970s and 1980s, local recurrence rates following surgical resection of rectal cancer often were in excess of 50% and resulted in tremendous morbidity. Since, Heald et al.³ demonstrated in 1982 better oncologic outcome by using a total mesorectal excision (TME), which resulted in lower local recurrence rates, the TME has become the standard surgical approach for treating rectal cancer at present. Parallel to improvements in surgical technique, adjuvant therapy

¹ AIRTUM. I tumori in Italia. Epidemiol Prev 2014;38(6)Suppl1:1-144.

² Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013;63:11-30.

³ Heald RJ, Husband EM, Ryall RD. The mesorectum in rectal cancer surgery: the clue to pelvic recurrence? Br J Surg 1982;69:613-6.

regimens have been tested in clinical trials in an effort to reduce the local recurrence rate. Clinical trials demonstrating that postoperative radiation decreased rates of local recurrence and that 5-fluorouracil (5-FU)-based adjuvant chemotherapy could improve results ushered in the era of modern rectal cancer therapy. Since a consensus statement published in 1990, trimodality therapy with surgery, radiation, and systemic chemotherapy has been the standard approach to treatment of locally advanced rectal cancers (stage II and III)⁴. During the intervening near quarter century, considerable advances have been made.

1.1 Pretreatment evaluation

Rectal cancer staging is an essential component of treatment planning and determines whether trimodality therapy with chemotherapy, radiation and surgery is necessary. Careful staging provides critical information about the likelihood of achieving a complete resection (R0) and treatment modalities: early-stage disease (T1N0 or T2N0) can be managed with surgery alone; locally advanced disease includes T3 and T4 tumours, as well as T2 tumours with evidence of regional lymph node involvement.

Preoperative histopathologic diagnosis is essential. Although most rectal tumours are adenocarcinoma, squamous cell tumours and carcinoids and less commonly melanomas also are found and require different management strategies. Ascertainment of microsatellite instability may provide useful information about inherited cancer susceptibility, particularly for pts (pts) diagnosed at early age.

Digital rectal exam is important. It identifies distal tumours that are likely to require an abdominal perineal resection, which includes the anorectal sphincter and thus a permanent colostomy. In addition, rectal exam identifies tumours that are fixed and potentially adherent to local structures, such as prostate, seminal vesicles, or vagina.

Proctoscopy is important to establish the precise location of the primary tumour within the rectum. It is important to know the location of the tumour with respect to the peritoneal reflection and the rectal sphincter.

Staging should include a full colonoscopy to the cecum to identify synchronous polyps or second primary tumours. Their identification can

⁴ NIH consensus conference. Adjuvant therapy for patients with colon and rectal cancer. JAMA 1990;264:1444-50.

influence surgical planning. Metastatic disease should be ruled out with a contrast-enhanced CT scan of the chest, abdomen and pelvis. It is especially important to include the chest because of the propensity for rectal cancer to bypass the portal circulation and spread to the lung. Although CT scan may identify the primary rectal cancer and regional lymph nodes, it has limited accuracy for establishing the extent of tumour penetration into the rectal wall or lymph node involvement.

Routine bloodwork, including complete blood count, establishes the ability to withstand the myelosuppressive effects of chemoradiation. Liver function tests help to rule out metastatic disease and the ability to withstand systemic treatment. Finally, a carcinoembryonic antigen test (CEA) is a tumour marker that often is a useful indicator of disease burden⁵.

Endorectal ultrasound (ERUS) and MRI are the most accurate strategies for evaluating T and N stage. ERUS is highly dependent on operator skill and expertise⁶. MRI is less subject to operator expertise but to achieve high-caliber images requires pts to minimize motion during the scanning procedure. The use of MRI is increasing as are technological advances in imaging techniques, and as a result it is better able to delineate encroachment on the mesorectal fascia and thereby the potential for a positive radial margin⁷. Together with MRI, also PET has a role in the prediction of pathologic complete response after radiochemotherapy⁸.

⁵ National Comprehensive Cancer Network. NCCN guidelines rectal cancer version 2. 2015.

⁶ Marone P, de Bellis M, D'Angelo V et al. Role of endoscopic ultrasonography in the loco-regional staging of patients with rectal cancer. *World J Gastrointest Endosc* 2015;7(7):688-701.

⁷ Beets-Tan RG, Beets GL, Vliegen RF et al. Accuracy of magnetic resonance imaging in prediction of tumour-free resection margin in rectal cancer surgery. *Lancet* 2001;357:497-504.

MERCURY Study Group. Diagnostic accuracy of preoperative magnetic resonance imaging in predicting curative resection of rectal cancer: prospective observational study. *BMJ* 2006;333:779.

⁸ Maffione AM, Marzola MC, Capirci C et al. Value of (18)F-FDG PET for predicting response to neoadjuvant therapy in rectal cancer: systematic review and meta-analysis. *Am J Roentgenol* 2015;204(6):1261-8.

Joye I, Deroose CM, Vandecaveye V et al. The role of diffusion-weighted MRI and 18F-FDG PET/CT in the prediction of pathologic complete response after radiochemotherapy for rectal cancer: a systematic review. *Radiotherapy and Oncology* 2014;113:158-65.

van Stiphout R, Valentini V, Buijsen J et al. Nomogram predicting response after chemoradiotherapy in rectal cancer using sequential PETCT imaging: a multicentric prospective study with external validation. *Radiotherapy and Oncology* 2014;113:215-22.

It is important to note that no imaging modality is able to predict lymph node involvement perfectly. Even very small lymph nodes less than 5 mm have potential to contain tumour. As a result, clinical staging of rectal cancer involves estimation⁹.

1.2 Preoperative versus postoperative radiation

Advantages that have often been associated with preoperative radiotherapy (RT), as opposed to RT given postoperatively, are related to both tumour response and preservation of normal tissue. First of all, reducing tumour volume may facilitate resection and increase the likelihood of a sphincter-sparing procedure¹⁰. Second, irradiating tissue that is surgery-naive and thus better oxygenated may result in increased sensitivity to RT. Tumour cells are significantly more sensitive to an equivalent dose of RT in the presence of oxygen as opposed to hypoxic conditions¹¹. Third, preoperative RT can avoid the occurrence of radio-induced injury to the small bowel trapped in the pelvis by postsurgical adhesions¹². Finally, the anastomosis remains unaffected by the effects of RT because irradiated tissue is resected. Preoperative RT that includes structures that will be resected increases the likelihood that an anastomosis with a healthy colon can be performed. However, one disadvantage of using preoperative RT is the possibility of over-treating early-stage tumours that do not require adjuvant RT. Recent improvements in preoperative staging techniques have allowed for more accurate staging, but the risk of over-staging the disease has not been eliminated¹³.

An interval analysis at a median follow-up of 1 year of the first 116 pts enrolled in the NSABP R-03 trial showed an increase in sphincter preservation favoring the preoperative arm (44% vs. 34%), with a similar incidence of postoperative toxicities in pre- and postoperative chemoradiotherapy (CRT)

⁹ Schrag D. Evolving role of neoadjuvant therapy in rectal cancer. *Current Treatment Options in Oncology* 2013;14:350-64.

¹⁰ Wagman R, Minsky BD, Cohen AM et al. Sphincter preservation in rectal cancer with preoperative radiation therapy and coloanal anastomosis: long term follow-up. *Int J Radiat Oncol Biol Phys* 1998;42:51-7.

¹¹ Kachnic LA. Should preoperative or postoperative therapy be administered in the management of rectal cancer? *Semin Oncol* 2006;33(6Suppl11):S64-9.

¹² Sauer R, Becker H, Hohenberger W et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004;351:1731-40.

¹³ Madoff RD. Chemoradiotherapy for rectal cancer-when, why and how? *N Engl J Med* 2004;351:1790-2.

arms¹⁴. Wagman et al.¹⁵ demonstrated that preoperative RT allowed sphincter preservation in 77% of selected pts who would otherwise have required an abdominoperineal resection and that 85% of those pts had good to excellent sphincter function. In the CAO/ARO/AIO 94 study, Sauer et al.¹⁶ randomly assigned 805 pts with clinical stage II or III rectal cancer to preoperative or postoperative regimens of CRT. With a median follow-up of 4 years, no significant differences between preoperative and postoperative CRT were reported in the primary endpoint of 5-year overall survival (OS) (74% vs. 76%, $p=0.32$). However, treatment compliance (92% vs. 54%; $p<0.001$), grades 3–4 acute and late toxicity profiles (27% vs. 40%, $p=0.001$), tumour (8% vs. 0%, $p<0.001$) and nodal (25% vs. 40%, $p=0.001$) downstaging, and rates of pelvic recurrence (6% vs. 13%, $p=0.006$), all favored the preoperative CRT arm. In recently published long-term follow-up data of this trial, the improvement in local control persisted, with the 10-year cumulative incidence of local relapse at 7.1% and 10.1% in the preoperative and the postoperative arms, respectively ($p=0.048$). Also, in a recent trial, no significant differences were detected for the 10-year cumulative incidences of distant metastases (29.8% vs. 29.6%, $p=0.9$), disease-free survival (DFS) (68.1% vs. 67.8%, $p=0.65$), and OS (59.6% vs. 59.9%, $p=0.85$)¹⁷.

Based on above results, preoperative CRT is associated with enhanced sphincter-preservation, significant tumour and nodal downstaging, improved acute and late tolerability, improved local control and at least similar survival. Therefore, preoperative CRT is now considered the standard of care for pts with stages II and III rectal cancer.

1.3 Long-course or short-course radiation

¹⁴ Hyams DM, Mamounas EP, Petrelli N et al. A clinical trial to evaluate the worth of preoperative multimodality therapy in patients with operable carcinoma of the rectum: a progress report of National Surgical Breast and Bowel Project Protocol R-03. *Dis Colon Rectum* 1997;40:131-9.

¹⁵ Wagman R, Minsky BD, Cohen AM et al. Sphincter preservation in rectal cancer with preoperative radiation therapy and coloanal anastomosis: long term follow-up. *Int J Radiat Oncol Biol Phys* 1998;42:51-7.

¹⁶ Sauer R, Becker H, Hohenberger W et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004;351:1731-40.

¹⁷ Sauer R, Liersch T, Merkel S et al. Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. *J Clin Oncol* 2012;30:1926-33.

The best course of neoadjuvant treatment has not yet been determined. Many European investigators over the past two decades have investigated preoperative RT alone for stages II and III rectal cancer, most commonly as a short, high-dose-per-fraction course. However, the United States has not adopted a short-course RT approach because the potential for late radiation morbidity and anorectal dysfunction remains a significant concern with hypofractionation. In the United States and Europe, stage II or higher rectal cancers are more commonly treated with preoperative CRT consisting of 45 to 50.4 Gy of RT in conjunction with infusion 5-FU-based chemotherapy. The RT is delivered over a period of 5 to 6 weeks, and surgery (low anterior resection, LAR, or abdominal perineal resection, APR) is done 6 to 10 weeks after completion of the radiation therapy. The combination of preoperative RT with infusion 5-FU-leucovorin (LV) often results in a dramatic reduction in tumour size (or downstaging) and may result in an apparent complete eradication of the tumour in up to 25% of the cases. Neoadjuvant CRT may increase the ability of the surgeon to preserve continence by downstaging the cancer, in some cases shrinking tumour size to permit the achievement of a cancer-free margin at the distal extent of the resection, when a clear margin that will permit an anastomosis in the anal canal cannot be achieved without such shrinkage¹⁸.

In Europe, a short course of RT followed by extirpative surgery (LAR or APR) remains a possible approach. Several European studies have looked at the efficacy of a shorter course of preoperative RT (25 Gy over 5 days), not combined with chemotherapy, for the treatment of rectal cancer. In a Swedish rectal cancer trial, the results showed a survival advantage and a decreased rate

¹⁸ Dahlberg M, Glimelius B, Graf W et al. Preoperative irradiation affects functional results after surgery for rectal cancer: results from a randomized study. *Dis Colon Rectum* 1998;41:543-9.

King M, Tolan S, Giridharan S et al. Late toxicity after short course preoperative radiotherapy and total mesorectal excision for resectable rectal cancer. *Clin Oncol (R Coll Radiol)* 2003;15:233-6.

Peeters KC, van de Velde CJ, Leer JW et al. Late side effects of short-course preoperative radiotherapy combined with total mesorectal excision for rectal cancer: increased bowel dysfunction in irradiated patients: a Dutch colorectal cancer group study. *J Clin Oncol* 2005;23:6199-206.

Bujko K, Rutkowski A, Chang GJ et al. Is the 1-cm rule of distal bowel resection margin in rectal cancer based on clinical evidence? A systematic review. *Ann Surg Oncol* 2012;19:801-8.

Pollack J, Holm T, Cedermark B et al. Long-term effect of preoperative radiation therapy on anorectal function. *Dis Colon Rectum* 2006;49:345-52.

of local recurrence with this approach compared with surgery alone¹⁹. However, a follow-up study showed that short-course preoperative RT had caused relatively increased risk for postoperative hospitalization due to bowel obstructions and other gastrointestinal (GI) complications²⁰. Despite improvements in local control of disease, some studies have demonstrated that preoperative short-course RT for rectal cancer pts does not affect their overall survival significantly²¹. A recent multicenter, randomized study of 1,350 pts with rectal cancer compared short-course preoperative preoperative RT and no postoperative treatment with no preoperative RT and a postoperative approach that included CRT in selected pts (i.e., those with a positive circumferential margin) and no RT in pts without evidence of residual disease following surgery²². Results indicated that pts in the preoperative RT arm had significantly lower local recurrence rates and a 6% absolute improvement in 3-year disease free survival ($p=0.03$), although no difference in overall survival was observed between the arms of the study²³. In a long-term (12-year) follow-up of a Dutch TME trial, preoperative short-course RT reduced the 10-year local recurrence by more than 50% relative to surgery alone, but without an overall survival benefit. This study showed that for pts with TNM stage III cancer with a negative circumferential resection margin, the 10-year survival was 50% in the preoperative RT group versus 40% in the surgery-alone group ($p=0.032$). However, this long-term follow-up showed that secondary malignancies and other non-rectal-cancer causes of death were more frequent in the RT than in

¹⁹ Swedish Rectal Cancer Trial Group. Improved survival with preoperative radiotherapy in resectable rectal cancer. *N Engl J Med* 1997;336:980-7.

²⁰ Birgisson H, Pahlman L, Gunnarsson U et al. Adverse effects of preoperative radiation therapy for rectal cancer: long-term follow-up of the Swedish Rectal Cancer Trial. *J Clin Oncol* 2005;23:8697-705.

²¹ Peeters KC, Marijnen CA, Nagtegaal ID et al. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. *Ann Surg* 2007;246:693-701.

Siegel R, Burock S, Wernecke KD et al. Preoperative short-course radiotherapy versus combined radiochemotherapy in locally advanced rectal cancer: a multi-centre prospectively randomised study of the Berlin Cancer Society. *BMC Cancer* 2009;9:50.

²² Sebag-Montefiore D, Stephens RJ, Steele R et al. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009;373:811-20.

²³ Stephens RJ, Thompson LC, Quirke P et al. Impact of short-course preoperative radiotherapy for rectal cancer on patients' quality of life: data from the Medical Research Council CR07/National Cancer Institute of Canada Clinical Trials Group C016 randomized clinical trial. *J Clin Oncol* 2010;28:4233-9.

the control group, negating any survival advantage in the node-negative subpopulation. Nevertheless, the authors concluded that preoperative short-term RT significantly improved the 10-year survival in pts with a negative circumferential margin and TNM stage III cancer²⁴. Results from a Polish rectal cancer trial showed that short-term RT was as effective as long-course CRT in the aspects of local recurrence and survival²⁵. Similarly, in the Trans-Tasman Radiation Oncology Group Trial 01.04 that randomized 326 pts to short-course RT or long-course CRT, the 3-year local recurrence rates (cumulative incidence) were 7.5% for short-course RT and 4.4% for long-course CRT (p=0.240). The 5-year distant recurrence rates were 27% for short-course RT and 30% for long-course CRT (p=0.920). The overall survival rates at 5 years were 74% for short-course RT and 70% for long-course CRT (p=0.620). The late toxicity rates were not substantially different (p=0.530)²⁶. Additionally, results from an interim analysis of the Stockholm III trial showed that short-course RT in combination with delayed surgery was feasible and had a downstaging effect²⁷. Based on the above results, short-course RT appears to provide effective local control and the same OS as more long-course CRT schedules and, therefore, may be an appropriate choice in some situations.

1.4 Concurrent chemoradiotherapy

In the early 1990s, preoperative RT was considered in most European countries as the standard treatment for T3–4 rectal cancers. Conversely, a National Institutes of Health Consensus Conference stated that postoperative CRT should be regarded as the standard treatment for pts with stages II and III rectal cancer. Thus, the evaluation of concurrent chemotherapy and RT had become an attractive field of research. The putative benefits of the addition of

²⁴ van Gijn W, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomized controlled TME trial. *Lancet Oncol* 2011;12:575-82.

²⁵ Bujko K, Nowacki MP, Nasierowska-Guttmejer A et al. Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative conventionally fractionated chemoradiation for rectal cancer. *Br J Surg* 2006;93:1215-23.

²⁶ Ngan SY, Burmeister B, Fisher RJ et al. Randomized trial of short-course radiotherapy versus long-course chemoradiation comparing rates of local recurrence in patients with T3 rectal cancer: Trans-Tasman Radiation Oncology Group trial 01.04. *J Clin Oncol* 2012;30:3827-33.

²⁷ Pettersson D, Cedermark B, Holm T et al. Interim analysis of the Stockholm III trial of preoperative radiotherapy regimens for rectal cancer. *Br J Surg* 2010;97:580-7.

chemotherapy concurrent with either pre- or postoperative RT include local RT sensitization and systemic control of disease (eradication of micrometastases). Also, preoperative CRT has the potential to increase the rates of pathologic complete response and sphincter preservation²⁸.

In 1993, the European Organization for Research and Treatment of Cancer (EORTC) initiated a four-arm, randomized trial (EORTC 22921) to examine the value of preoperative CRT versus preoperative RT alone and the value of additional chemotherapy versus none with respect to overall survival and progression-free survival. In preliminary results, after preoperative CRT, tumours were smaller ($p=0.0001$), had less advanced pT ($p=0.001$) and pN stages ($p=0.001$), had small numbers of examined nodes ($p=0.046$) and had less frequent lymphovascular or perineural invasions ($p=0.008$). Mucinous tumours increased after preoperative CRT ($p=0.001$)²⁹. However, more mature results from EORTC 22921 showed no significant difference in OS between the groups that received chemotherapy preoperatively ($p=0.840$) and those that received it postoperatively ($p=0.120$), and the 5-year cumulative incidence rates for local recurrences were 8.7%, 9.6%, and 7.6% in the groups that received chemotherapy preoperatively, postoperatively, or both, respectively, and 17.1% in the group that did not receive chemotherapy ($p=0.002$). The authors indicated that, in pts with rectal cancer who receive preoperative RT, adding 5-FU-based chemotherapy preoperatively or postoperatively had no significant effect on survival. However, they concluded that chemotherapy, regardless of whether it

²⁸ Latkauskas T, Pauzas H, Gineikiene I et al. Initial results of a randomized controlled trial comparing clinical and pathological downstaging of rectal cancer after preoperative short-course radiotherapy or long-term chemoradiotherapy, both with delayed surgery. *Colorectal Dis* 2012;14:294-8.

Gerard JP, Conroy T, Bonnetain F et al. Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3-4 rectal cancers: results of FFCO 9203. *J Clin Oncol* 2006;24:4620-5.

McCarthy K, Pearson K, Fulton R et al. Pre-operative chemoradiation for non-metastatic locally advanced rectal cancer. *Cochrane Database Syst Rev* 2012;12:CD008368.

De Caluwe L, Van Nieuwenhove Y, Ceelen WP. Preoperative chemoradiation versus radiation alone for stage II and III resectable rectal cancer. *Cochrane Database Syst Rev* 2013;2:CD006041.

²⁹ Bosset JF, Calais G, Mineur L et al. Enhanced tumorocidal effect of chemotherapy with preoperative radiotherapy for rectal cancer: preliminary results-EORTC 22921. *J Clin Oncol* 2005;23:5620-7.

was administered before or after surgery, conferred a significant benefit with respect to local control³⁰.

The FFCO 9203 trial for pts with T3–4 rectal cancer without evidence of distant metastases showed no difference in sphincter preservation between the preoperative RT alone and the preoperative CRT with 5-FU/LV groups. However, complete sterilization of the operative specimen was more frequent with CRT (11.4% vs. 3.6%, $p=0.050$). The 5-year incidence of local recurrence was lower with CRT (8.1% vs. 16.5%; $p=0.050$), but the 5-year OSs in the two groups were not different³¹. Also, some systematic reviews³² concluded that the addition of chemotherapy to preoperative RT enhanced the pathologic response and improved local control, but had no effect on DFS and OS.

Many chemotherapy regimens have been examined in the adjuvant therapy of rectal cancer, although virtually all have been based on 5-FU. A previously-reported GI intergroup trial of continuous-infusion 5-FU during radiation therapy in an attempt to maximize local control demonstrated no significant improvement in local tumour control, but statistically-significant improvements in DFS and OS compared with bolus 5-FU³³. However, most of the pts in that study had node-positive disease. On the other hand, the final reports of the Intergroup 0114³⁴ and the GI INT 0144³⁵ trials demonstrated that similar outcomes with respect to OS and relapse-free survival were observed when an infusion 5-FU or bolus 5-FU/LV was administered concurrently with postoperative RT. Till now, there has been no clinically-meaningful difference

³⁰ Bosset JF, Collette L, Calais G et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006;355:1114-23.

³¹ Gerard JP, Conroy T, Bonnetain F et al. Preoperative radiotherapy with or without concurrent fluorouracil and leucovorin in T3-4 rectal cancers: results of FFCO 9203. *J Clin Oncol* 2006;24:4620-5.

³² McCarthy K, Pearson K, Fulton R et al. Pre-operative chemoradiation for non-metastatic locally advanced rectal cancer. *Cochrane Database Syst Rev* 2012;12:CD008368.

De Caluwe L, Van Nieuwenhove Y, Ceelen WP. Preoperative chemoradiation versus radiation alone for stage II and III resectable rectal cancer. *Cochrane Database Syst Rev* 2013;2:CD006041.

³³ O'Connell MJ, Martenson JA, Wieand HS et al. Improving adjuvant therapy for rectal cancer by combining protracted-infusion fluorouracil with radiation therapy after curative surgery. *N Engl J Med* 1994;331:502-7.

³⁴ Tepper JE, O'Connell M, Niedzwiecki D et al. Adjuvant therapy in rectal cancer: analysis of stage, sex, and local control: final report of intergroup 0114. *J Clin Oncol* 2002;20:1744-50.

³⁵ Twelves C, Wong A, Nowacki MP et al. Capecitabine as adjuvant treatment for stage III colon cancer. *N Engl J Med* 2005;352:2696-704.

in outcome based on FU-only dose schedule. Also, whether 5-FU is biochemically modulated by LV or administered as protracted venous infusion during part of or the entirety of treatment, outcomes were equivalent.

When postoperative CRT is recommended, a “sandwich” approach in which chemotherapy (typically 5-FU-based) is administered before and after the CRT regimen may be commonly used³⁶. This is because postoperative pelvic radiation may compromise the colorectal or coloanal anastomosis and the function of the neorectum.

1.5 Addition of capecitabine

Capecitabine is an oral fluoropyrimidine derivative that is as effective as 5-FU plus folinic acid for adjuvant treatment of stage III colon cancer. It is also not inferior to infusion 5-FU in combination with oxaliplatin for first-line treatment of metastatic colorectal cancer³⁷. Recent studies have shown that capecitabine is equivalent to 5-FU in perioperative CRT therapy. Sanghera et al.³⁸ found similar pathologic complete response rates with capecitabine (17%) and infusion 5-FU (20%) in a meta-analysis of 71 trials with a total of 4,732 pts. In 2012, one randomized trial in which 401 pts with stage II or III rectal cancer received capecitabine or 5-FU-based CRT either pre- or postoperatively showed that capecitabine was not inferior to 5-FU in perioperative CRT therapy³⁹. The 5-year OS in the capecitabine group was not inferior to that in the 5-FU group (76% in the capecitabine group vs. 67% in the 5-FU group, non-inferiority $p=0.0004$). The effect of capecitabine relative to 5-FU was noted for both cohorts, although it was slightly smaller in the neoadjuvant cohort than in the adjuvant cohort (hazard ratio [HR], 1.28; 95% confidence interval [CI], 0.69–2.37 vs. HR, 1.62; 95% CI, 0.92–2.86, respectively). Furthermore, in that

³⁶ Smalley SR, Benedetti JK, Williamson SK et al. Phase III trial of fluorouracil-based chemotherapy regimens plus radiotherapy in postoperative adjuvant rectal cancer: GI INT 0144. *J Clin Oncol* 2006;24:3542-7.

³⁷ Cassidy J, Clarke S, Diaz-Rubio E et al. Randomized phase III study of capecitabine plus oxaliplatin compared with fluorouracil/folinic acid plus oxaliplatin as first-line therapy for metastatic colorectal cancer. *J Clin Oncol* 2008;26:2006-12.

³⁸ Sanghera P, Wong DW, McConkey CC et al. Chemoradiotherapy for rectal cancer: an updated analysis of factors affecting pathological response. *Clin Oncol (R Coll Radiol)* 2008;20:176-83.

³⁹ Hofheinz RD, Wenz F, Post S et al. Chemoradiotherapy with capecitabine versus fluorouracil for locally advanced rectal cancer: a randomised, multicentre, non-inferiority, phase 3 trial. *Lancet Oncol* 2012;13:579-88.

study, the 3-year DFS was higher in the capecitabine group than in the 5-FU group (75% vs. 67%, $p=0.070$)⁴⁰. Based on the above results, capecitabine is an acceptable alternative to infusion 5-FU in those pts who are able to manage the responsibilities inherent in self-administered oral chemotherapy.

1.6 Addition of oxaliplatin

With optimized local treatment, which can be achieved with preoperative RT or CRT and TME surgery, local recurrence rates have been markedly reduced. Another main cause for failure in the treatment of rectal cancer is distant metastases. Any improvement in overall survival will require better control of systemic disease while keeping the rate of local recurrences below 5%–10%. Along these lines, several randomized trials have addressed the addition of oxaliplatin to the chemotherapy regimen.

The National Surgical Adjuvant Breast and Bowel Project (NSABP) Trial R-04 compared protracted venous infusion 5-FU with capecitabine for preoperative treatment of rectal cancer. Addition of oxaliplatin to either regimen was investigated using a two-by-two factorial design. Preliminary data showed that no differences in the pathologic complete response rates, numbers of sphincter-saving surgery, or surgical downstagings were seen between regimens with capecitabine and with 5-FU while toxicity was increased with the inclusion of oxaliplatin.

In the pathologic results of the STAR-01 trial, grades 3 to 4 adverse events during preoperative treatment were more frequent with oxaliplatin plus 5-FU and RT than with RT and 5-FU alone (24% vs. 8%, $p<0.001$) while there were no differences in the sphincter-saving rates, the numbers of pathologically positive lymph nodes, the tumour depths or the pathologically positive circumferential margins. That study reported that adding oxaliplatin to 5-FU-based preoperative CRT significantly increased toxicity without affecting primary tumour response. The OS which is the primary end point of the study will be reported in the future⁴¹.

⁴⁰ Hofheinz RD, Wenz F, Post S et al. Chemoradiotherapy with capecitabine versus fluorouracil for locally advanced rectal cancer: a randomised, multicentre, non-inferiority, phase 3 trial. *Lancet Oncol* 2012;13:579-88.

⁴¹ Aschele C, Cionini L, Lonardi S et al. Primary tumor response to preoperative chemoradiation with or without oxaliplatin in locally advanced rectal cancer: pathologic results of the STAR-01 randomized phase III trial. *J Clin Oncol* 2011;29:2773-80.

The ACCORD 12 trial, in which CRT with capecitabine was compared to CRT with capecitabine and oxaliplatin, showed that at 3 years there were no significant differences in cumulative incidences of local recurrence (6.1% vs. 4.4%), OS (87.6% vs. 88.3%), and DFS (67.9% vs. 72.7%, $p=0.390$)⁴². The initial results of the German CAO/ARO/AIO-04 randomized phase III trial also assessed the addition of oxaliplatin to a 5-FU RT regimen. In contrast to other trials, that study demonstrated that a pathological complete response was achieved in 103 of 591 pts (17%) who underwent surgery in the 5-FU and oxaliplatin group and in 81 of 606 pts (13%) who underwent surgery in the 5-FU group ($p=0.038$). However, that finding might have resulted from the differences in the 5-FU schedule between the arms, and long-term follow-up is needed to assess the DFS⁴³. Based on the above results, concurrent administration of oxaliplatin and RT is not recommended at this time.

1.7 Addition of targeted agents

The epidermal growth factor receptor (EGFR)-targeted monoclonal antibodies cetuximab and panitumumab have shown efficacy as monotherapy in phase III studies in pts with chemotherapy-refractory metastatic colorectal cancer⁴⁴. Preliminary data suggest that EGFR-targeted agents in combination with RT may be synergistic as RT increases EGFR expression within tumour cells while EGFR blockade sensitizes the cells to the effects of RT⁴⁵. In the

⁴² Gerard JP, Azria D, Gourgou-Bourgade S et al. Clinical outcome of the ACCORD 12/0405 PRODIGE 2 randomized trial in rectal cancer. *J Clin Oncol* 2012;30:4558-65.

⁴³ Rodel C, Liersch T, Becker H et al. Preoperative chemoradiotherapy and postoperative chemotherapy with fluorouracil and oxaliplatin versus fluorouracil alone in locally advanced rectal cancer: initial results of the German CAO/ARO/AIO-04 randomised phase 3 trial. *Lancet Oncol* 2012;13:679-87.

⁴⁴ Cunningham D, Humblet Y, Siena S et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004;351:337-45.

Douillard JY, Siena S, Cassidy J et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010;28:4697-705.

Jonker DJ, O'Callaghan CJ, Karapetis CS et al. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007;357:2040-8.

⁴⁵ Bonner JA, Maihle NJ, Folven BR et al. The interaction of epidermal growth factor and radiation in human head and neck squamous cell carcinoma cell lines with vastly different radiosensitivities. *Int J Radiat Oncol Biol Phys* 1994;29:243-7.

Liang K, Ang KK, Milas L et al. The epidermal growth factor receptor mediates radioresistance. *Int J Radiat Oncol Biol Phys* 2003;57:246-54.

setting of locally advanced head and neck cancer, addition of cetuximab to RT enhanced locoregional control and survival⁴⁶. In the multicenter randomized phase II EXPERT-C trial, a significant improvement in OS was seen in pts with KRAS exon 2/3 wild-type tumours treated with cetuximab (p=0.034). However, in those pts, the addition of cetuximab did not improve the primary endpoint of complete response (p=1.000); further evaluation of this regimen is warranted⁴⁷. Another randomized phase II trial, SAKK 41/07, evaluated the efficacy and the safety of panitumumab in combination with capecitabine and external beam RT as a neoadjuvant regimen for pts with wild-type KRAS locally advanced rectal cancer. In that study, although the addition of panitumumab to neoadjuvant CRT in pts with KRAS wild-type rectal cancer resulted in a high pathologic nearly complete or complete rate, it increased toxicity⁴⁸. Thus, more well-designed and large-scaled research on the addition of targeted agents to the CRT regimen required.

1.8 Technique of radiation therapy

The rectum is defined inferiorly from the lowest level of the ischial tuberosities; it ends superiorly before it loses its round shape in the axial plane and connects anteriorly with the sigmoid. Part of the anus may unintentionally be included when using this definition, which is of no concern. Besides rectum, the mesorectum is also a target structure in radiation planning for rectal cancers. With respect to administration of RT, multiple RT fields should include the tumour or tumour bed with a margin, presacral nodes, and the internal iliac nodes. The external iliac nodes should also be included for T4 tumours involving anterior structures; inclusion of the inguinal nodes for tumours invading the distal anal canal can also be considered. In long-course RT,

⁴⁶ Bonner JA, Harari PM, Giralt J et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med* 2006;354:567-78.

Bonner JA, Harari PM, Giralt J et al. Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival. *Lancet Oncol* 2010;11:21-8.

⁴⁷ Dewdney A, Cunningham D, Tabernero J et al. Multicenter randomized phase II clinical trial comparing neoadjuvant oxaliplatin, capecitabine, and preoperative radiotherapy with or without cetuximab followed by total mesorectal excision in patients with high-risk rectal cancer (EXPERT-C). *J Clin Oncol* 2012;30:1620-7.

⁴⁸ Helbling D, Bodoky G, Gauschi O et al. Neoadjuvant chemoradiotherapy with or without panitumumab in patients with wild-type KRAS, locally advanced rectal cancer (LARC): a randomized, multicenter, phase II trial SAKK 41/07. *Ann Oncol* 2013;24:718-25.

recommended doses of RT are typically 45 to 50 Gy in 25 to 28 fractions to the pelvis using multiple fields. Any boost clinical target volumes extend to the entire mesorectum and the presacral region at the involved levels, including 2-cm cephalad and caudad in the mesorectum and 2-cm on a gross tumour within the anorectum. Positioning and other techniques to minimize radiation to the small bowel are encouraged⁴⁹. In preoperative short-course RT, the tumour dose is 25 Gy administered in 5 fractions over 1 week⁵⁰.

1.9 Preoperative restaging after neoadjuvant therapy

Some reports in the literature have questioned whether routine imaging for restaging after preoperative CRT is needed or not. Radiological modalities for staging primary rectal tumours include CT, ERUS and pelvic MR images. At present, fluorine-18-fluorodeoxyglucose PET is widely used in colorectal cancer staging because of its good sensitivity in detecting abnormal metabolism of cancer cells⁵¹. However, many investigators have demonstrated that the accuracy of restaging with imaging modalities, including CT, ERUS, MRI, or PET-CT, after CRT is very low⁵². They proposed that the low accuracy after CRT might be attributed to the effects of radiation on the rectal wall or to alterations of the histopathologic morphology in and around the tumour site. Marked fibrosis of the bowel wall resulting from radiation is easily overestimated by images. Another reason for these results might be the peritumoral desmoplastic reaction. Peritumoral infiltration with inflammatory cells or vascular proliferation is found in and around the tumour site. These alterations in histopathologic morphology were correlated with perilesional enhancement by

⁴⁹ Gay HA, Barthold HJ, O'Meara E et al. Pelvic normal tissue contouring guidelines for radiation therapy: a Radiation Therapy Oncology Group consensus panel atlas. *Int J Radiat Oncol Biol Phys* 2012;83:e353-62.

⁵⁰ Swedish Rectal Cancer Trial Group. Initial report from a Swedish multicentre study examining the role of preoperative irradiation in the treatment of patients with resectable rectal carcinoma. *Br J Surg* 1993;80:1333-6.

Glimelius B, Graffman S, Pahlman L et al. Preoperative irradiation with high-dose fractionation in adenocarcinoma of the rectum and rectosigmoid. *Acta Radiol Oncol* 1982;21:373-9.

⁵¹ Guillem JG, Ruby JA, Leibold T et al. Neither FDG-PET Nor CT can distinguish between a pathological complete response and an incomplete response after neoadjuvant chemoradiation in locally advanced rectal cancer: a prospective study. *Ann Surg* 2013;258:289-95.

⁵² Hanly AM, Ryan EM, Rogers AC et al. Multicenter Evaluation of Rectal cancer ReImaging pOst Neoadjuvant (MERRION) Therapy. *Ann Surg* 2014;259:723-7.

images, often leading to stage overestimation⁵³. However, a need exists to restage tumours for a less invasive approach. Downstaging may permit sphincter- or even organ-preserving approaches that include diligent surveillance without resection⁵⁴. Local tumour upstaging may necessitate more aggressive procedures, such as a multivisceral resection or pelvic exenteration⁵⁵.

1.10 Timing of surgery after preoperative radiation

One of the unresolved questions concerning preoperative CRT for rectal cancer is the timing of surgery. The colorectal surgeon is faced with a dilemma of having to choose between offering immediate radical surgery and interrupting possible ongoing necrosis and further tumour downstaging or offering the possibility of complete tumour regression and nonsurgical management, but with the risk of significantly delaying necessary radical surgery. Therefore, evaluating the outcomes of pts managed by using delayed surgical therapy is fundamental to determine the safety and the potential benefits or harms of this treatment strategy. In terms of tumour downstaging, the Lyon trial showed increased downstaging in the group of pts with delayed

⁵³ Kuo LJ, Chiou JF, Tai CJ et al. Can we predict pathologic complete response before surgery for locally advanced rectal cancer treated with preoperative chemoradiation therapy? *Int J Colorectal Dis* 2012;27:613-21.

⁵⁴ Janjan NA, Khoo VS, Abbruzzese J et al. Tumor downstaging and sphincter preservation with preoperative chemoradiation in locally advanced rectal cancer: the M. D. Anderson Cancer Center experience. *Int J Radiat Oncol Biol Phys* 1999;44:1027-38.

Martin ST, Heneghan HM, Winter DC. Systematic review of outcomes after intersphincteric resection for low rectal cancer. *Br J Surg* 2012;99:603-12.

Kim CJ, Yeatman TJ, Coppola D et al. Local excision of T2 and T3 rectal cancers after downstaging chemoradiation. *Ann Surg* 2001;234:352-8.

Bonnen M, Crane C, Vauthey JN et al. Long-term results using local excision after preoperative chemoradiation among selected T3 rectal cancer patients. *Int J Radiat Oncol Biol Phys* 2004;60:1098-105.

Smith FM, Chang KH, Sheahan K et al. The surgical significance of residual mucosal abnormalities in rectal cancer following neoadjuvant chemoradiotherapy. *Br J Surg* 2012;99:993-1001.

Martin ST, Heneghan HM, Winter DC. Systematic review and meta-analysis of outcomes following pathological complete response to neoadjuvant chemoradiotherapy for rectal cancer. *Br J Surg* 2012;99:918-28.

⁵⁵ Smith JD, Nash GM, Weiser MR et al. Multivisceral resections for rectal cancer. *Br J Surg* 2012;99:1137-43.

Gannon CJ, Zager JS, Chang GJ et al. Pelvic exenteration affords safe and durable treatment for locally advanced rectal carcinoma. *Ann Surg Oncol* 2007;14:1870-7.

RT-to-surgery interval (6–8 weeks from the end of preoperative RT), although this finding did not lead to significantly increased sphincter-preservation rates⁵⁶. Although longer intervals have been shown to be associated with an increase in pathologic complete response rate, whether such longer intervals are associated with clinical benefit is unclear⁵⁷. Specific reasons may exist for planning a resection at a shorter or a longer interval from end of radiation. A large, bulky tumour showing a good response at evaluation at the end of radiation may be a reason for postponing the resection whereas a progressive disease would necessitate early surgery. Although monitoring the tumour response is difficult, response monitoring may be helpful for tailoring patient management regarding the timing of surgery⁵⁸. Especially, this may be helpful for identifying progressive disease requiring early surgery. In general, for pts treated with preoperative CRT, most surgeons recommend an interval of 5 to 12 weeks following the completion of full-dose CRT (45–50 Gy) prior to surgical resection in order to allow the patient to recuperate from CRT-associated toxicities⁵⁹.

1.11 Surgery

The operation for distal tumours, typically those lower than 4-5 cm from the anal verge, is an APR that necessitates a permanent ostomy. The operation for tumours at 5 cm and above is typically LAR, which is often performed with a temporary diverting ostomy to optimize anastomotic healing and to minimize the risk of leak and resulting pelvic abscess.

⁵⁶ Francois Y, Nemoz CJ, Baulieux J et al. Influence of the interval between preoperative radiation therapy and surgery on downstaging and on the rate of sphincter-sparing surgery for rectal cancer: the Lyon R90-01 randomized trial. *J Clin Oncol* 1999;17:2396.

⁵⁷ Habr-Gama A, Perez RO, Proscurshim I et al. Interval between surgery and neoadjuvant chemoradiation therapy for distal rectal cancer: does delayed surgery have an impact on outcome? *Int J Radiat Oncol Biol Phys* 2008;71:1181-8.

Moore HG, Gittleman AE, Minsky BD et al. Rate of pathologic complete response with increased interval between preoperative combined modality therapy and rectal cancer resection. *Dis Colon Rectum* 2004;47:279-86.

Tulchinsky H, Shmueli E, Figer A et al. An interval >7 weeks between neoadjuvant therapy and surgery improves pathologic complete response and disease-free survival in patients with locally advanced rectal cancer. *Ann Surg Oncol* 2008;15:2661-7.

⁵⁸ Sloothak DA, Geijsen DE, van Leersum NJ et al. Optimal time interval between neoadjuvant chemoradiotherapy and surgery for rectal cancer. *Br J Surg* 2013;100:933-9.

⁵⁹ Kye BH, Cho HM. Overview of radiation therapy for treating rectal cancer. *Ann Coloproctol* 2014;30(4):165-74.

The preferred surgical approach includes removal of the rectum with its fascial covering known as the mesorectum. The TME approach reduces the likelihood of having pathologic involvement of the circumferential (radial) margin, which corresponds to decreased rates of pelvic recurrence⁶⁰.

Rectal cancer operations should be performed by surgeons who have been trained in TME. Considerable evidence suggests that high-volume surgeons achieve more favorable outcomes, and as a result, in many countries, rectal cancer pts are triaged to regional specialty centers for surgical care⁶¹. A U.K. study suggests that laparoscopic and open surgical approaches yield similar outcomes in rectal cancer surgery; however, pts in this study did not have neoadjuvant chemoradiation⁶².

Ongoing studies comparing laparoscopic and open approaches for pts who receive neoadjuvant chemoradiation suggest no differences in the rates of involve circumferential margins, but long-term results are not yet available. Surgery employing robotic assisted approaches has been adopted recently for rectal cancer, but there are not yet any studies demonstrating the superiority of this approach in terms of either short- or long-term outcomes.

1.12 Pathological evaluation of tumour response and new perspectives

Although pretreatment staging is the standard for planning the treatment regimen for pts with rectal cancer, pathologic stage may be the better prognostic determinant of cancer-related survival. Therefore, improving the understanding of tumour response in the natural history of rectal cancer on the basis of post-CRT pathology will provide practical information for pts and practitioners who are considering prognosis or who are planning adjuvant treatment. Many investigators demonstrated that 50%-60% of pts are

⁶⁰ Heald RJ, Husband EM, Ryall RD. The mesorectum in rectal cancer surgery-the clue to pelvic recurrence? Br J Surg. 1982;69:613-6.

Heald RJ, Moran BJ, Ryall RD, et al. Rectal cancer: the Basingstoke experience of total mesorectal excision, 1978-1997. Arch Surg 1998;133:894-9.

⁶¹ Schrag D, Panageas KS, Riedel E, et al. Hospital and surgeon procedure volume as predictors of outcome following rectal cancer resection. Ann Surg. 2002;236:583-92.

⁶² Jayne DG, Thorpe HC, Copeland J, et al. Five-year follow-up of the Medical Research Council CLASICC trial of laparoscopically assisted versus open surgery for colorectal cancer. Br J Surg. 2010;97:1638-45.

downstaged following preoperative therapy, with 10%-25% of pts showing a pathologic complete response⁶³.

The response after preoperative CRT in rectal cancer pts may be associated with their oncologic outcomes. One study in the United State reported that pts who failed to respond to preoperative 5-FU-based chemotherapy given concomitantly with RT had higher rates of distant metastases with adjuvant 5-FU therapy⁶⁴. Another retrospective study demonstrated that the pathologically determined response to preoperative treatment correlated with long-term outcomes. In that study, the 5-year recurrence-free survival rates were 90.5%, 78.7%, and 58.5% for pts with complete, intermediate, and poor responses, respectively ($p < 0.001$). Distant metastases and local recurrences also correlated with the level of response⁶⁵. Preoperative treatment response may serve as not only an indicator of prognosis but also an indicator of subsequent response to the same chemotherapeutic agents used for radiosensitization among pts with good responses and of the need for expanded therapeutic options for pts with poor response⁶⁶. Controversy exists as to whether adjuvant chemotherapy should be required for pts with good response to preoperative CRT. One study reported that in pts with ypN0 status, the continuation of adjuvant chemotherapy did not improve the prognosis because the prognosis was

⁶³ Das P, Skibber JM, Rodriguez-Bigas MA et al. Clinical and pathologic predictors of locoregional recurrence, distant metastasis, and overall survival in patients treated with chemoradiation and mesorectal excision for rectal cancer. *Am J Clin Oncol* 2006;29:219-24.

Das P, Skibber JM, Rodriguez-Bigas MA et al. Predictors of tumor response and downstaging in patients who receive preoperative chemoradiation for rectal cancer. *Cancer* 2007;109:1750-5.

Fietkau R, Barten M, Klautke G et al. Postoperative chemotherapy may not be necessary for patients with ypN0-category after neoadjuvant chemoradiotherapy of rectal cancer. *Dis Colon Rectum* 2006;49:1284-92.

Silberfein EJ, Kattepogu KM, Hu CY et al. Long-term survival and recurrence outcomes following surgery for distal rectal cancer. *Ann Surg Oncol* 2010;17:2863-9.

Smith KD, Tan D, Das P et al. Clinical significance of acellular mucin in rectal adenocarcinoma patients with a pathologic complete response to preoperative chemoradiation. *Ann Surg* 2010;251:261-4.

⁶⁴ Janjan NA, Crane C, Feig BW et al. Improved overall survival among responders to preoperative chemoradiation for locally advanced rectal cancer. *Am J Clin Oncol* 2001;24:107-12.

⁶⁵ Park IJ, You YN, Agarwal A et al. Neoadjuvant treatment response as an early response indicator for patients with rectal cancer. *J Clin Oncol* 2012;30:1770-6.

⁶⁶ Bosset JF, Collette L, Calais G et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006;355:1114-23.

excellent independent of the adjuvant chemotherapy⁶⁷. However, a subgroup analysis of the EORTC 22921 trial showed that pts downstaged to ypT0-2 were more likely to benefit from adjuvant chemotherapy than pts with ypT3-4 staging⁶⁸. Much to our regret, there are no prospective data to predict the benefit of adjuvant therapy in pts with tumour downstaging or a pathologic complete response.

In 2004, Habr-Gama et al.⁶⁹ retrospectively compared the outcomes of 71 pts who were observed without surgery following a complete clinical response to the outcomes of 22 pts who had incomplete clinical responses but complete pathologic responses after a TME. With re-evaluation using proctoscopy examination by an experienced colorectal surgeon, the absence of significant residual ulcer or positive biopsies performed during proctoscopy were considered as a clinical complete response. The OS and the DFS rates at 5 years were 100% and 92% in the non-operative group compared to 88% and 83% in the surgery group. In 2011, a prospective study that used very strict criteria, including MRI and endoscopy plus biopsies, to determine the clinical complete response was published. In that study, only one patient of 21 pts with clinical complete responses who were then observed with careful follow-up developed a local recurrence after a mean follow-up of 25 months; that patient underwent successful salvage surgery. The cumulative probabilities for the 2-year DFS and OS rates were 89% and 100%, respectively in the wait-and-see group and 93% and 91% in the 20 pts with a complete pathologic response after resection⁷⁰.

Despite their impressive results, many investigators still believe that longer follow-up, larger sample sizes, and additional careful observational studies are needed before pts with a clinical complete response are routinely managed by a wait-and-see approach. The rationale of a wait-and-see policy relies mainly on

⁶⁷ Fietkau R, Barten M, Klautke G et al. Postoperative chemotherapy may not be necessary for patients with ypN0-category after neoadjuvant chemoradiotherapy of rectal cancer. *Dis Colon Rectum* 2006;49:1284-92.

⁶⁸ Collette L, Bosset JF, den Dulk M et al. Patients with curative resection of cT3-4 rectal cancer after preoperative radiotherapy or radiochemotherapy: does anybody benefit from adjuvant fluorouracil-based chemotherapy? A trial of the European Organisation for Research and Treatment of Cancer Radiation Oncology Group. *J Clin Oncol* 2007;25:4379-86.

⁶⁹ Habr-Gama A, Perez RO, Nadalin W et al. Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results. *Ann Surg* 2004;240:711-7.

⁷⁰ Maas M, Beets-Tan RG, Lambregts DM et al. Wait-and-see policy for clinical complete responders after chemoradiation for rectal cancer. *J Clin Oncol* 2011;29:4633-40.

retrospective observations from a single series. Proof of principle in small, low rectal cancers, where clinical assessment is easy, should not be extrapolated uncritically to more advanced cancers where nodal involvement is common. Long-term prospective observational studies with more uniform inclusion criteria are required to evaluate the risk versus benefit.

2. Molecular basis of chemoradiosensitivity in rectal cancer

The predominant local effects of chemoradiotherapy (CRT), which is designed to achieve tumour cell damage, are primarily elicited by irradiation, whereas concomitant chemotherapy may serve as a radiosensitizer, most often without or with only small direct effects on tumour cell killing. The effects of CRT are largely the result of DNA damage, which either occurs directly through ionization within the DNA molecule or indirectly from the action of chemical radicals, which are also formed during irradiation (fig. 1). Through these mechanisms, several alterations, like base damage, DNA-protein cross-links, and single-strand or double-strand breaks, are generated and contribute to the antitumour effects and side effects¹.

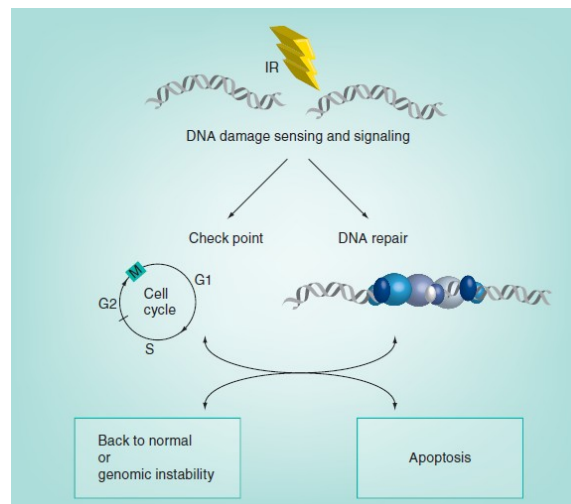


Fig. 1- Schematic mechanisms of cell death by ionizing radiation.

Despite the clinical importance of preoperative CRT in multimodal treatment concepts for patients (pts) with locally advanced rectal cancer (LARC), our understanding of both the genetic basis of chemoradiosensitivity and the molecular events leading to chemoradioresistance remains relatively sparse. From a systematic point of view, high-throughput analyses (whole-genome analysis) can be distinguished from low-throughput analyses (single-

¹ Grade M, Wolff HA, Gaedcke J et al. The molecular basis of chemoradiosensitivity in rectal cancer: implications for personalized therapies. *Langenbecks Arch Surg* 2012;397:543-555.

biomarker or multibiomarker analysis). A plethora of potential biomarkers has already been evaluated using whole-genome and singlemarker or multimer analysis, some of which have great potential to stratify rectal cancer pts for multimodal treatment regimens and to implement targeted therapeutics (fig. 2).

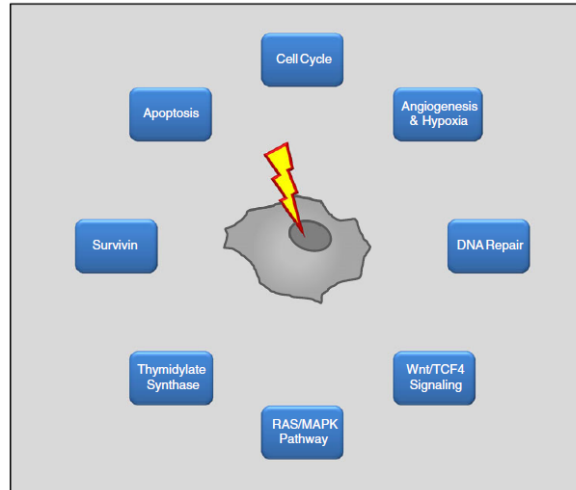


Fig. 2- Potential pathways and proteins regulating and mediating resistance of rectal cancer cells to CRT.

2.1 Whole-genome analyses

2.1.1 Gene expression profiling

2.1.1.1 Microarrays in tumour tissue

The first study on the application of a genetic signature to predict response to neoadjuvant treatment in rectal cancer appeared in 2005². Based on downsizing or tumour shrinkage they identified 54 genes expressed differently between responders versus non-responders in tumour samples extracted prior to neoadjuvant therapy. By using these genes they attained 83% precision in the prediction, both for responders and non-responders, thus proving that the study of genetic expression through microarrays was useful in predicting a reduction in tumour size in response to preoperative CRT therapy. These 54 genes are involved in many biological functions, including repairing damage to cellular DNA (SMC1), organizing microtubules (CLMN and CDC42BPA), and cellular signaling (FLNB).

² Ghadimi BM, Grade M, Difilippantonio MJ et al. Effectiveness of gene expression profiling for response prediction of rectal adenocarcinomas to preoperative chemoradiotherapy. *Journal of Clinical Oncology* 2005;23(9):1826-38.

The following year a Japanese group with a similar objective, published a microarray analysis of DNA³, that analyzed a total of 52 pts. A group of 33 differentially expressed genes was established among responders and non-responders: 20 were overexpressed genes related to apoptosis such as lumican (LUM), thrombospondin 2 (THBS2), and galectin-1 (LGALS1), while 13 were repressed in the responder-group, such as cyclophilin 40 (CYP40) and glutathione peroxidase 2 (GPX2). A protein structure prediction was then done on 33 genes from 17 pts included in the validation group, which found 82.4% exactness for determining class, 50% sensibility, 100% specificity, a positive predictive value of 100%, and a negative predictive value of 76.6%.

Kim and colleagues conducted a study in 2007 using samples from 46 pts⁴. They identified a group of 95 genes and found that this group of genes enabled tumour response to be predicted with 84% precision, 64% sensibility, 95% specificity, an 88% positive predictive value and an 87% negative predictive value. Two of the 95 genes stood out: thymidylate synthase, TYMS, involved in DNA synthesis, which was highly expressed in responding tumours, and RAD23B, involved in nucleotide excision repair, which was elevated in non-responders and has previously been associated with pts resistant to treatment with 5FU. These two genes could be used to evaluate response to treatment with 5FU.

Rimkus et al.⁵ also studied the tumour biopsies of pts in stage T3. They found 42 statistically significant genes that were expressed differently among responders and non-responders. Five of them (FREM1, M-RIP, SDHC, TDE1, and USP42) had a reduced expression in the group of responders, while the rest of the genes were overexpressed and involved in apoptosis (CASP1), transport (SLC35E1), cellular signaling (STAT2 and ETS2), and cellular cycle (CCNK). Sensibility was 71%, specificity was 86%, positive predictive value was 71%, and negative predictive value was 86%.

³ Watanabe T, Komuro Y, Kiyomatsu T et al. Prediction of sensitivity of rectal cancer cells in response to preoperative radiotherapy by DNA microarray analysis of gene expression profiles. *Cancer Research* 2006;66(7):3370-7.

⁴ Kim IJ, Lim SB, Kang HC et al. Microarray gene expression profiling for predicting complete response to preoperative chemoradiotherapy in patients with advanced rectal cancer. *Diseases of the Colon and Rectum* 2007;50(9):1342-53.

⁵ Rimkus C, Friederichs J, Boulesteix AL et al. Microarray-based prediction of tumor response to neoadjuvant radiochemotherapy of patients with locally advanced rectal cancer. *Clinical Gastroenterology and Hepatology* 2008;6(1):53-61.

More recently, a group formed by Nishioka and colleagues⁶ included 20 pts. A microarray of 132 genes related to a response to 5FU was used in addition to other chemotherapeutics. Researchers identified 17 genes expressed differently among the two patient subgroups (responders versus non-responders). Of them, five were metalloproteinases (MMP1, MMP7, MMP9, MMP14, and MMP16). It is worth emphasizing that in the non-responder case none of the genes were overexpressed.

Palma et al.⁷ identified a 4-gene profile (C-MYC, GNG4, POLA, and RRM1) associated with response to preoperative CRT in 43 rectal cancer pts. Using this gene set, a new model for predicting the response to CRT in rectal cancer was established with a sensitivity of 60% and 100% specificity.

Gantt published a study in 2014 using high-throughput nucleotide microarrays to develop a genetic profile associated with CRT-resistant rectal cancer: 33 pts were incorporated in the study⁸. They identified a unique gene expression profile composed of 812 genes associated with rectal cancer that had a poor response to CRT. The top 10 up-regulated genes included APOA2, AHSG, DBH, APOA1, APOB, APOC3, LMX1A, SOAT2, SLC7A9, and TF. The top 10 down-regulated genes included LOC729399, SERINC5, SCNN1B, ZC3H6, SLC4A4, DTWD2, MS4A12, BEX5, MMRN1, and CLCA4. Functional analysis of differentially expressed genes with IPA software (Ingenuity Pathways Analysis) revealed “DNA repair by homologous recombination” as a statistically significant canonical pathway in this study with RAD50 as the most significant differentially expressed gene in this pathway. RAD50 is a member of the MRE11-RAD50-NBS1 (MRN) complex that detects double-stranded DNA breaks and regulates DNA damage repair primarily through homologous recombination. A number of apolipoprotein genes were upregulated in non-responders (APOA2, APOA1, APOB, and APOC3). AHSG is a serum glycoprotein involved in endocytosis, brain development, and the formation of bone tissue previously associated with resistance to neoadjuvant chemotherapy in pts with advanced breast cancer.

⁶ Nishioka M, Shimada M, Kurita N et al. Gene expression profile can predict pathological response to preoperative chemoradiotherapy in rectal cancer. *Cancer Genomics & Proteomics* 2011;8(2):87-92.

⁷ Palma P, Cano C, Conde-Muino R et al. Expression profiling of rectal tumors defines response to neoadjuvant treatment related genes. *PLoS ONE* 2014;9(11):e112189.

⁸ Gantt GA, Chen Y, DeJulius K et al. Gene expression profile is associated with chemoradiation resistance in rectal cancer. *Colorectal Disease* 2014;16(1):57-66.

LMX1A is known to be involved in insulin gene transcription and the embryogenesis of dopamine-producing neurons. In cancer, LMX1A has been shown to be a poor prognostic indicator in ovarian and pancreatic tumours but LMX1A was also recently shown to inhibit cell proliferation, migration, invasion, and colony formation in vitro.

Recently, Watanabe conducted a new study to establish a prediction model for response to CRT in rectal cancer⁹. First, gene expression profiles were determined by DNA microarray analysis on 46 training samples. They identified 24 probes that were differentially expressed between responders and non-responders. Twenty genes showed higher and four genes showed lower expression in non-responders compared with responders. Microarray expression levels showed significant differences in 16 genes between responders and non-responders. Based on the 16 genes and their combination, the predictive accuracies of the 2500 different sets of predictor genes were calculated. The highest accuracy rate (89.1%) was obtained with a 4-gene set including LRR1Q3, FRMD3, SAMD5, and TMC7.

Although tissue gene microarray profiling has led to promising data in cancer, to date, none of the identified signatures or molecular markers in LARC has been successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice. Moreover, there has been little agreement between signatures published, with scarce overlap in the reported genes. Only three genes, MMP4, FLNA and RRM1, have been reported in more than one paper¹⁰.

2.1.1.2 Microarrays in peripheral blood

Peripheral blood mononuclear cells have emerged recently as pathology markers of cancer and other diseases, making their use as therapy predictors possible. Furthermore, the importance of the immune response in radiosensitivity of solid organs led Palma et al.¹¹ to hypothesize that microarray gene expression profiling of peripheral blood mononuclear cells could identify pts with response to CRT: 35 pts with LARC were recruited initially to perform

⁹ Watanabe T, Kobunai T, Akiyoshi T et al. Prediction of response to preoperative chemoradiotherapy in rectal cancer by using reverse transcriptase polymerase chain reaction analysis of four genes. *Diseases of the Colon and Rectum* 2014;57(1):23-31.

¹⁰ Conde-Muino R, Cuadros M, Zambudio N et al. Predictive biomarkers to chemoradiation in locally advanced rectal cancer. *BioMed Res Int* 2015:e921435.

¹¹ Palma P, Cuadros M, Conde-Muino R et al. Microarray profiling of mononuclear peripheral blood cells identifies novel candidate genes related to chemoradiation response in rectal cancer. *PLoS ONE* 2013;8(9):e74034.

the study. Peripheral blood samples were obtained before neoadjuvant treatment. RNA was extracted and purified to obtain cDNA and cRNA for hybridization of microarrays included in Human WG CodeLink bioarrays. Quantitative real-time PCR was used to validate microarray experiment data. The authors performed a multiple t-test using Significance Analysis of Microarrays to find those genes differing significantly in expression between responders (n=11) and non-responders (n=16) to CRT. The differently expressed genes were BC 035656.1, CIR, PRDM2, CAPG, FALZ, HLADPB2, NUPL2, and ZFP36. The measurement of FALZ gene expression level showed statistically significant differences between the two groups (p=0.029). They postulated the idea that gene expression profiling reveals novel genes in peripheral blood samples of mononuclear cells that could predict responders and non-responders to CRT in pts with LARC. The authors hypothesized the importance of mononuclear cells' mediated response in the neoadjuvant treatment of rectal cancer.

2.1.1.3 Microarrays of microRNA

RNA expression profiling has been used to assess microRNAs (miRNA) that are small non-coding RNA molecules that can be up- or down-regulated and influence activity of signaling pathways that may be associated with prognosis and response to CRT. Differential expression of 53 miRNAs was demonstrated between pathological complete response (pCR) and non-pCR by Della Vittoria Scarpati et al. The greatest differential expression was found in 14 miRNAs. miRNA-622 and miRNA-630 demonstrated an impressive 100% specificity and sensitivity to predict pCR¹². The authors concluded that miRNA influences genes and signaling pathways involved in cell repair following CRT. In the case of miRNA 630, it has previously been shown to impair a cell's ability to repair DNA damage caused by cisplatin-based chemotherapy in non-small cell lung cancer. This may explain the benefit seen in this patient cohort receiving oxaliplatin-based CRT, which may not, however, be transferable to the more standard 5-FU based neoadjuvant treatment. In direct contrast to these

¹² Della Vittoria Scarpati G, Falcetta F, Carlomagno C et al. A specific miRNA signature correlates with complete pathological response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* 2012;83:1113-9.

findings, a study in rectal cancer cells lines by Ma et al. identified miR-622 as a marker of radioresistance and not of pCR¹³.

Kheirleiseid et al. extracted miRNA from twelve formalin fixed paraffin embedded rectal cancer specimens and found that miRNA-16, miRNA-590-5p and miRNA-153 predicted pCR with 100% accuracy¹⁴.

Lopes-Ramos et al. investigated miRNA expression in 43 pts following LCCRT. Pts were divided into three groups: clinical complete response (cCR), clinical incomplete response and those with an initial cCR who developed early recurrence. They identified four miRNA with differential expression that predicted cCR miR-21-5p, miR-1246, miR1290-3p and miR-205-5p. The sensitivity and specificity of miR-21-5p to predict complete response was calculated to be 100% and 85%. Importantly those with cCR who developed an early recurrence had levels of expression of miR-21-5p that was similar to those with an incomplete response and statistically significantly lower to those with sustained cCR¹⁵. While these results are promising individually, the lack of concordance between studies highlights the inconsistencies in the molecular prediction of complete response¹⁶.

2.1.2 Chromosomal aberrations

Chromosomal aneuploidy is a defining feature of colorectal carcinomas¹⁷. This is reflected by tumour- and stage specific genomic copy number aberrations, which are virtually identical in colon and rectal cancers¹⁸.

¹³ Ma W, Yu J, Qi X et al. Radiation-induced microRNA-622 causes radioresistance in colorectal cancer cells by down-regulating Rb. *Oncotarget* 2015;6(18):15984-94.

¹⁴ Kheirleiseid EA, Miller N, Chang KH et al. miRNA expressions in rectal cancer as predictors of response to neoadjuvant chemoradiation therapy. *Int J Colorectal Dis* 2013;28:247-60.

¹⁵ Lopes-Ramos CM, Habr-Gama A, Quevedo Bde S et al. Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients. *BMC medical genomics* 2014;7:68.

¹⁶ Ryan JE, Warriar SK, Lynch AC et al. Predicting pathological complete response to neoadjuvant chemoradiotherapy in locally advanced rectal cancer: a systemic review. *Colorectal Dis.* 2015;Epub ahead of print.

¹⁷ Grade M, Becker H, Liersch T et al. Molecular cytogenetics: genomic imbalances in colorectal cancer and their clinical impact. *Cell Oncol* 2006;28(3):71-84.

¹⁸ Grade M, Hormann P, Becker S et al. Gene expression profiling reveals a massive, aneuploidy-dependent transcriptional deregulation and distinct differences between lymph node-negative and lymph node-positive colon carcinomas. *Cancer Res* 2007;67(1):41-56.

Accordingly, it may be speculated that differences in treatment responses can be correlated with differences on the DNA level.

In one of the first studies to address this question, pre-therapeutic biopsies from 42 pts with LARC were analyzed using metaphase comparative genomic hybridization (CGH). Based on downsizing of the T-category, chromosomal gains of 7q32–q36 and 7q11–q31 as well as amplifications of 20q11–q13 were associated with responsiveness to preoperative CRT¹⁹. However, the authors reported a high probability that these genomic copy number changes were detected by chance, therefore requiring independent validation in a larger patient population and with a higher resolution.

In a more recent study, Chen and colleagues used oligonucleotide array-based CGH to screen for chromosomal copy number alterations correlated with pathologic complete response (pCR). Analyzing DNA from 95 rectal cancers, the authors observed that chromosomal loss of 15q11.1–q26.3 was associated with non-pCR, while loss of 12p13.31 was associated with pCR²⁰.

2.2 Single-biomarker and multibiomarker analyses

2.2.1 Single-nucleotide polymorphisms

Single-nucleotide polymorphisms (SNPs) are sites in the genome sequence where individuals differ by a single base²¹. The total number of these sites in the human genome is estimated to be roughly 10 million, and these SNPs are distributed at an overall frequency of 1 in every 300 to 1,000 base pairs²². Importantly, it has been demonstrated that specific polymorphisms are associated with clinical phenotypes. For instance, the presence of a G allele within the SNP rs6983267, located on chromosome 8q24, confers an increased

¹⁹ Grade M, Gaedcke J, Wangsa D et al. Chromosomal copy number changes of locally advanced rectal cancers treated with preoperative chemoradiotherapy. *Cancer Genet Cytogenet* 2009;193(1):19-28.

²⁰ Chen Z, Liu Z, Li W et al. Chromosomal copy number alterations are associated with tumor response to chemoradiation in locally advanced rectal cancer. *Genes Chromosomes Cancer* 2011;50(9):689-699.

²¹ Manolio TA, Brooks LD, Collins FS. A HapMap harvest of insights into the genetics of common disease. *J Clin Invest* 2008;118(5):1590-605.

²² Botstein D, Risch N. Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nat Genet* 2003;33(Suppl):228-37.

risk for the development of colorectal cancer²³. Due to the growing body of evidence suggesting that genetic variation between individuals can account for differences in drug response²⁴, it has been speculated that genetic polymorphisms in genes encoding drug- or radiation-related responses may influence the individual's response to CRT²⁵.

Most prominently, thymidylate synthase (TS) has been analyzed in this respect, but the results are conflicting. Villafranca and colleagues were the first to correlate polymorphisms in the TS promoter and tumour response to preoperative CRT²⁶. Other investigators failed to demonstrate any association between TS genotype and relevant clinical parameters such as local response, tumour regression grading, or disease-free and overall survival²⁷. In contrast, there are studies demonstrating that the TS genotype has a significant impact on histopathological tumour regression and complete pathological response following preoperative CRT²⁸.

²³ Tomlinson I, Webb E, Carvajal-Carmona L et al. A genome-wide association scan of tag SNPs identifies a susceptibility variant for colorectal cancer at 8q24.21. *Nat Genet* 2007;39(8):984-8.

Zanke BW, Greenwood CM, Rangrej J et al. Genome-wide association scan identifies a colorectal cancer susceptibility locus on chromosome 8q24. *Nat Genet* 2007;39(8):989-94.

Haiman CA, Le Marchand L, Yamamoto J et al. A common genetic risk factor for colorectal and prostate cancer. *Nat Genet* 2007;39(8):954-6.

²⁴ Weinshilboum R. Inheritance and drug response. *N Engl J Med* 2003;348(6):529-37.

Evans WE, McLeod HL. Pharmacogenomics-drug disposition, drug targets, and side effects. *N Engl J Med* 2003;348(6):538-49.

²⁵ Barnett GC, Coles CE, Elliott RM et al. Independent validation of genes and polymorphisms reported to be associated with radiation toxicity: a prospective analysis study. *Lancet Oncol* 2012;13(1):65-77.

²⁶ Villafranca E, Okruzhnov Y, Dominguez MA et al. Polymorphisms of the repeated sequences in the enhancer region of the thymidylate synthase gene promoter may predict downstaging after preoperative chemoradiation in rectal cancer. *J Clin Oncol* 2001;19(6):1779-86.

²⁷ Terrazzino S, Agostini M, Pucciarelli S et al. A haplotype of the methylenetetrahydrofolate reductase gene predicts poor tumor response in rectal cancer patients receiving preoperative chemoradiation. *Pharmacogenet Genomics* 2006;16(11):817-24.

Conradi LC, Bleckmann A, Schirmer M et al. Thymidylate synthase as a prognostic biomarker for locally advanced rectal cancer after multimodal treatment. *Ann Surg Oncol* 2011;18(9):2442-52.

²⁸ Spindler KL, Nielsen JN, Lindebjerg J et al. Germline polymorphisms may act as predictors of response to preoperative chemoradiation in locally advanced T3 rectal tumors. *Dis Colon Rectum* 2007;50(9):1363-9.

Tan and colleagues reported the prospective use of TS genotyping to direct preoperative CRT in a single-institution phase II study²⁹. As a second example, recent data indicated that germline polymorphisms in the TGFB1 gene are associated with quality of life-impairing acute organ toxicity in pts with LARC. Analyzing DNA from two independent cohorts of pts participating in the CAO/ARO/AIO-94 and -04 trials (n088 and n075), Schirmer and colleagues demonstrated that all pts carrying the TGFB1 Pro25 variant developed high-grade acute organ toxicity during preoperative 5-FU-based CRT³⁰. The positive predictive value for acute toxicity in the presence of this SNP is 100%, which highlights the potential clinical importance of this observation.

2.2.2 Immunohistochemistry

A plethora of studies has been published which focused on a single immunohistochemical marker or a combination of a few. Interesting comprehensive reviews have been published³¹. Primary focus was the analysis of proteins involved in cell cycle, proliferation, apoptosis and angiogenesis. However, for most marker studies, the results are conflicting and still remain inconclusive.

Stoehlmacher J, Goekkurt E, Mogck U et al. Thymidylate synthase genotypes and tumour regression in stage II/III rectal cancer patients after neoadjuvant fluorouracil-based chemoradiation. *Cancer Lett* 2008;272(2):221-5.

Hur H, Kang J, Kim NK et al. Thymidylate synthase gene polymorphism affects the response to preoperative 5-fluorouracil chemoradiation therapy in patients with rectal cancer. *Int J Radiat Oncol Biol Phys* 2011;81(3):669-76.

²⁹ Tan BR, Thomas F, Myerson RJ et al. Thymidylate synthase genotype-directed neoadjuvant chemoradiation for patients with rectal adenocarcinoma. *J Clin Oncol* 2011;29(7):875-83.

³⁰ Schirmer MA, Nadine Mergler CP, Rave-Frank M et al. Acute toxicity of radiochemotherapy in rectal cancer patients: a risk particularly for carriers of the TGFB1 Pro25 variant. *Int J Radiat Oncol Biol Phys* 2012;83(1):149-57.

³¹ Kuremsky JG, Tepper JE, McLeod HL. Biomarkers for response to neoadjuvant chemoradiation for rectal cancer. *Int J Radiat Oncol Biol Phys* 2009;74(3):673-88.

Berardi R, Maccaroni E, Onofri A et al. Locally advanced rectal cancer: from molecular profiling to clinical practice. A literature review: part 2. *Expert Opin Pharmacother* 2009;10(15):2467-78.

Kapur P. Tailoring treatment of rectal adenocarcinoma: immunohistochemistry for predictive biomarkers. *Anticancer Drugs* 2011;22(4):362-70.

2.2.2.1 Cell cycle

With regard to the cell cycle, p53 and the cyclin-dependent kinase inhibitors (CDKIs: p21 and p27) have been extensively studied in rectal cancer and ionizing radiation.

p53

Preclinical studies have demonstrated that wild-type p53 was required for mouse thymocytes to undergo apoptosis induced by ionizing radiation. Thymocytes derived from p53-homozygous mutants were exquisitely resistant and p53 heterozygous thymocytes were relatively resistant to 5 Gy; while p53 wild-type cells were highly sensitive to the same dose of ionizing radiation. However, when apoptosis was induced by chemotherapeutic agents and not by ionizing radiation, both p53-mutant and wild-type cells displayed the same sensitivity to cell death, which suggested that p53 was required for ionizing radiation cell death in thymocytes but not for all forms of apoptosis³². p53 mechanisms of cell cycle arrest following irradiation of colon cancer cells have also been described³³.

These results have been mirrored in models of colorectal cancer in vitro and in vivo³⁴, but have been in disagreement with a few others³⁵. Furthermore, other studies have suggested that p53 mutations may render cells a more radiosensitive phenotype owing to a reduction in p53-DNA-dependent repair mechanisms³⁶.

Examination of p53 protein positivity in irradiated tumours demonstrated that nuclear expression of p53 in rectal cancers predicted treatment failure and expression of nuclear p53 protein by immunohistochemistry (IHC) signified

³² Lowe SW, Schmitt EM, Smith SW et al. p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 1993;362:847-9.

³³ Bracey TS, Miller JC, Preece A et al. Radiation-induced apoptosis in human colorectal adenoma and carcinoma cell lines can occur in the absence of wild type p53. *Oncogene* 1995;10:2391-6.

³⁴ Spitz FR, Nguyen D, Skibber J. M et al. Adenoviral-mediated wild-type p53 gene expression sensitizes colorectal cancer cells to ionizing radiation. *Clin Cancer Res* 1996;2:1665-71.

³⁵ Hendry JH, Cai WB, Roberts SA et al. p53 deficiency sensitizes clonogenic cells to irradiation in the large but not the small intestine. *Radiat Res* 1997;148:254-9.

³⁶ Ribeiro JC, Barnetson AR, Fisher RJ et al. Relationship between radiation response and p53 status in human bladder cancer cells. *Int J Radiat Biol* 1997;72:11-20.

resistance to preoperative ionizing radiation³⁷. Mutant p53 indicated resistance to apoptosis in rectal cancers compared with wild-type rectal tissues by IHC analysis³⁸.

By contrast, p53 status was not useful as a preoperative prognostic marker in rectal cancer in 100 pre-irradiated tumours, in which 55% of tumours were positive for p53³⁹. Another study showed that p53 status evaluated by IHC or by mutational analysis had no correlation between tumour regression and p53⁴⁰.

The conflicting results between preclinical and ex vivo studies with regard to p53 status as a predictor of radiosensitivity might stem from the mutation status of p53 and the half-lives of wildtype compared with mutant p53. The half-life of wild-type p53 is short and may not be detected by a single point in time by IHC. Conformational changes of the p53 protein resulting from mutations lead to protein stability and a longer half-life, which may allow for increased detection by IHC⁴¹.

Furthermore, molecular phenotypic differences that confer variable response rates to ionizing radiation in pts with rectal cancer is also mirrored by mutations along the p53 gene. While most mutations are localized to exons 5-8 of the p53 gene, it is the mutations of codon 288 in exon 8 that seem to affect rectal cancers and lead to a worse prognosis⁴². Thus, these specific mutations may lead to a more resistant phenotype in pts with rectal cancer.

In summary, although not universal, p53 has been demonstrated to play an essential role in cellular response to radiation in vitro. That is, wild-type p53 renders a radiosensitive phenotype in cultured cells. Ex vivo studies have failed to provide predictive or prognostic information as a result of the low number of

³⁷ Adell G, Sun XF, Stal O et al. p53 status: an indicator for the effect of preoperative radiotherapy of rectal cancer. *Radiother Oncol* 1999;51:169-74.

³⁸ Hamada M, Fujiwara T, Hizuta A et al. The p53 gene is a potent determinant of chemosensitivity and radiosensitivity in gastric and colorectal cancers. *J Cancer Res Clin Oncol* 1996;122:360-5.

³⁹ Nehls O, Klump B, Holzmann K et al. Influence of p53 status on prognosis in preoperatively irradiated rectal carcinoma. *Cancer* 1999;85:2541-8.

⁴⁰ Elsaleh H, Robbins P, Joseph D et al. Can p53 alterations be used to predict tumour response to pre-operative chemoradiotherapy in locally advanced rectal cancer? *Radiother Oncol* 2000;56:239-44.

⁴¹ Baas IO, van den Berg FM, Mulder JW et al. Potential false-positive results with antigen enhancement for immunohistochemistry of the p53 gene product in colorectal neoplasms. *J Pathol* 1996;178:264-7.

⁴² Vidaurreta M, Maestro ML, Sanz-Casla MT et al. Colorectal carcinoma prognosis can be predicted by alterations in gene p53 exons 5 and 8. *Int J Colorectal Dis* 2008;23:581-6.

subjects included in the studies, the techniques utilized, the ability of the antibody to recognize the mutated versus the wild-type form of p53, or a combination of these.

p21

p21 is the protein product of the WAF1 (also known as CIP1) gene, which is transcriptionally activated by p53 via one of two binding sites on its promoter⁴³. The main activation of p21 occurs via p53⁴⁴. p21 is a classic prototype that inhibits CDKs. DLD-1 colorectal cancer cells bearing mutations of the p53 gene expressed low levels of p21 following DNA damage with irradiation or chemotherapeutic agents⁴⁵. Colon cancer-p21-deficient cells were resistant to irradiation-induced cell death⁴⁶.

Pre-irradiated tissue biopsies obtained from 49 pts demonstrated that 49% of these tumours were positive for p53 and 29% for p21. In this study, p53-negative tumours and p21-positive status predicted a good response to ionizing radiation⁴⁷. This study also showed that 92% of p53-positive and 80% of p21-negative tumours were radioresistant; while 64% of p53 and 80% of p21 tumours were radiosensitive.

In a study of 72 resected specimens following neoadjuvant radiotherapy, preoperatively assessed stage II/III tumours were compared with their final pathological counterparts. This study examined microsatellite instability (MSI), microvessel count and protein expression of p53, p21 and p27. Only p21 expression correlated with good pathological response⁴⁸.

Examination of 27 pre-irradiated and postirradiated tumour samples by IHC demonstrated positive tumours for p53, Bcl-2 and p21 in 78, 48 and 52% of the cases, respectively. This was compared with postirradiated residual tumours,

⁴³ Namba H, Hara T, Tukazaki T et al. Radiation-induced G1 arrest is selectively mediated by the p53-WAF1/Cip1 pathway in human thyroid cells. *Cancer Res* 1995;55:2075-80.

⁴⁴ el-Deiry WS, Tokino T, Velculescu VE et al. WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817-25.

⁴⁵ Waldman T, Lengauer C, Kinzler KW et al. Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* 1996;381:713-6.

⁴⁶ Brugarolas J, Chandrasekaran C, Gordon JI et al. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* 1995;377:552-7.

⁴⁷ Fu CG, Tominaga O, Nagawa H et al. Role of p53 and p21/WAF1 detection in patient selection for preoperative radiotherapy in rectal cancer patients. *Dis Colon Rectum* 1998;41:68-74.

⁴⁸ Qiu H, Sirivongs P, Rothenberger M et al. Molecular prognostic factors in rectal cancer treated by radiation and surgery. *Dis Colon Rectum* 2000;43:451-9.

which demonstrated a rate of protein expression in 70, 52 and 26% of the cases, respectively. In this study, p21 expression was substantially reduced in radioresistant tumour cells. Thus, a decreased rate of 50% protein expression for p21 in the post-irradiated tumours compared with irradiated biopsies was associated with a radioresistant phenotype⁴⁹. The findings of this study are in contrast with the results of a similar protocol where the investigators aimed to determine whether p53, p21, p27 and Bcl-2 predicted tumour response of rectal cancer to neoadjuvant therapy in biopsies from tumours prior to ionizing radiation⁵⁰. The investigators examined 70 tumours by IHC. The percentage of tumour expression for p53, p21 and p27 prior to and after chemoradiation differed from that reported by Qiu. In their study, Lin et al. reported a level of expression of p27 in 63% of biopsies prior to irradiation and 69% in tumours after radiation. In this study, tumours with fair responses were identified to be p53 negative and p27 positive in the pre-irradiated biopsies. p27-positive tumours had a better response to ionizing radiation with an odds ratio of 3.3. These same markers, however, were not useful in the postirradiated tumour samples in determining a good response to ionizing radiation.

Altogether, the information derived from these reports highlights the wide difference in results obtained from similar experimental protocols. Tissue collection, timing of collection, antibodies used, differences in treatment modalities and differences between patient populations examined might account for these variations.

p27

The absence of p53 and p27 along with young age was associated with poor response to ionizing radiation in 38 biopsies of pts with rectal tumours prior to treatment. In this study, pCR was observed in 16% of this cohort of pts. The pro-apoptotic activity of these genes was suggested to be responsible for the positive association between p53 and p27 and tumour response.

The levels of p53 and p27 increased in tumour samples compared with biopsies following chemoradiation treatment, which was thought to be the result of protein induction as a result of DNA damage. An alternative hypothesis to explain the positivity of p53 and p21 following ionizing radiation

⁴⁹ Palazzo JP, Kafka NJ, Grasso L et al. The role of p53, p21WAF1/C1PI, and bcl-2 in radioresistant colorectal carcinoma. *Hum Pathol* 1997;28:1189-95.

⁵⁰ Lin LC, Lee HH, Hwang WS et al. p53 and p27 as predictors of clinical outcome for rectal-cancer patients receiving neoadjuvant therapy. *Surg Oncol* 2006;15:211-6.

treatment suggested that the development of mutations in these proteins made them more stable and detectable by IHC, but that these proteins still retained their pro-apoptotic properties⁵¹.

2.2.2.2 Proliferation markers

The effect of preoperative irradiation was examined in 122 pts with LARC with regards to tumour proliferation measured by the extent of Ki-67, proliferating-cell nuclear antigen (PCNA) immunostaining, as well as the number of mitoses examined under the microscope. In this study, 13 out of 122 pts (11%) had pCR. The pre-irradiated biopsies from these pts all had high-to-moderate levels of Ki-67 and PCNA immunostaining⁵². Evaluation of postirradiated tumours revealed that all indices for proliferation had decreased following surgical intervention (pre-irradiated Ki-67, PCNA and mean mitotic count of 92%, 81% and 19.4 number of mitoses compared with 73%, 58% and 10.7 number of mitoses, after surgery respectively). In this study, ionizing radiation was associated with a decrease in tumour size by 50%. Final pathological stage demonstrated that tumours that were smaller also had a significant increase in all markers.

Only a few small-sampled studies have reported high Ki-67 staining to be correlated with a positive response to ionizing radiation⁵³. However, most studies have uniformly shown that proliferating nuclear antigen labeling index does not correlate with response to ionizing radiation⁵⁴.

⁵¹ Esposito G, Pucciarelli S, Alaggio R et al. P27kip1 expression is associated with tumor response to preoperative chemoradiotherapy in rectal cancer. *Ann Surg Oncol* 2001;8:311-8.

⁵² Willett CG, Warland G, Hagan MP et al. Tumor proliferation in rectal cancer following preoperative irradiation. *J Clin Oncol* 1995;13: 1417-24.

⁵³ Rodel C, Grabenbauer GG, Papadopoulos T et al. Apoptosis as a cellular predictor for histopathologic response to neoadjuvant radiochemotherapy in patients with rectal cancer. *Int J Radiat Oncol Biol Phys* 2002;52:294-303.

⁵⁴ Desai GR, Myerson RJ, Higashikubo R et al. Carcinoma of the rectum. Possible cellular predictors of metastatic potential and response to radiation therapy. *Dis Colon Rectum* 1996;39:1090-6.

Qiu H, Sirivongs P, Rothenberger M et al. Molecular prognostic factors in rectal cancer treated by radiation and surgery. *Dis Colon Rectum* 2000;43:451-9.

Tannapfel A, Nusslein S, Fietkau R et al. Apoptosis, proliferation, bax, bcl-2 and p53 status prior to and after preoperative radiochemotherapy for locally advanced rectal cancer. *Int J Radiat Oncol Biol Phys* 1998;41:585-91.

2.2.2.3 Apoptosis

Cells unable to undergo apoptosis accumulate DNA errors, which translate into tumorigenic potential and resistance to radio-immunochemotherapeutic interventions. The process of apoptosis has been reviewed in detail⁵⁵, as has its role in colon carcinogenesis⁵⁶. Apoptosis is an important mechanism by which ionizing radiation exerts its therapeutic response. Faulty apoptosis is a known mechanism that renders resistance to radiation therapy in rectal cancer.

The pathological effects of CRT of 24 pre-irradiated rectal tumour biopsies were examined to determine the efficacy of treatment in the level of apoptosis, mitosis, p53 and Bcl-2 protein expression. pCR was observed in 25% of the cases in this study. The main finding of this report was that tumours with the best histopathological response to treatment had a significantly higher rate of spontaneous apoptosis as determined by the apoptotic index (AI). Mitotic index, p53 and Bcl-2 status were shown to not correlate with response to ionizing radiation⁵⁷.

In 44 biopsies obtained prior to ionizing radiation, pts who achieved a complete response and a good response to ionizing radiation had an AI of 2.06% compared with pts who experienced moderate or minimal response to ionizing radiation and had an AI of 1.44%. Thus, spontaneous apoptosis in the pretreatment biopsies was a good predictor of pathological response⁵⁸.

The role of both intrinsic apoptosis and radiation-induced apoptosis as markers for prognosis in rectal cancer was examined in 1198 tumour samples from the Dutch Total Mesorectal Excision trial by tissue microarray. The rate of recurrence in pts who received irradiation was 5% compared with 10% in pts who did not. Non-irradiated pts with high apoptosis had a decrease in local

⁵⁵ Huerta S, Goulet EJ, Huerta-Yepez S et al. Screening and detection of apoptosis. *J Surg Res* 2007;139:143-56.

⁵⁶ Huerta S. Recent advances in the molecular diagnosis and prognosis of colorectal cancer. *Expert Rev Mol Diagn* 2008;8:277-88.

⁵⁷ Scott N, Hale A, Deakin M et al. A histopathological assessment of the response of rectal adenocarcinoma to combination chemo-radiotherapy: relationship to apoptotic activity, p53 and bcl-2 expression. *Eur J Surg Oncol* 1998;24:169-73.

⁵⁸ Rodel C, Haas J, Groth A et al. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. *Int J Radiat Oncol Biol Phys* 2003;55:1341-7.

recurrence by 1.7-fold. While there was an increase in apoptosis in the irradiated tumours, this did not correlate with prognosis⁵⁹.

Bcl-2

Increased expression of Bcl-2 occurs in 33-67% of all colorectal cancers⁶⁰. Most studies have found no association between Bcl-2 and radiation response⁶¹. Bcl-2 protein expression by IHC did not change following irradiation treatment in rectal tumours⁶². Another study found only a weak association between Bcl-2 staining and radiosensitivity assessed by IHC⁶³. Fu et al. found no association between Bcl-2 expression and histologic radiosensitivity⁶⁴.

In the classical form of apoptosis, activation of executioner proteolytic enzymes called cysteinyl aspartate-specific proteases (caspases) destines the cell to undergo programmed cell death. Activation of executioner caspases (i.e., caspases 3, 6 and 7) is a point of no return for a cell to undergo apoptosis. Thus, activation of these caspases is under a great degree of cellular control. Caspases are negatively regulated by the inhibitors of apoptosis (IAPs) and positively by second mitochondrial activator of caspases (Smac-Diablo). The former is under the regulation of NF- κ B, while the latter is secreted by the mitochondria upon stimulation of apoptosis. NF- κ B controls the synthesis and activation of IAPs. In most cases, NF- κ B couples in an inactive form with I- κ B in the cytoplasm. Stimuli such as TNF- α or ionizing radiation cause phosphorylation, ubiquitination and subsequent degradation of I- κ B by the proteasome. These molecular modifications enable I- κ B to release either one or two subunits of NF- κ B,

⁵⁹ de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. *Clin Cancer Res* 2006;12:6432-6.

⁶⁰ Sinicrope FA, Ruan SB, Cleary KR et al. Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. *Cancer Res* 1995;55:237-41.

⁶¹ Rodel C, Grabenbauer GG, Papadopoulos T et al. Apoptosis as a cellular predictor for histopathologic response to neoadjuvant radiochemotherapy in patients with rectal cancer. *Int J Radiat Oncol Biol Phys* 2002;52:294-303.

⁶² Palazzo JP, Kafka NJ, Grasso L et al. The role of p53, p21WAF1/C1PI, and bcl-2 in radioresistant colorectal carcinoma. *Hum Pathol* 1997;28:1189-95.

⁶³ Qiu H, Sirivongs P, Rothenberger M et al. Molecular prognostic factors in rectal cancer treated by radiation and surgery. *Dis Colon Rectum* 2000;43:451-9.

⁶⁴ Fu CG, Tominaga O, Nagawa H et al. Role of p53 and p21/WAF1 detection in patient selection for preoperative radiotherapy in rectal cancer patients. *Dis Colon Rectum* 1998;41:68-74.

allowing for translocation to the nucleus and the ensuing transcription of IAP mRNAs⁶⁵.

NF- κ B

Nuclear factor- κ B induction by ionizing radiation exposure was inhibited by transfection of colorectal cancer cells WiDR, KM1214 and HT-29 with AdCMV I- κ B α (a mutated transcript that prevents NF- κ B activity) or pretreatment with the proteasome inhibitor PS-341. TUNEL assays in these cell lines demonstrated that NF- κ B inhibition was associated with an increase in ionizing radiation-induced apoptosis. Similarly, clonogenic survival assays showed that NF- κ B inhibition was associated with a reduction of cell growth.

In an elegant study, Cusack's group demonstrated that by inhibiting radiation-induced NF- κ B, apoptosis was increased and cell growth decreased in colorectal cancer cells. Radiosensitivity was demonstrated in colorectal cancer LOVO, WiDR and KM12L4 cells pretreated with the proteasome inhibitor PS-341 or AdCMV I- κ B. LOVO xenografts pretreated with the proteasome inhibitor PS-341 followed by 6Gy ionizing radiation experienced an 84% reduction in tumour growth⁶⁶.

Inhibitors of apoptosis

Survivin belongs to a family of eight members called the IAPs, which are under the regulation of NF- κ B. The IAPs act by directly inhibiting caspases 9, 3 and 7. Examination of three cell lines with different sensitivities to ionizing radiation demonstrated higher spontaneous and ionizing radiation-induced apoptosis in radiosensitive SW48 compared with radioresistant SW480 colorectal cancer cells, which was the result of higher levels of survivin in SW480 versus SW48 cells. The HCT15 cell line had intermittent responses in terms of both spontaneous apoptosis and survivin expression⁶⁷. Survivin expression was interrogated by quantitative RT-PCR in these cell lines. Survivin transcriptional activity increased substantially in SW480 cells, was

⁶⁵ Anguiano-Hernandez YM, Chartier A, Huerta S. Smac/DIABLO and colon cancer. *Anticancer Agents Med Chem* 2007;7:467-73.

⁶⁶ Russo SM, Tepper JE, Baldwin AS Jr et al. Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF- κ B. *Int J Radiat Oncol Biol Phys* 2001;50:183-93.

⁶⁷ Rodel C, Haas J, Groth A et al. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. *Int J Radiat Oncol Biol Phys* 2003;55:1341-7.

intermediate for HCT15 cells and did not change in SW48 cells treated with the same doses of ionizing radiation. Thus, there was a dose-dependent increase in both survivin mRNA and protein expression in radioresistant SW480. This study suggested that survivin acted as a constitutive radioresistant factor in colorectal cancer cells⁶⁸.

Inhibition of survivin transcription by siRNA studies demonstrated an increase in apoptosis and reduced survival in radioresistant SW480 and HCT15 colorectal cancer cells⁶⁹. Transfection of these cells with survivin siRNA decreased cell viability, induced cell cycle arrest at G2/M, increased DNA double-strand breaks and resulted in an increase in radiosensitivity by clonogenic assays. Inhibition of survivin by specific synthetic single-stranded DNA antisense oligonucleotide demonstrated substantial radiosensitization of SW480 cells as well as established SW480 xenografts. SW480 cells transfected with antisense oligonucleotide had higher spontaneous and ionizing radiation-induced apoptosis and an increase of cell accumulation at the G2/M phase, as well as in DNA double-stranded breaks.

These findings suggested that survivin inhibition improved ionizing radiation-induced cell death by mechanisms beyond caspase-mediated pathways, which included mitotic arrest, cell-cycle redistribution and impairment of DNA repair mechanisms⁷⁰.

2.2.2.4 Angiogenesis

Radiation-induced damage is mediated in part by formation of O₂ radicals, which rely on adequate blood supply. The relationship between tumour response to ionizing radiation and angiogenesis remains ill defined.

HIF-1

Tumour progression is highly dependent on angiogenesis. The transcription factor HIF-1 plays an important role in the upregulation of genes involved in angiogenesis and in facilitating metastasis and resistance to oxidative stress.

⁶⁸ Rodel F, Hoffmann J, Grabenbauer GG et al. High survivin expression is associated with reduced apoptosis in rectal cancer and may predict disease-free survival after preoperative radiochemotherapy and surgical resection. *Strahlenther Onkol* 2002;178:426-35.

⁶⁹ Rodel F, Hoffmann J, Distel L et al. Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res* 2005;65:4881-7.

⁷⁰ Rodel F, Frey B, Leitmann W et al. Survivin antisense oligonucleotides effectively radiosensitize colorectal cancer cells in both tissue culture and murine xenograft models. *Int J Radiat Oncol Biol Phys* 2008;71:247-55.

Ionizing radiation improves tumour oxygenation. However, by activation of stress genes and free radical production, ionizing radiation also increases the levels of HIF-1. Up-regulation of HIF-1 protects cells against cytotoxic therapy.

Ionizing radiation induces cell death in the most radiosensitive cells. This effect results in a decrease in oxygen consumption, but tumour cells reoxygenate following treatment.

After ionizing radiation treatment of tumour cells, HIF-1 stabilization may occur via three mechanisms: ionizing radiation-increased radical formation, stress granule formation, macrophage tumour infiltration and nitric oxide production⁷¹.

In this context, HIF-1 or VEGF should be inversely correlated to ionizing radiation sensitivity and prognosis in rectal cancers. Clinical data regarding HIF-1 as a predictor of tumour response or prognosis is currently lacking.

VEGF

High microvessel count and angiogenesis, as determined by VEGF expression, are desirable for tumour growth such that high expression of these two factors has been associated with tumour aggressiveness, metastasis and poor prognosis⁷².

VEGF overexpression has been correlated with poor response to ionizing radiation, and preclinical studies have shown that anti-angiogenic drugs improve oxygenation, as well as the response to ionizing radiation⁷³. Limited clinical studies have demonstrated no correlation with VEGF and tumour response to ionizing radiation⁷⁴.

Bevacizumab is a humanized (immunoglobulin) monoclonal antibody that interferes with the VEGF signaling pathway⁷⁵. Bevacizumab is the first anti-angiogenesis drug used in colorectal cancer. Bevacizumab improved tumour

⁷¹ Dewhirst MW, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer* 2008;8:425-37.

⁷² Tanigawa N, Amaya H, Matsumura M et al. Tumor angiogenesis and mode of metastasis in patients with colorectal cancer. *Cancer Res* 1997;57:1043-6.

⁷³ Citrin D, Menard C, Camphausen K. Combining radiotherapy and angiogenesis inhibitors: clinical trial design. *Int J Radiat Oncol Biol Phys* 2006;64:15-25.

⁷⁴ Qiu H, Sirivongs P, Rothenberger M et al. Molecular prognostic factors in rectal cancer treated by radiation and surgery. *Dis Colon Rectum* 2000;43:451-9.

⁷⁵ Presta LG, Chen H, O'Connor SJ et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997;57:4593-9.

blood flow, reduced tumour interstitial pressure and decreased mean vessel density when it was administered in the neoadjuvant setting in pts with rectal cancers⁷⁶. These factors are thought to be responsible for the radiosensitizing properties of bevacizumab⁷⁷.

⁷⁶ Czito BG, Bendell JC, Willett CG et al. Bevacizumab, oxaliplatin, and capecitabine with radiation therapy in rectal cancer: Phase I trial results. *Int J Radiat Oncol Biol Phys* 2007;68:472-8.

⁷⁷ Willett CG, Boucher Y, Duda DG et al. Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. *J Clin Oncol* 2005;23:8136-9.

3. The MAPK network: KRAS and BRAF

Cancer cells rely on signaling networks that are self-sufficient in providing growth signals and are refractory to growth inhibitory or apoptosis signals. This is due to multiple activating mutations in proto-oncogenes and functional loss of tumour suppressor genes¹. KRAS and BRAF are major oncogenic drivers of colorectal cancer (CRC).

KRAS, a small GTPase, acts as a central relay for signals originating at receptor tyrosine kinases such as the EGFR family in the intestinal epithelium and in many other tissues². Receptor tyrosine kinases stimulate KRAS activity via guanine nucleotide exchange factors, which activate KRAS by favoring GTP binding. The negative control is exerted through GTPase-activating proteins, which promote hydrolysis of GTP and thus KRAS inactivation. BRAF is a serine-threonine kinase that can be activated by KRAS and represents the top level element of the RAF-MEK-ERK (MAPK) kinase cascade³. MAPK signals regulate proliferation, differentiation, cell motility and further aspects of cellular activity via phosphorylation of many ERK substrates, such as cytoskeletal components and transcription factors. KRAS can also activate other signaling pathways in addition to the MAPK cascade. One of these is the PIK3CA-AKT-mTOR axis, which regulates protein translation and cell survival⁴. Together, the MAPK cascade and intersecting signaling pathways form a highly connected oncogenic network in CRC.

Approximately 40% of CRCs display activating missense mutations in KRAS⁵ (the COSMIC database reports 36%⁶, while TCGA reports 42% of

¹ Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-74.

² Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011;11:761-74.

³ Röring M, Brummer T. Aberrant B-Raf Signaling in Human Cancer-10 Years from Bench to Bedside. *Critical Reviews in Oncogenesis* 2012;17:97-121.

⁴ Castellano E, Downward J. RAS interaction with PI3K: more than just another effector pathway. *Genes Cancer* 2011;2:261-74.

⁵ Bos JL, Fearon ER, Hamilton SR et al. Prevalence of ras gene mutations in human colorectal cancers. *Nature* 1987;327:293-7.

Forrester K, Almoguera C, Han K et al. Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. *Nature* 1987;327:298-303.

Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319:525-32.

⁶ Forbes SA, Beare D, Gunasekaran P et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* 2015;43:805-11.

KRAS mutations⁷; fig. 3). These affect hotspots in codons 12 and 13 (80% of all KRAS mutations, of these are G12D>G12V>G13D>G12C>G12A), codon 61 (4% of all KRAS mutations, of these are Q61H>Q61L>Q61R) and 146 (1-2% of all KRAS mutations, mostly A146T and A146V). Furthermore, additional mutations in KRAS at various positions (e.g. 68, 117) are cataloged in the databases, yet their functional impact on KRAS protein function is largely unknown.

Structural analyses have presented a rationale for how the most frequent mutations activate KRAS: the glycine residues at positions 12 and 13 are important sites for interaction of KRAS with GAPs, while the glutamine at position 61 is a crucial site for the hydrolysis of GTP⁸. Therefore, mutations at either site lock KRAS in an active GTP-bound conformation constitutively presenting a docking surface for RAF kinases, including BRAF and CRAF (RAF1).

BRAF mutations are less frequent in CRC⁹ (COSMIC and TCGA report 11% and 10% of CRCs with activating mutations in BRAF; fig. 3)¹⁰. BRAF mutations in CRC are mostly V600E amino acid substitutions, although various other mutations at codon 600 or neighboring positions within the kinase domain are documented, too. Structural studies of RAF proteins have identified the valine at position 600 as a crucial site within the conserved kinase domain, which is required for BRAF to maintain an inactive conformation in the

⁷ Gao J, Aksoy BA, Dogrusoz U et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 2013;6:1.

Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330-7.

⁸ Scheffzek K, Ahmadian MR, Kabsch W et al. The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* 1997;277:333-8.

Scheidig AJ, Burmester C, Goody RS. The pre-hydrolysis state of p21 (ras) in complex with GTP: new insights into the role of water molecules in the GTP hydrolysis reaction of ras-like proteins. *Structure* 1999;7:1311-24.

Buhrman G, Holzapfel G, Fetics S et al. Allosteric modulation of Ras positions Q61 for a direct role in catalysis. *Proc Natl Acad Sci USA* 2010;107:4931-6.

⁹ Davies H, Bignell GR, Cox C et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949-54.

¹⁰ Forbes SA, Beare D, Gunasekaran P et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. *Nucleic Acids Res* 2015;43:805-11.

Gao J, Aksoy BA, Dogrusoz U et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 2013;6:1.

Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330-7.

absence of KRAS-BRAF interaction¹¹. Mechanistically, mutations at this site likely render mutated BRAF independent from dimerization with BRAF or RAF1, which is normally a prerequisite for activation. Consequently, the V600E mutation is strongly activating, resulting in constitutive MEK binding, phosphorylation and therefore BRAF signal transduction.

KRAS and BRAF mutations occur in a mutually exclusive manner in CRC¹². This may suggest that the mutations are functionally redundant during CRC development, i.e. no further selective advantage is provided for a cell by the second mutation when the first is already present.

Another explanation for the mutual exclusivity is that mutations in KRAS and BRAF may be functionally incompatible; BRAF mutations would thus have unfavorable effects in KRAS-mutant CRC and vice versa, consequently leading to elimination of cells that have acquired both mutations sequentially.

As a further explanation, KRAS or BRAF mutations could provide specific selective advantages that co-depend on the presence of other mutations. In support of this latter scenario, APC and KRAS mutations frequently co-occur, while APC and BRAF mutations show a significant trend towards mutual exclusivity.

In contrast, mutations in the ubiquitin ligase FBXW7 often co-occur with BRAF mutations, but are less frequent in KRAS-mutant or KRAS/ BRAF-wildtype CRC (fig. 3). This suggests that KRAS, but not BRAF mutations provide a selective advantage specifically in APC-mutant CRC precursor cells, whereas FBXW7 mutations provide the greatest advantage for CRC cells harboring activated BRAF.

¹¹ Wan P, Garnett MJ, Roe SM et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* 2004;116:855-67.

¹² Rajagopalan H, Bardelli A, Lengauer C et al. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 2002;418:934.

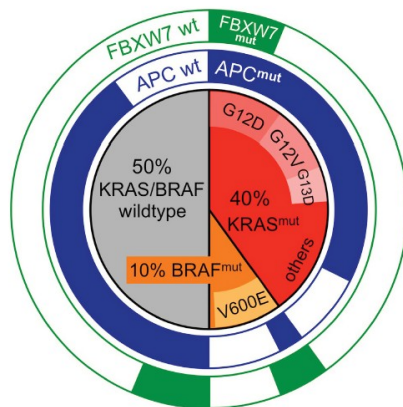


Fig. 3- Mutational spectra of KRAS, BRAF and the Wnt effector genes APC and FBXW7 in CRC.

Activation of the EGFR-RAS-RAF and the Wnt-APC- β -Catenin signaling axes represent key steps in initiation and early progression of CRC¹³. Indeed, EGFR signals, together with Wnt and Notch signals, form part of a larger signaling network controlling the maintenance of stem cells and the proliferative compartment of the normal intestinal epithelium¹⁴. Pathway-activating mutations represent essential steps during the early phases of CRC development, because they favor stem cell and proliferative characteristics independently of ligands provided by the microenvironment¹⁵.

During tumour progression, genetic (and epigenetic) alterations accumulate in an evolutionary manner via consecutive cycles of mutation and selection. Multiple mutations ultimately contribute to the formation of an oncogenic network sustaining the transformed cancer phenotype. In the oncogenic signal networks of advanced CRC, mutated KRAS and BRAF have been shown to

¹³ Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990;61:759-67.

Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol* 2011;6:479-507.

¹⁴ Sato T, van Es JH, Snippert HJ et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature* 2010;469:415.

Wong VWY, Stange DE, Page ME et al. Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nat Cell Biol* 2012;14:401-8.

¹⁵ Barker N, Ridgway RA, van Es JH et al. Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature* 2009;457:608-11.

Zhu L, Gibson P, Currle DS et al. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* 2009;457:603-7.

Matano M, Date S, Shimokawa M et al. Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids. *Nat Med* 2015;256-62.

serve many functions beyond maintaining cellular proliferation and growth factor-independent growth. Indeed, both oncoproteins have been shown to contribute to angiogenesis, cell differentiation, epithelial-mesenchymal transition, adaptations of cellular metabolism and circadian rhythm networks and many further traits of tumour cells.¹⁶

The essential role of the hyperactivated EGFR-KRAS-BRAF signaling cascade in CRC has spurred the development of therapeutic approaches to inhibit the cascade on several levels, specifically targeting EGFR, KRAS and BRAF (fig. 4).

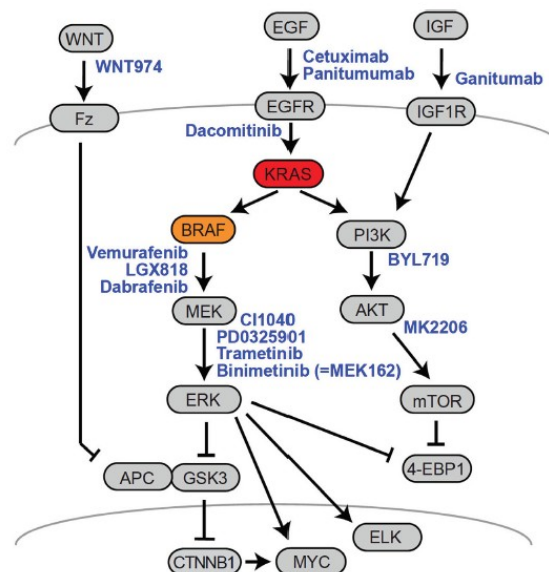


Fig. 4- Schematic representation of the EGFR-RAS-MAPK, PI3K and Wnt-APC- β -Catenin signaling axes. Drugs are given in blue, next to their targets.

Inhibition of the transmembrane tyrosine kinase receptor EGFR has proven to be beneficial for a considerable subset of patients (pts) with metastatic CRC.

¹⁶ Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 2011; 1:761-74.

Röring M, Brummer T. Aberrant B-Raf Signaling in Human Cancer-10 Years from Bench to Bedside. *Critical Reviews in Oncogenesis* 2012;17:97-121.

Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005; 7:548-58.

Relógio A, Thomas P, Medina-Pérez P et al. Ras-mediated deregulation of the circadian clock in cancer. *PLoS Genet* 2014;10:e1004338.

Herr R, Köhler M, Andrlová H et al. B-Raf inhibitors induce epithelial differentiation in BRAF-mutant colorectal cancer cells. *Cancer Res* 2015;75:216-29.

Upon treatment with EGFR-inhibiting antibodies such as cetuximab or panitumumab, pts showed an overall survival benefit of 3–5 months when the cancer was wildtype for KRAS, but no benefit when the cancer was KRAS-mutated¹⁷. Therefore, KRAS, and now also NRAS mutations are considered negative predictive markers for anti-EGFR therapy. Presently, cetuximab and panitumumab are recommended as first-line therapy in combination with chemotherapy for pts with wildtype configurations in KRAS and NRAS according to European (ESMO) and American (AJCC) standards. Other targeted therapies currently available in clinical routine, such as the VEGF inhibitor bevacizumab, seem to act independently of both KRAS and BRAF¹⁸.

In contrast to the RAS mutations, mutant BRAF has not been identified as an independent predictive marker for first-line anti-EGFR therapy in a dedicated clinical study. This is most likely due to the fact that BRAF mutations occur at rather low frequencies and thus no clinical study harbors enough pts to reach statistical significance. Furthermore, pts with BRAF mutations have a poor outcome, which is independent of the applied therapy¹⁹. However, a recent meta-analysis investigating the outcome of more than 400 RASwt/BRAFmut pts from 10 different trials clearly showed that pts harboring BRAF mutations do not benefit from EGFR-directed therapy and thus should be tested prior to the administration of either cetuximab or panitumumab²⁰.

It is important to note that even responders to anti-EGFR therapy routinely develop secondary resistance during anti-EGFR therapy, often by selection of KRAS/ NRAS or BRAF-mutant clones arising from a RAS-wildtype cancer²¹.

¹⁷ Karapetis CS, Khambata-Ford S, Jonker DJ et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008;359:1757-65.

Van Cutsem E, Köhne C-H, Hitre E et al. Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer. *N Engl J Med* 2009;360:1408-17.

Douillard J-Y, Oliner KS, Siena S et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 2013;369:1023-34.

¹⁸ Heinemann V, Douillard JY, Ducreux M et al. Targeted therapy in metastatic colorectal cancer - An example of personalised medicine in action. *Cancer Treat Rev* 2013;39:592-601.

¹⁹ Phipps AI, Limburg PJ, Baron JA et al. Association between molecular subtypes of colorectal cancer and patient survival. *Gastroenterology* 2015;148:77–87.

²⁰ Pietrantonio F, Petrelli F, Coinu A et al. Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: A meta-analysis. *Eur J Cancer* 2015;51:587-94.

²¹ Misale S, Yaeger R, Hobor S et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012;486:532-6.

Indeed, mathematical modeling has suggested that targeted monotherapy will invariably lead to the selection of resistant cells once a cancer has grown beyond a certain size²².

It appears to be a rational strategy to target mitogenic signaling downstream of mutated KRAS and BRAF, since both mutations are prevalent in primary and resistant CRC and the mutations have a negative predictive and prognostic value. However, inhibition of oncogenic BRAF(V600E) using vemurafenib, or of the MEK kinase using CI1040, has proven to be ineffective in CRC²³. The major reason for this disappointing outcome of kinase inhibition within the MAPK kinase cascade is the existence of multiple levels of feedback control, and regulatory intersections with further pathways such as PI3K-AKT²⁴. Indeed, several levels of feedback exist between the RAF-MEK-ERK axis and upstream receptor tyrosine kinases such as EGFR (fig. 5).

Diaz LA, Williams RT, Wu J et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012;486:537-40.

Misale S, Arena S, Lamba S et al. Blockade of EGFR and MEK intercepts heterogeneous mechanisms of acquired resistance to anti-EGFR therapies in colorectal cancer. *Sci Transl Med* 2014;6:224-6.

²² Bozic I, Allen B, Nowak MA. Dynamics of targeted cancer therapy. *Trends Mol Med* 2012;18:311-6.

²³ Rinehart J, Adjei AA, Lorusso PM et al. Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer. *J Clin Oncol* 2004;22:4456-62.

²⁴ Morkel M, Riemer P, Blaker H et al. Similar but different: distinct roles for KRAS and BRAF oncogenes in colorectal cancer development and therapy resistance. *Oncotarget* 2015;6(25):20785-800.

(fig.5). First results suggest that a limited clinical response exists in pts that have failed in first-line therapies²⁶.

It is important to note that KRAS- versus BRAF-mutant CRCs likely display characteristic differences in response to therapeutic interference in the MAPK cascade, due to mechanistic differences in signal transduction (fig. 5). For one, BRAF mutations disallow the critical feedback from ERK to RAF to occur, while KRAS mutations leave this feedback intact²⁷. As a consequence, higher levels of MEK inhibitor are required in KRAS mutated CRC cells as compared to BRAF mutated cells to suppress MEK/ERK activation. Furthermore, highlighting an important difference between KRAS- and BRAF-mutant cancer cells, ATP-competitive RAF inhibitors were found to block MEK-ERK signal transduction in BRAF-mutant cancer cells, but unexpectedly activated MEK-ERK signaling in cancer cells harboring mutant RAS and wildtype BRAF²⁸. On a molecular level, this paradoxical activation could be

²⁶ Capalbo C, Marchetti P, Coppa A et al. Vemurafenib and panitumumab combination tailored therapy in BRAF-mutated metastatic colorectal cancer: a case report. *Cancer Biol Ther* 2014;15:826-31.

Yaeger R, Cercek A, O'Reilly EM et al. Pilot Trial of Combined BRAF, and EGFR Inhibition in BRAF-Mutant Metastatic Colorectal Cancer Patients. *Clin Cancer Res* 2015;21:1313-20.

Corcoran RB, Atreya CE, Falchook GS et al. Phase 1–2 trial of the BRAF inhibitor dabrafenib (D) plus MEK inhibitor trametinib (T) in BRAF V600 mutant colorectal cancer (CRC): Updated efficacy and biomarker analysis. *J Clin Oncol* 2014;32:5s.

Bendell JC, Atreya CE, André T et al. Efficacy and tolerability in an open-label phase I/II study of MEK inhibitor trametinib (T), BRAF inhibitor dabrafenib (D), and anti-EGFR antibody panitumumab (P) in combination in patients (pts) with BRAF V600E mutated colorectal cancer (CRC). *J Clin Oncol* 2014;32:5s.

Hong DS, Van Karlyle M, Fu S et al. Phase 1B study of vemurafenib in combination with irinotecan and cetuximab in patients with BRAF-mutated advanced cancers and metastatic colorectal cancer. *J Clin Oncol* 2014;32:5s.

Tabernero J, Chan E, Baselga J et al. VE-BASKET, a Simon 2-stage adaptive design, phase II, histology-independent study in nonmelanoma solid tumors harboring BRAF V600 mutations (V600m): Activity of vemurafenib (VEM) with or without cetuximab (CTX) in colorectal cancer (CRC). *J Clin Oncol* 2014;32:5s.

²⁷ Fritsche-Guenther R, Witzel F, Sieber A et al. Strong negative feedback from Erk to Raf confers robustness to MAPK signalling. *Mol Syst Biol* 2011;7:489.

²⁸ Poulidakos PI, Zhang C, Bollag G et al. RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature* 2010;464:427-30.

Heidorn SJ, Milagre C, Whittaker S et al. Kinase-dead BRAF, and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* 2010;140:209-21.

Hatzivassiliou G, Song K, Yen I et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 2010;464:431-5.

explained by different propensities of BRAF and RAF-1 to form homo- versus heterodimers²⁹. It was also found that inhibition of active MEK has different constraints downstream of oncogenic KRAS versus BRAF³⁰: while KRAS-driven cancer cells are sensitive towards inhibitors interacting with MEK-Serine212 (a site critical for feedback between MEK and wildtype BRAF), BRAF-mutant cancer cells required another class of MEK inhibitor that blocks phosphorylated active MEK. Taken together, the aforementioned studies underline the necessity to develop specific and effective diagnostics and therapies for pts with BRAF mutated CRC. An unusual approach to exploit specific traits of BRAF-mutated cells was recently presented by exploiting the finding that synthesis of BRAF is dependent on chaperone action. Thus, interference with the BRAF chaperone TRAP1 was shown to effectively inhibit proliferation of BRAF mutated CRC cells³¹.

²⁹ Röring M, Brummer T. Aberrant B-Raf Signaling in Human Cancer-10 Years from Bench to Bedside. *Critical Reviews in Oncogenesis* 2012;17:97-121.

Cox AD, Der CJ. The RAF Inhibitor Paradox Revisited. *Cancer Cell* 2012;21:147-9.

³⁰ Hatzivassiliou G, Haling JR, Chen H et al. Mechanism of MEK inhibition determines efficacy in mutant KRAS- versus BRAF-driven cancers. *Nature* 2013;501:232-6.

³¹ Condelli V, Piscazzi A, Sisinni L et al. TRAP1 is involved in BRAF regulation and downstream attenuation of ERK phosphorylation and cell-cycle progression: a novel target for BRAF-mutated colorectal tumors. *Cancer Res* 2014;74:6693-704.

4. Materials and methods

4.1 Patients and chemoradiotherapy

Between October 2006 and December 2013 (minimum follow-up: 2 years), 184 patients (pts) affected by rectal cancer underwent radiation therapy at Radiotherapy Department of University Hospital in Foggia (Italy). 116 pts with local advanced (cT3-4 or cN1-2) or distal T2 (located ≤ 2 cm from the anorectal transition) tumours were treated by chemoradiation followed by surgery, with neoadjuvant intent. Exclusion criteria were: inflammatory bowel disease, pregnancy, previous radiation to the pelvis, contraindication to chemotherapy. The pathological results of 72 pts undergoing surgery after chemoradiation were available for analysis. Baseline characteristics are shown in tab. 3.

Age	62 (48-86)
Sex	M: 47 (65%); F: 25 (35%)
Clinical stage	T2N0: 3 (4%); T2N1-2: 3 (4%) T3N0: 35 (49%); T3N1-2: 27 (38%) T4N0: 3 (4%); T4N1-2: 1 (1%)
Distance from anal verge (cm)	5.5 (0-15)

Tab. 3- Baseline characteristics of patients treated by neoadjuvant chemoradiation.

Treatment consisted of 50.4 Gy (28×1.8 Gy on weekdays) combined with 5-fluorouracil or capecitabine. The radiation dose was delivered by 3D-conformal radiation therapy (3DCRT), to the planned target volume (PTV), which comprises the gross tumor volume (GTV) and clinical target volume (CTV). Target volumes were delineated on computed tomography (CT) scans. The CTV follows the mesorectal fascia up to the rectosigmoid curvature and stretches maximally to 4 cm caudal from the tumour. The lymph node regions (internal iliac and obturator) stretch from the caudal end of the v. iliaca communis downward to the crossing of the internal iliac vessels under the m. piriformis, laterally limited by the pelvic muscles. The obturator region stretches from the m. obturatorius to the m. elevator, ventrally limited by the ureter or dorsal side of the neurovascular bundle without inclusion of the vesiculae, uterus and vagina. Lateral and dorsal border are marked by the pelvic muscles and ventral iliac region. In addition, the presacral region stretches from

the upper level of the iliac vessels to mesorectum, ventrally limited 2 cm from the sacrum, including the a. rectalis superior and excluding the neuroforamina.

The PTV is a non-uniform margin around the CTV consisting of an expansion for internal margin and set-up margin. The prescribed dose to the PTV was that 95% of the prescribed dose should cover $\geq 99\%$ of the PTV. Radiation was delivered with an Elekta linear accelerator, using high-energy photons ≥ 10 MV. Appropriate shielding of non-target volumes was performed with multileaf collimators. Individual three dimensional dose planning of the tumour target volume was used. For weekly position verification, electronic portal images were used until the implementation of cone-beam CT. Surgery was performed 6-8 weeks postradiation. The decision whether to perform total mesorectal excision surgery in form of low anterior resection (LAR) or abdomino-perineal resection (APR) was made on the basis of the location and extensiveness of the tumour. The standard operation included total mesorectal excision, defined as removal of the rectum with the entire mesorectum by sharp dissection along the mesorectal fascia down to the pelvic floor.

The primary endpoint of our study was pCR, which is a complete tumor regression (TRG1), that is a sterile specimen with absence of residual cancer cells¹. Experienced gastrointestinal pathologists use a standardized protocol to evaluate the specimens². Secondary endpoints included non-complete pathologic responses (TRG 2–5), acute and late toxicity, clinical response, disease-free and overall survival. The non-complete pathologic responses are categorized as partial responses (TRG 1–2) or stable diseases (TRG 3–5). Toxicity was assessed weekly during the radiation treatment, as well as at follow-up visits. Toxicity was recorded according to the RTOG criteria for adverse events³. Clinical response evaluation was based on CT or MRI and DRE. Disease-free survival was defined as the time in absence of a rectal cancer local recurrence or metastasis. Median follow-up was 66 months (IQR 56–73) for all patients.

¹ Mandard AM, Dalibard F, Mandard JC et al. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma: clinicopathologic correlations. *Cancer* 1994;73:2680-6.

² Quirke P, Morris E. Reporting colorectal cancer. *Histopathology* 2007;50:103-12.

³ National Cancer Institute, National Institutes of Health. Common Terminology Criteria for Adverse Events (CTCAE) v.4 data files. <http://evs.nci.nih.gov/ftp1/CTCAE/About.html>. Accessed 14 Feb 2015.

4.2 Cell cultures, siRNAs and chemicals

Human CRC HCT116, HT29, COLO320 cells were purchased from American Type Culture Collection (ATCC). Cell line authentication was verified before starting this study by short tandem repeat (STR) profiling, according to ATCC product description. HCT116 and HT29 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1.5 mM glutamine, and 100 U/ml penicillin and streptomycin. Stable clones of HCT cells in which TRAP1 was silenced (shTRAP1) were used as well. COLO320 cells were grown in Rosewell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% FBS, 0.75 mM glutamine, and 100 U/ml penicillin and streptomycin. Cells were incubated in humidified air at 37°C with 5% CO₂. The growth medium was replaced every 48 hours, thus enabling a rapid multiplication of the number of cells.

5-Fluorouracil (5-FU) was purchased from Sigma–Aldrich (fig. 6). It was used at doses of 5, 15, 25, 100 and 500 nM, for 24 and 48 hours. Doses and timing of 5-FU were chosen based on published data⁴.

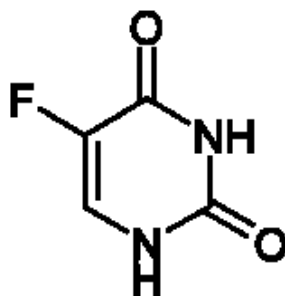


Fig. 6- 5-Fluorouracil. Chemical structure.

PLX4720 (fig. 7) is a potent inhibitor of B-Raf-V600E with IC₅₀ of 13 nM. The selectivity of the molecule for B-Raf protein with V600E mutation resulted

⁴ Urick ME, Chung EJ, Shield WP et al. Enhancement of 5-Fluorouracil-induced in vitro and in vivo radiosensitization with MEK inhibition. *Clin Cancer Res* 2011;17(15):5038-47.

Deng J, Lei W, Fu JC et al. Targeting miR-21 enhances the sensitivity of human colon cancer HT-29 cells to chemoradiotherapy in vitro. *Biochemical and Biophysical Research Communications* 2014;443:789-95.

Srimuangwong K, Tocharus C, Chintana PY et al. Hexahydrocurcumin enhances inhibitory effect of 5-fluorouracil on HT-29 human colon cancer cells. *World J Gastroenterol* 2012;18(19):2383-9.

10 times higher than the selectivity shown by the wild-type form. PLX4720 was purchased from Selleck Chemicals and used at a dose of 10 μ M.

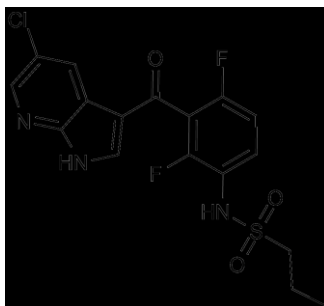


Fig. 7- PLX4720. Chemical structure.

HSP990 (or NVP-HSP990), the inhibitor of Hsp90/TRAP1, was provided by Novartis and used at doses of 75 nM and 150 nM (fig. 8).

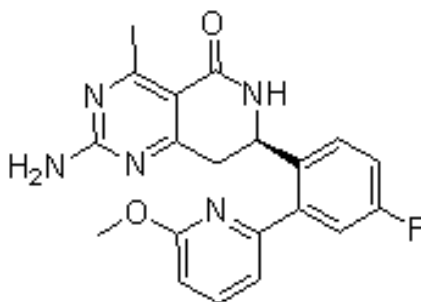


Fig. 8- HSP990. Chemical structure.

To evaluate the effects of gene silencing, short interfering RNAs (siRNAs) were used. SiRNAs specific for TRAP1 and BRAF were purchased from Qiagen. For control experiments, cells were transfected with a similar amount of control siRNA (Qiagen). For knock-down experiments, siRNAs were diluted to a final concentration of 40 nM and transiently transfected by the HiPerFect Transfection Reagent (Qiagen), according to manufacturer protocol.

4.3 Cell culture irradiation

Cell lines were irradiated in plexiglass plates (12x8 cm) using Elekta Synergy Linear Accelerator; 6 MV photon energy was used with a 400-MU/min dose rate. Because the maximum aperture of the linac field is 40 \times 40cm at source-surface distance (SSD) of 100, at most six plates were irradiated at a time.

A phantom was constructed to minimize build-up effect and therefore improve scatter conditions in the medium and allow isodose coverage of 95–107%. The phantom was made of plexiglas plates, due to its tissue equivalent characteristics. The size of the phantom (40×40x1 cm) allowed sufficient scatter material around the radiation field to cover all plates; it was put under the plates to allow a uniform posterior-anterior irradiation, avoiding the presence of air between the top of plates and cell culture.

Before starting our experiments, plates with cell lines were placed on the phantom and were CT scanned. The CT data were imported into a treatment planning system (TPS), contoured and planned with Oncentra Masterplan TPS (Elekta) (fig. 9).

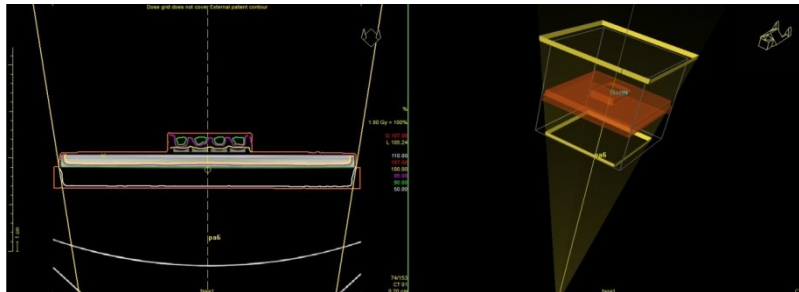


Fig. 9- Treatment planning for cell plates.

From CT information the spatial coordinates of set-up were established and with TPS the optimal geometry of the field was identified. A SSD of 100 cm and a gantry rotation of 180° and the interposition of the plexiglass bolus were set. This arrangement was necessary to remedy the build-up phenomenon: the layer of the target absorbing most energy is not located superficially but more deeply. The depth of maximum absorption varies in relation to the energy of the radiation and the characteristics of the material. Build-up causes an underdosing of the first milliliters compared to the underlying layers. The gantry rotation of 180° and the interposition of the bolus overcome the build-up phenomenon allowing a good coverage also in the upper layers of cell culture (fig. 10).

Cell samples were irradiated with 1.8 to 24 Gy. Control samples were carried to the linac bunker, but not irradiated, in order to be exposed to the same conditions of transport and temperature.



Fig. 10- Cell plates on linac table.

4.4 Colorimetric MTT (tetrazolium) assays

MTT assays are well known techniques to study chemosensitivity⁵ or toxicity⁶ of drugs in human tumor cell lines. The assay is less common to study survival of cancer cells after irradiation. We performed MTT assays to compare them with the well-established clonogenic assays. Cell viability was evaluated by tetrazolium colorimetric MTT assay. The assay is based on the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), into formazan by mitochondrial dehydrogenases in viable cells⁷. MTT (5 mg/ml) was dissolved in phosphate buffered saline and filtered to sterilize and remove the small amount of insoluble residue present in some batches of MTT. Stock MTT solution (10 μ l per 100 μ l medium) was added to all wells, and plates were incubated at 37°C for 4 hours. Acid isopropanol (100 μ l of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly

⁵ Kratzke RA, Kramer BS. Evaluation of in vitro chemosensitivity using human lung cancer cell lines. *J Cell Biochem Suppl* 1996;24:160-4.

⁶ Foldbjerg R, Dang DA, Autrup H. Cytotoxicity and genotoxicity of silver nanoparticles in the human lung cancer cell line, A549. *Arch Toxicol* 2011;85(7):743-50.

⁷ Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.

to dissolve the dark blue crystals. After few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a spectrophotometer within 1 hour of adding the isopropanol. Colour intensity was measured at the test wavelength of 570 nm using a reference wavelength of 655 nm.

4.5 Apoptosis assays

Radio-induced apoptosis was evaluated by a cytofluorimetric analysis 48 h after radiation. Apoptosis was evaluated by cytofluorimetric analysis of annexin-V and 7-amino-actinomycin-D (7-AAD) positive cells using the fluorescein isothiocyanate-(FITC)-annexin-V/7-AAD Kit (Beckman Coulter, Milan, Italy). Treated and control cells were grown and then stained with FITC-annexin-V (0.25 ng/ μ l) and 7-AAD (50 ng/ μ l). Stained cells were analyzed by "EPICS XL" Flow Cytometer (Beckman Coulter).

Ten thousand events were collected per sample. Positive staining for annexin-V as well as double staining for annexin-V and 7-AAD were interpreted as signs of early and late apoptosis, respectively⁸. H₂O₂ was used as positive control. Data were processed with "System II" software (Beckman Coulter) and results expressed as percentage of positive cells (%).

4.6 Colony forming assays

Clonogenic assays are commonly used to investigate survival of irradiated cancer cells, since they determine cell reproductive death after treatment with ionizing radiation. Commonly, plating densities for clonogenic assays were adapted paying attention to the situation that the endpoint analysis of this assay is determined optically by counting clones. Therefore, plating density must not be too high or clones will coalesce and counting of single colonies will become impossible. So starting from 600 cells/well, cells were then seeded at a density of 300 cells/well in 6-well plates and treated with the above-mentioned cytotoxic agents. Cell lines were routinely monitored by microscopic morphology. We used the protocol by Franken et al.⁹. Cells were incubated in a CO₂ incubator at 37°C until cells in control plates formed colonies with

⁸ George TC, Basiji DA, Hall BE et al. Distinguishing modes of cell death using the imagestream multispectral imaging flow cytometer. *Cytometry A* 2004;59(2):237-45.

⁹ Franken NA, Rodermond HM, Stap J et al. Clonogenic assay of cells in vitro. *Nat Protoc* 2006;1(5):2315-9.

substantially good size; 50 cells per colony was considered the minimum for scoring (fig. 11). It took nearly 15 days, with medium changes every 2 days.

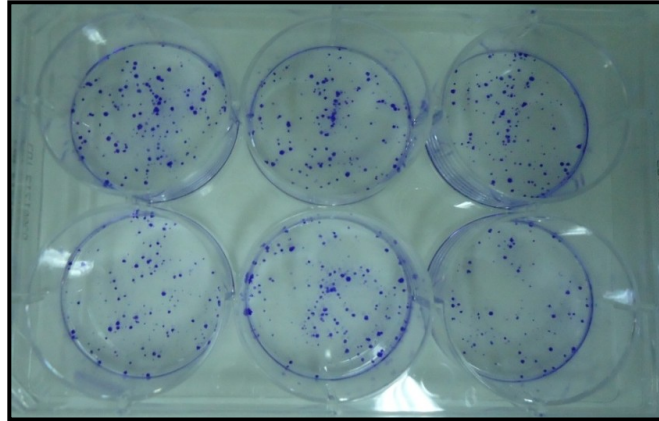


Fig. 11- 6-well plate containing HCT116 colonies after 4Gy irradiation.

Then medium was removed and cells rinsed with 10ml phosphate-buffered saline (PBS). After removal of PBS, 2-3ml of fixation solution were added and plates left at room temperature for 5 min. Acetic acid/methanol (1:7) was used (Sigma-Aldrich) as colony fixation solution. After removal of fixation solution, 0.5% crystal violet solution was added (Sigma-Aldrich); plates were incubated at room temperature for 2 hours. Plates were then immersed in tap water to rinse off crystal violet and finally air-dried at room temperature for up to a few days.

The number of colonies was counted with a stereomicroscope. Average number of colonies among all wells, plating efficiency (PE) in control cells and surviving fraction (SF) in treated cells were calculated according the following formulas:

$$\text{PE} = \text{no. of colonies formed} / \text{no. of cells seeded} \times 100\%;$$

$$\text{SF} = \text{no. of colonies formed after treatment} / (\text{no. of cells seeded} \times \text{PE}).$$

4.7 Immunoblot analysis

The protein profile of CRC cell lines was characterized by Western blotting, in order to evaluate the expression of BRAF and TRAP1 genes. Total cell lysates were obtained by homogenization of cell pellets in a cold lysis buffer (20 mM Tris pH 7.5 containing 300 mM sucrose, 60 mM KCl, 15 mM NaCl, 5% glycerol, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2% deoxycholate) for 2 min at 4°C and

further sonication for 30 sec on ice. Samples were resolved on SDS-PAGE, transferred on nitrocellulose membrane (Bio-Rad Laboratories GmbH, Munchen, Germany). Specific proteins were detected by using the following mouse monoclonal antibodies from Santa Cruz Biotechnology: anti-HSP75, anti-BRAF and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Specific bands were revealed using the Clarity Western ECL Substrate (Bio-Rad Laboratories GmbH, Munchen, Germany).

4.8 Statistical analysis

All experiments were independently performed at least three times. The average of all determinations are reported. Two-sided Student's t-test was used to establish the statistical significance between different levels of cell death in two groups. One-way variance analysis (ANOVA) was applied to test the differences in cell survival among more groups, and Duncan and Tuckey post-hoc tests were performed after detecting the significance. Analyses of frequencies were conducted with Chi-square tests. A probability value less than 0.05 ($p < 0.05$) was regarded as statistically significant. R statistical program was used for data analysis¹⁰.

¹⁰ R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

5. Results

The above mentioned methods allowed to evaluate:

- a) neoadjuvant chemoradiation outcomes in a sample of pts affected by rectal cancer;
- b) the radiation doses to be used in in-vitro experiments;
- c) the radioresistance of COLO320, HCT116 and HT29 cell lines after exclusive radiation treatment;
- d) the clonogenic capacity of COLO320, HCT116 and HT29 cell lines after pre-treatment with 5-fluorouracil and subsequent irradiation;
- e) the effects of transfection of siRNAs specific for BRAF and pre-treatment with PLX4720 on HT29 radiosensitivity;
- f) the effects of transfection of siRNAs specific for TRAP1 and pre-treatment with HSP990 on HT29 radiosensitivity;
- g) the radio-induced response in stable clones of HCT cells in which TRAP1 was silenced (shTRAP1);
- h) the role of TRAP1 in the adaptive response to radiation.

5.1 Pathological and clinical outcomes of chemoradiation

The pathological results of 72 pts undergoing surgery were available for analysis. 48 pts had a LAR, 17 underwent APR, 4 a transanal resection; 2 pts did not have surgery. Of these 72 pts, 15 (21%) had ypT0 tumours, 7 (10%) had ypT1 tumours, 15 (21%) had ypT2 tumours, 35 (48%) had ypT3 tumours (tab.4).

	T0		T1		T2		T3		T4	
c	0	0%	0	0%	6	8%	62	86%	4	6%
yp	15	21%	7	10%	15	21%	35	48%	0	0%

Tab. 4- Comparison of clinical and pathological stages.

Because of toxic effects, one patient was unable to complete the full course of chemoradiotherapy. Three pts had positive resection margins; the remaining 69 pts underwent neoadjuvant chemoradiotherapy and surgery and had negative resection margins. 15 pts (21%) achieved a pCR, 28 (39%) a partial response and 29 (40%) had a stable disease. The estimated 2-year disease-free survival

was 91.0% (95% CI 84.8-97.6). The estimated 2-year overall survival 96.2% (95% CI: 92.0-100).

All patients have been followed-up for a median 66 months (IQR 56-73) after surgery, with no treatment-related deaths. The proportions of all patients having adverse events during neoadjuvant chemoradiotherapy are presented in tab. 5.

	No toxicities		Grade 1-2		Grade 3		Grade 4	
Gastrointestinal	31	43%	21	29%	20	28%	0	0%
Pain	44	61%	17	24%	10	14%	1	1%
Dermatological	58	81%	8	11%	6	8%	0	0%
Haematological	50	69%	11	15%	9	13%	2	3%
Constitutional symptoms	48	67%	20	28%	4	5%	0	0%
Cardiovascular	65	90%	5	7%	2	3%	0	0%
Genitourinary	57	79%	13	18%	2	3%	0	0%
Hepatic	65	90%	7	10%	0	0%	0	0%

Tab. 5- Adverse events during neoadjuvant chemoradiotherapy.

Three pts died from non cancer-related causes 8–38 months after surgery. At the end of follow-up, 8 (11%) of 72 pts had developed recurrence: five (7%) had distant metastases and four (5.5%) had local recurrence as initial sites of failure. One pts had both distant metastases and local recurrence. The 8 pts with recurrent tumours received salvage treatment. On the other hand, 61 pts (85%) were alive with no evidence of disease and rectal preservation. Results are illustrated in tab 6.

Type of surgery	LAR	48	67%
	APR	17	24%
	Transanal resection	4	6%
	No surgery	2	3%
Pathological stage	ypT0	15	21%
	ypT1	7	10%
	ypT2	15	21%
	ypT3	35	48%
Resection margins	R0	69	96%
	R1	3	4%
Tumor regression	pCR	15	21%
	PR	28	39%
	SD	29	40%
Outcomes	DOD	0	0%
	DOC	3	4%
	AWD	8	11%
	NED	61	85%
Disease-free survival	2-year DFS	91.0% 95% CI: 84.8-97.6	
Overall survival	2-year OS	96.2% 95% CI: 92.0-100	

Tab. 6- Surgical, pathological and clinical outcomes. LAR = low anterior resection; APR = abdomino-perineal resection; pCR = pathological complete response; PR = partial response; SD = stable disease; DOD = dead of disease; DOC = dead of other causes; AWD = alive with disease; NED = non evidence of disease; DFS = disease-free survival; OS = overall survival.

These data suggest that chemoradiation is effective in the majority of pts with locally advanced rectal cancer, whereas a subgroup of pts do not achieve a clinically valuable response and is more likely to have recurrence of disease. Based on this premise, we further studied the radiosensitivity of rectal cancer cell lines with the aim to find determinants of response/resistance and strategies to overcome radioresistance.

5.2 Dose finding for in-vitro experiments

In preliminary experiments, the sensitivity of HCT116 cells to increasing doses of radiation was tested using MTT assays. Radiobiological effects of single dose irradiation were presented as absorbance values obtained spectrophotometrically, which correspond to the number of viable and metabolically active cells. It is clear that radiobiological effects differ depending on the applied irradiation regime.

Compared to non-irradiated control, a significant decrease of cell viability in HCT116 cells was found after each irradiation dose applied ($p < 0.05$, Duncan

test). A statistically significant difference in cell survival was found between 3 Gy and 4 Gy irradiation ($p < 0.05$, Duncan and Tukey tests), decreasing from 75 to 55% (fig. 12). Thus, we decided to start our experiments with a minimum single dose of 4 Gy. Lower doses were delivered only in experiments evaluating the association with 5-fluorouracil.

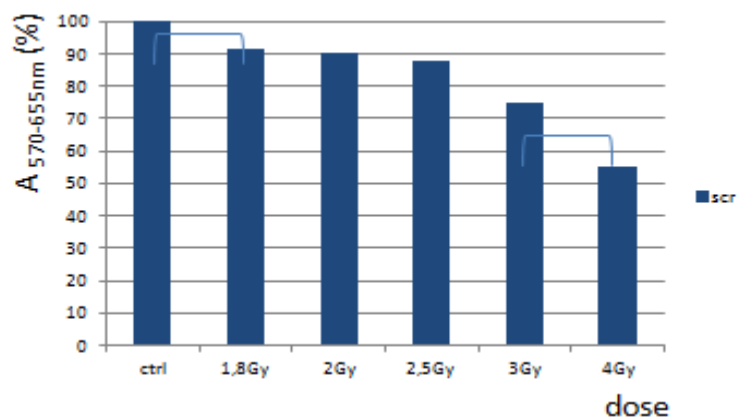


Fig. 12- Adsorbance values obtained from MTT tests in HCT116 cells.

Each bar represents the average of three determinations.

Solid lines connecting the columns represent statistically significant differences ($p < 0.05$).

5.3 Radioresistance after exclusive radiation treatment

Colony forming tests were performed in COLO320, HCT116 and HT29 cells after irradiation with the aim to establish whether different genotypes (RAS or BRAF mutations) correlate with radiosensitivity (fig. 13).

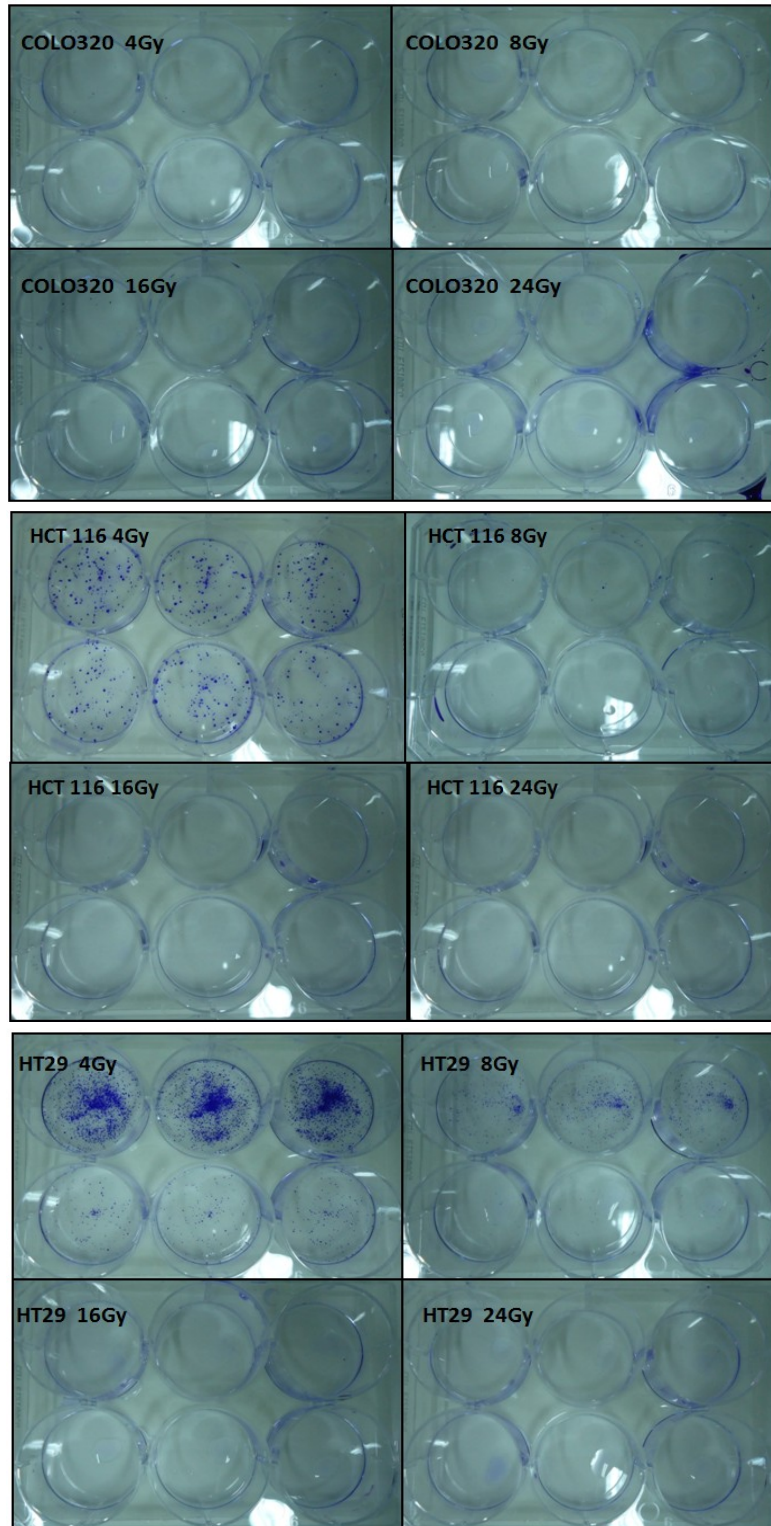


Fig. 13- Clonogenic assay of COLO320, HCT116 and HT29 CRC cells after 4, 8, 16 and 24 Gy.

Clonogenic assays evaluated the effects of growing doses of radiation on colony forming capacity of CRC cell lines. No colonies were detected with 16 Gy in all cell lines (fig. 14).

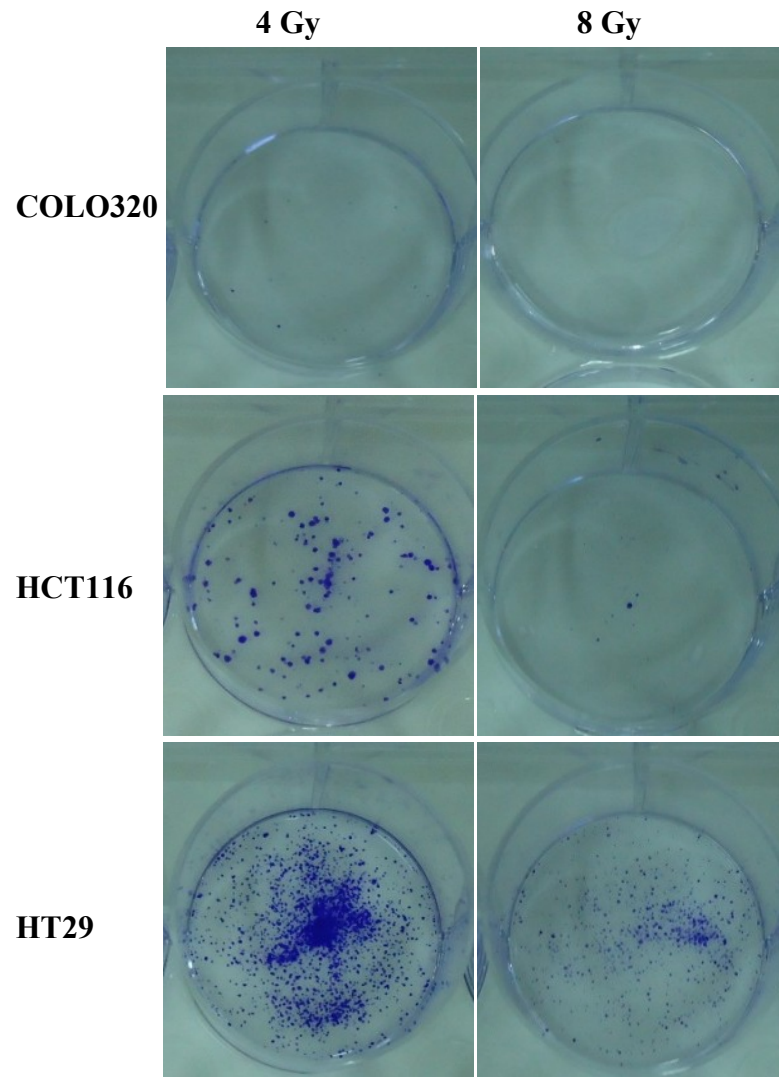


Fig. 14- Colonies after 4 and 8Gy irradiation.

Survival fraction (SF) data in all experimental conditions are shown at the end of the chapter (tab. 9). Average survival curves were obtained using different plating densities that were virtually identical.

2Gy single-dose irradiation reduced SFs of COLO320, HCT116 and HT29 cells to 35.7, 70.8 and 79.4%, respectively (fig. 15). A significant difference exists among cell lines ($p < 0.01$, ANOVA test).

4Gy single-dose irradiation reduced SFs of COLO320 and HCT116 cells to 12-13%, while HT29 SF remained as high as 46.5%. Even if a wide variability for HT29 cells was observed (fig. 16), analysis of variance showed a statistically significant difference among the three cell lines ($p < 0.01$). Compared to COLO320, a significant increase of cell viability in the HT29 cell line was found after 4Gy irradiation ($p < 0.001$, Duncan and Tukey tests).

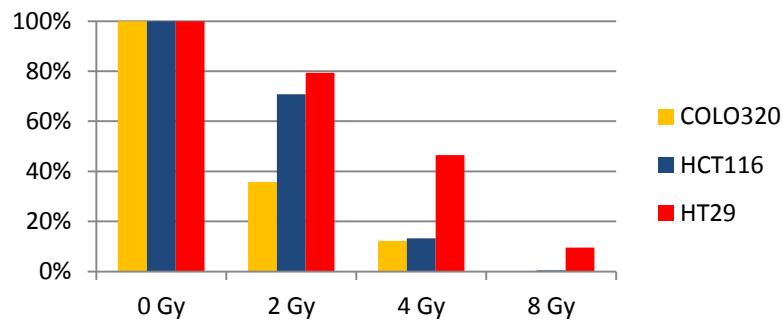


Fig. 15- SFs of COLO320, HCT116 and HT29 CRC cells after 2, 4 and 8Gy irradiation.

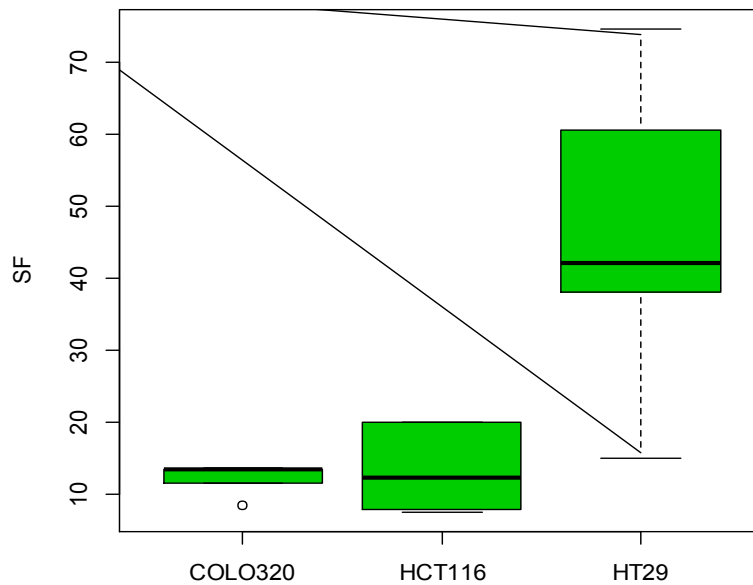


Fig. 16- Boxplots of SFs of COLO320, HCT116 and HT29 CRC cells after 4Gy irradiation.

Excluding the very low and not significant clonogenic capacity of HCT116 cells (SF=0.4%), 8Gy single-dose irradiation allowed only the growth of HT29 cells (SF=9.5%). Thus, the latter reveal to be more radioresistant than COLO320 and HCT116 CRC cells (figs. 14-15).

Student's t-tests comparing COLO320 and HCT116 cells found a statistical difference only after 2 Gy ($p < 0.01$). With higher doses this difference disappears.

In parallel experiments, apoptosis was evaluated in CRC cell lines upon exposure to radiation. In HCT116 cells apoptosis was assessed 24 and 48 hours after irradiation (figs. 17-18). The percentage of necrotic and early apoptotic cells did not differ among groups (not significant ANOVA), while late apoptotic cells increased and healthy cells significantly decreased with dose (fig. 19).

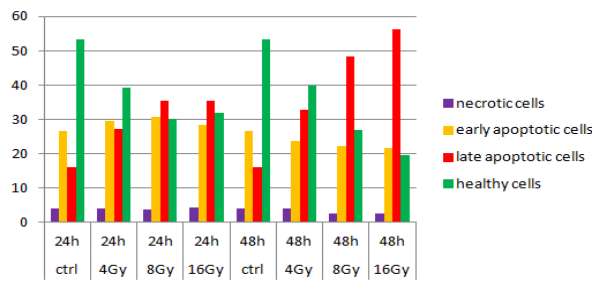


Fig. 17- Clustered bar plots of apoptosis assays in HCT116 after 24 and 48 hours.

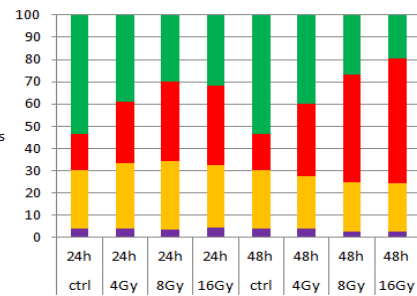


Fig. 18- Stacked bar plots of apoptosis assays in HCT116 after 24 and 48 hours.

Results were more evident after 48 hours (fig. 19), so we performed all following apoptosis assays 48 hours after irradiation. Injured and healthy cells resulted 46.6, 60.3, 73, 80.5% and 53.4, 39.7, 27, 19.5% at 0, 4, 8, 16 Gy, respectively.

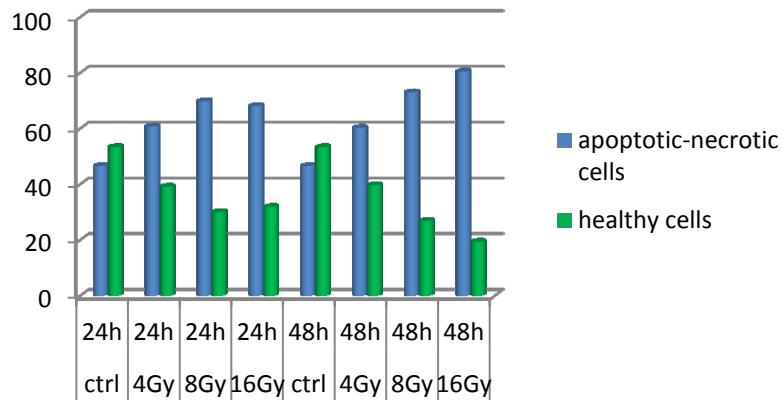


Fig. 19- Percentage of apoptotic/necrotic and vital HCT116 cells 24 and 48 hours after irradiation (0, 4, 8, 16 Gy).

Apoptosis assays were also conducted with HT29 cells exposed to radiation (figs. 20-21). Apoptotic/necrotic and vital cells resulted 11.3, 16.1, 21.9% and 88.7, 83.9, 78.1% at 0, 4, 8 Gy, respectively. HT29 cells resulted more resistant than HCT116 cells to radio-induced apoptosis (fig. 22).

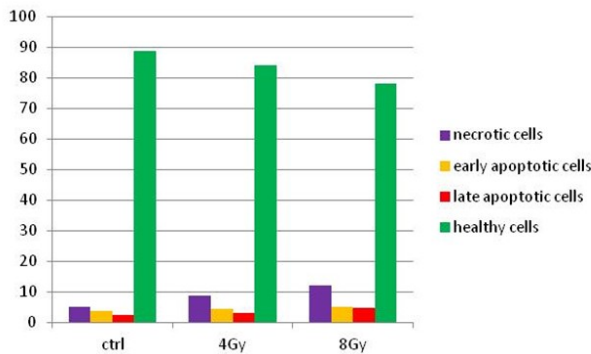


Fig. 20- Clustered bar plots of apoptosis assays in HT29.

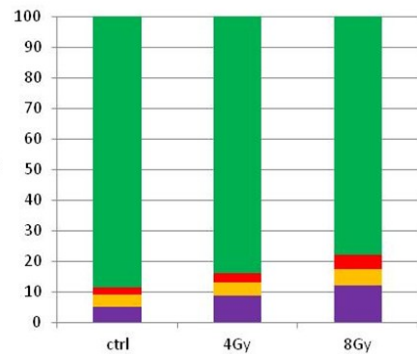


Fig. 21- Stacked bar plots of apoptosis assays in HT29.

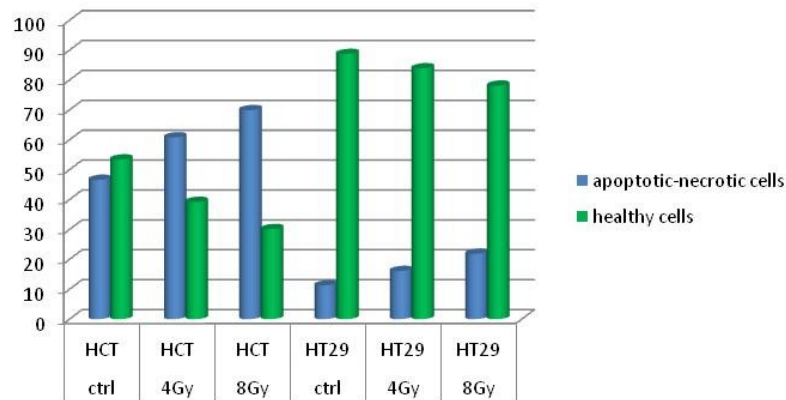


Fig. 22- Percentage of injured and healthy HCT116 and HT29 cells 48 hours after irradiation (0, 4, 8 Gy). Each bar is the average of three determinations.

5.4 The effects of chemotherapy on radiation resistance

Since BRAF-mutated colon carcinoma cells showed resistance to radiation, in further experiments we tested the sensitivity of our panel of colon carcinoma cell lines to combination of radiation and 5FU. Thus, 5FU pretreated COLO320, HCT116 and HT29 cells were irradiated. The association of 5-FU and radiation showed to arrest cell growth to a greater extent than radiation alone in all cell lines. COLO320 cells revealed to be the most sensitive (fig. 23): 15 nM 5-FU in combination with 4 Gy single-dose irradiation was able to completely eradicate cancer cells. Conversely, a higher dose of 5-FU (25 nM) in combination with 4 Gy single-dose irradiation was necessary to obtain HCT116 eradication (fig. 24).

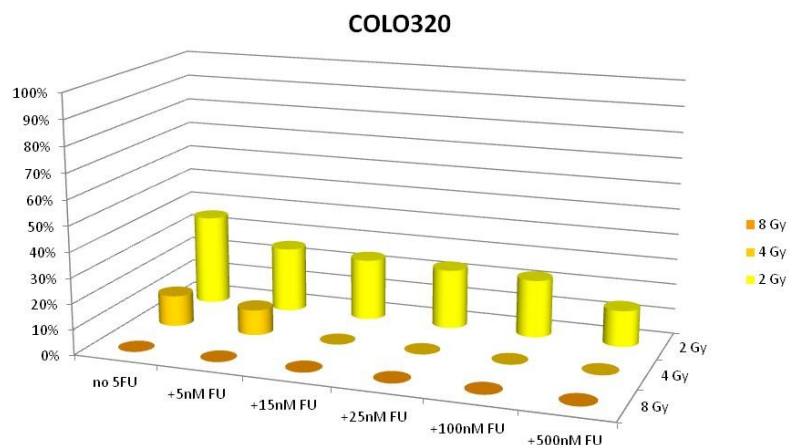


Fig. 23- SFs of COLO320 CRC cells after 5-fluorouracil (0, 5, 15, 25, 100, 500 nM) and radiation (2, 4, 8 Gy).

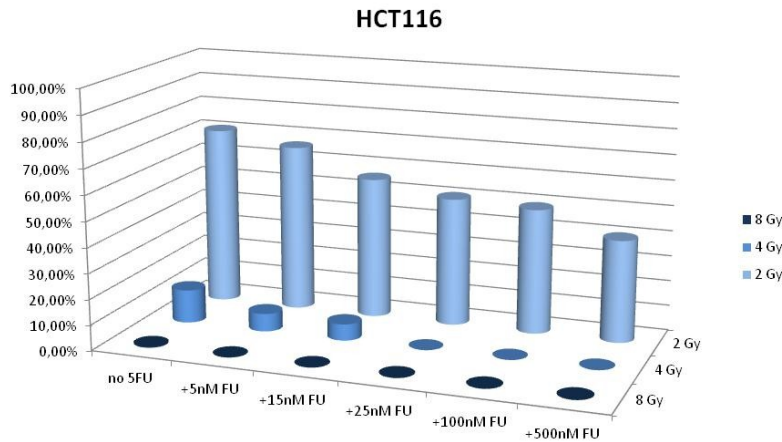


Fig. 24- SFs of HCT116 CRC cells after 5-fluorouracil (0, 5, 15, 25, 100, 500 nM) and radiation (2, 4, 8 Gy).

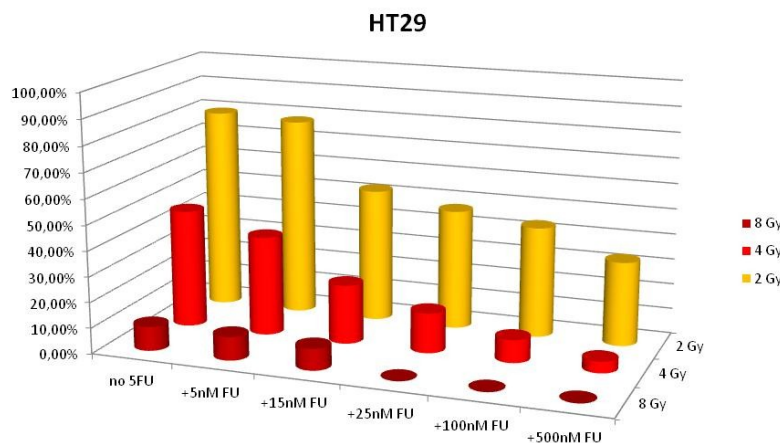


Fig. 25- SFs of HT29 CRC cells after 5-fluorouracil (0, 5, 15, 25, 100, 500 nM) and radiation (2, 4, 8 Gy).

HT29 resulted to be the most resistant cells to combination of 5-FU and radiation (fig. 25): 4.5% of BRAF V600E-mutated cells survived despite treatment with 500nM 5-FU and subsequent 4Gy irradiation; 8Gy single-dose irradiation was necessary in association with 5-FU (≥ 25 nM) to eradicate HT29 cells (fig 25).

5-FU confirmed its radiosensitizing activity at each dose level in all cell lines: the effect increased with growing concentrations of 5-FU, except for

COLO320 and HCT116 cells at 8 Gy where the maximum killing occurred even without drug (fig. 26).

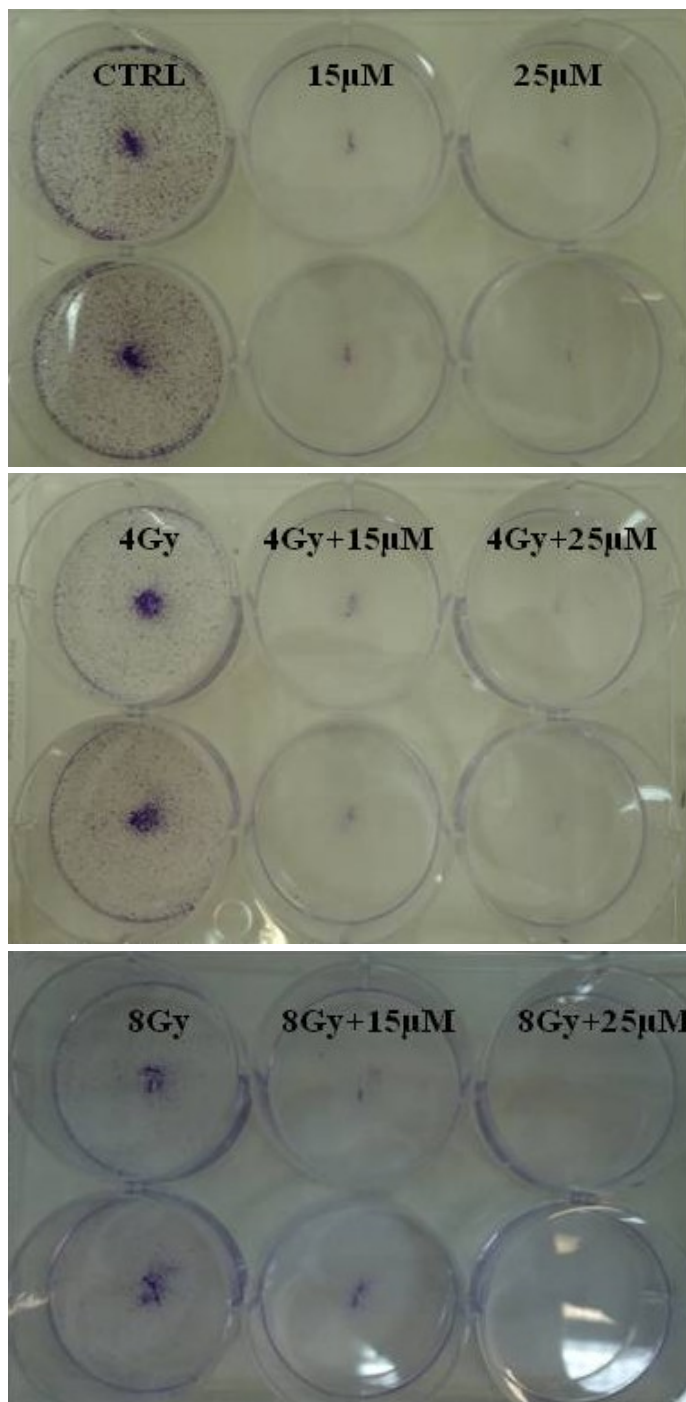


Fig. 26- Clonogenic assay: HT29 colonies after pre-treatment with 5-FU (15, 25 nM) and irradiation (0, 4, 8Gy).

5.5 BRAF silencing/inhibition

Since BRAF-mutated cells showed a high resistance to ionizing radiations, the effects of BRAF silencing/inhibition on radio-induced response were evaluated in HT29 cells using BRAF SiRNAs and PLX4720, a specific inhibitor of B-Raf-V600E mutant. Negative SiRNAs of BRAF were used as controls (fig. 27).

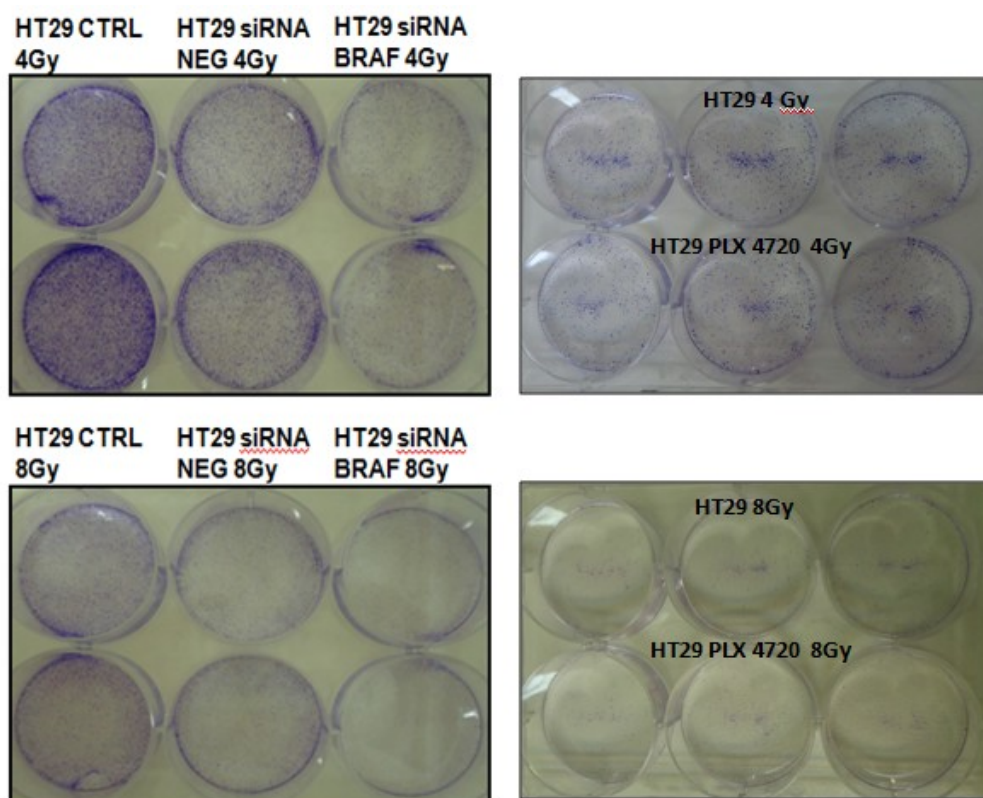


Fig. 27- Clonogenic assay: HT29 CRC colonies after transfection of BRAF siRNAs or treatment with PLX4720 (10 μ M) and radiation (4 and 8 Gy).

The expression of B-Raf protein was evaluated by Western blot analysis as control of siRNA transfection (fig. 28). Survival fraction data are shown in tab.7.

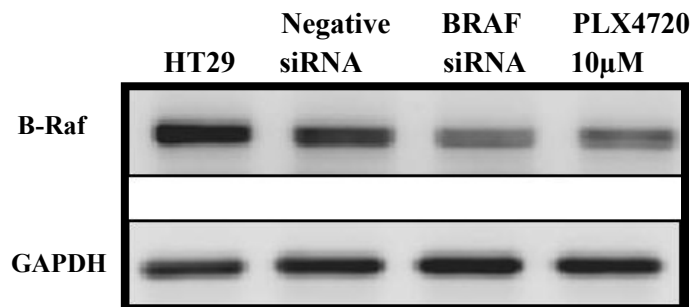


Fig. 28- Western blot. Expression of B-Raf in HT29 cells after transfection of BRAF siRNAs or treatment with PLX4720 (10 µM).

	HT29	Neg. BRAF siRNAs	BRAF siRNAs	+ PLX4720
0 Gy	100%	100%	100%	100%
4 Gy	46.5%	47.3%	24.6%	40.3%
8 Gy	9.5%	3.9%	0%	5.2%

Tab. 7- SFs of clonogenic assays after BRAF silencing/inhibition in HT29 cells (n≥3 for each experiment).

While PLX4720 had moderate but not significant sensitizing effects compared to untreated cells (Student's t-test), BRAF silenced cells resulted significantly more sensitive than control cells ($p < 0.01$, Student's t-test), both at 4 and 8 Gy (fig. 29).

4Gy single-fraction allowed the growth of 24.6% of BRAF-silenced cells versus 46.5% of control HT29 cells; 8 Gy single-fraction completely eradicated BRAF-silenced cells versus 9.5% of control HT29 cells. Student's t-tests comparing negative siRNA transfected cells and control cells did not show significant differences.

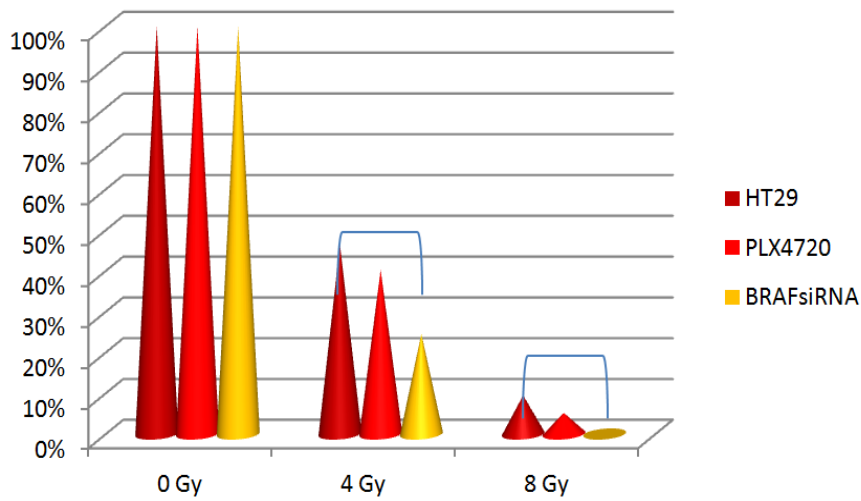


Fig. 29- SFs of HT29 CRC cells after treatment with PLX4720 (10 μ M) or transfection of BRAF siRNAs and radiation (0, 4, 8 Gy). Solid lines connecting cones represent statistically significant differences ($p < 0.01$).

5.6 TRAP1 silencing/inhibition

The effects of TRAP1 silencing/inhibition on radio-induced response were evaluated in HT29 cells using TRAP1 silencing and HSP990, a dual inhibitor of Hsp90/TRAP1. Two different concentrations of HSP990 were tested (75 and 150 nM). 4Gy single-dose irradiation caused the complete eradication of colonies; so, only for HSP990, we had to use 2Gy single-fractions. Transfection with negative SiRNAs was used as control (fig. 30).

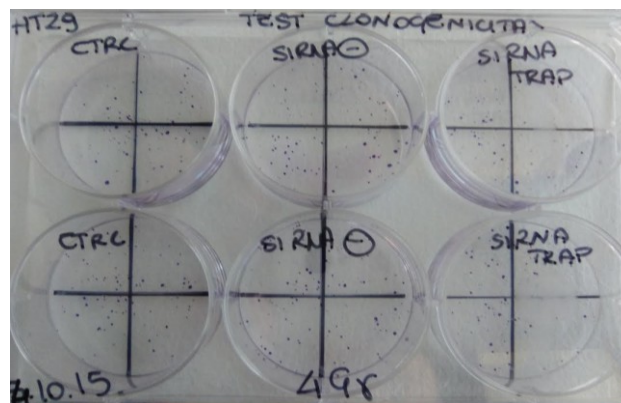


Fig. 30- Clonogenic assay: HT29 CRC colonies after transfection of TRAP1 siRNAs and radiation (4 Gy).

The expression of TRAP1 protein was evaluated by Western blot analysis (fig. 31). The inhibition of TRAP1 with HSP990 significantly downregulated

B-Raf protein expression (fig. 32), consistently with the chaperoning activity of Hsp90 chaperones toward BRAF. Survival fraction data are shown in tab. 8.

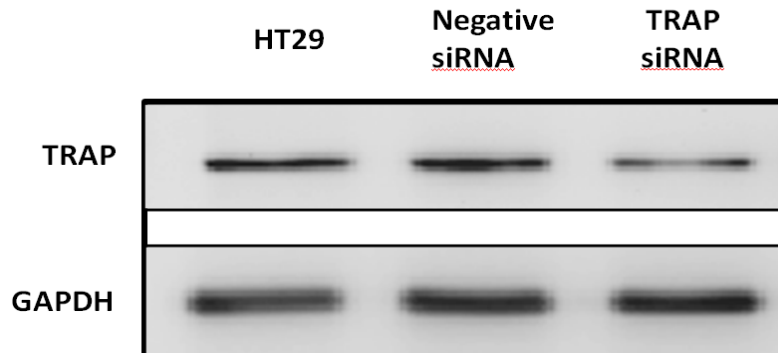


Fig. 31- Western blot. Expression of TRAP1 in HT29 cells after transfection of BRAF siRNAs.

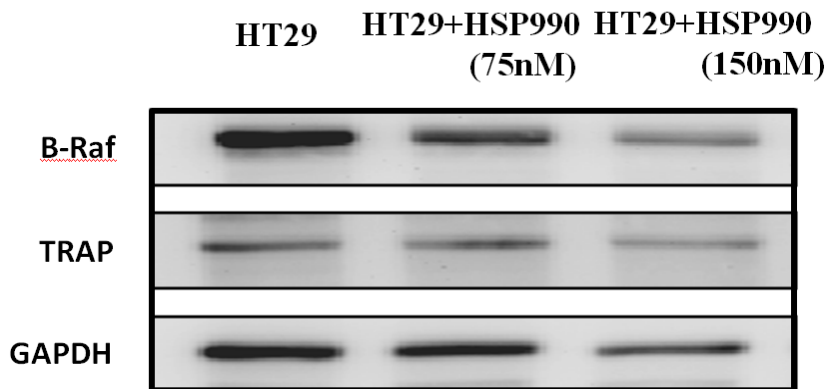


Fig. 32- Western blot. Expression of B-Raf and TRAP1 in HT29 cells after treatment with HSP990 (75 and 150 nM).

	HT29	Neg. siRNAs	TRAP siRNAs	HSP990 (75nM)	HSP990 (150nM)
0 Gy	100%	100%	100%	100%	100%
2 Gy	79.4%	n.d.	n.d.	20.0%	15.3%
4 Gy	46.5%	42.1%	32.0%	0%	0%
8 Gy	9.5%	6.9%	3.4%	0%	0%

Tab. 8- SFs of clonogenic assays after TRAP1 silencing/inhibition in HT29 cells ($n \geq 3$ for each experiment; n.d. = not determined).

Pre-treatment of HT29 cell lines with Hsp90/TRAP1 dual inhibitor showed to significantly increase the radiosensitivity of BRAF-mutated cells (fig. 33). HSP990 at concentrations of 75 and 150 nM improved the efficacy of radio-induced cancer cell killing even at the lowest dose of ionizing radiation (2 Gy).

2Gy single-dose irradiation allowed a survival fraction of 79.4% in control HT29 cells. SF was reduced to 20% and 15.3% in cells pretreated with 75nM and 150nM HSP990, respectively. SF was 46.5% in control cells, while no colonies grew among pretreated cells, at 4 Gy radiation dose. A very significant difference was found between control and inhibited cells, both at 2 and 4 Gy ($p < 0.001$, Student's t-test). A not significant difference was found between the effects of the inhibitor at 75 and 150 nM.

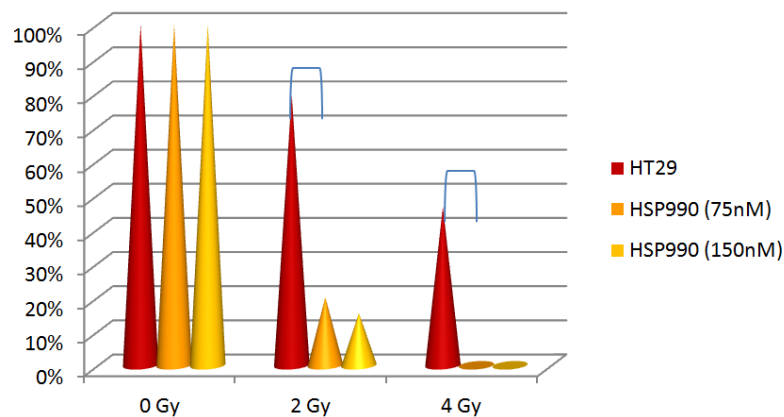


Fig. 33- SFs of HT29 CRC cells after treatment with HSP990 (75 and 150 nM) and radiation (0, 2, 4 Gy). Solid lines connecting cones represent statistically significant differences ($p < 0.001$).

The transfection of resistant cells with TRAP1 siRNAs increased their radiosensitivity, both at 4 and 8 Gy. However, no significant difference was found ($p = 0.08$ and $p = 0.07$ respectively, Student's t-test). The transfection with negative siRNAs of TRAP1 did not modify SFs (fig. 34).

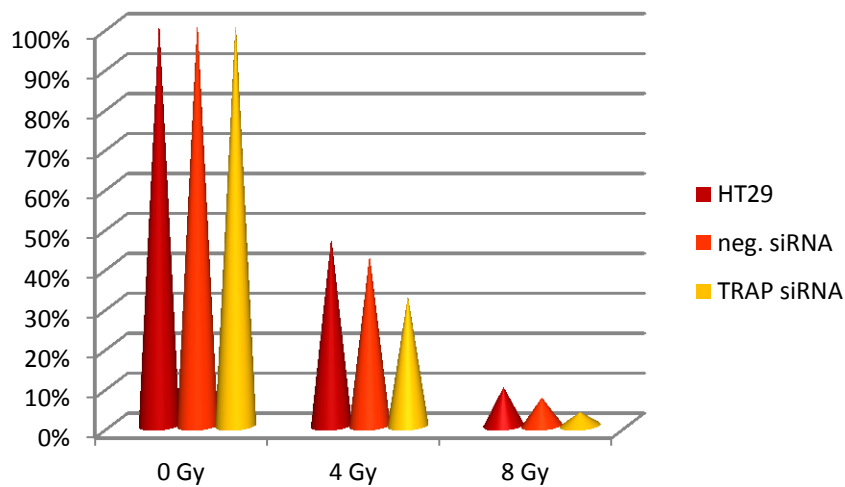


Fig. 34- SFs of HT29 CRC cells after transfection of TRAP1 siRNAs and radiation (0, 4, 8 Gy).

Apoptosis assays were carried out to confirm the role of siRNAs specific for TRAP1 in increasing radiosensitivity of HT29 cells; negative siRNAs were used as control. TRAP1 silenced cells revealed higher sensitivity to 4 and 8 Gy irradiation than control HT29 cells ($p < 0.01$, Chi-square tests). Surprisingly, 4Gy fractions in TRAP1 siRNA cells appear to be more effective than 8Gy fractions in control cells, even if this difference is not statistically significant (figs. 35-36).

Unfortunately, compared to negative siRNA cells, TRAP1 silencing did not show to significantly improve radio-induced effects, even if an increase of radiotoxicity was observed in TRAP1 siRNA cells, both at 4 and 8 Gy ($p = 0.07$ and $p = 0.08$ respectively, Chi-square tests). The efficiency of siRNA transfection, not optimal in each experiment, could be responsible for this interesting but not significant result.

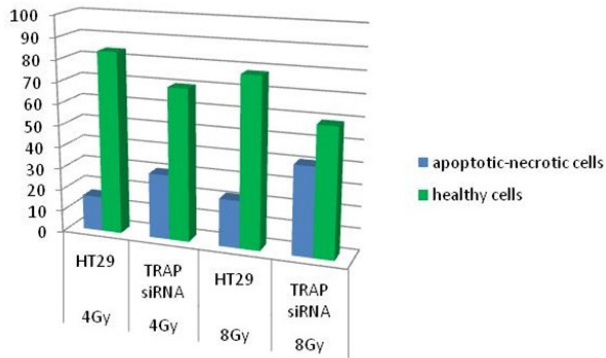


Fig. 35- Percentage of injured and healthy control and TRAP1 siRNA cells after irradiation.

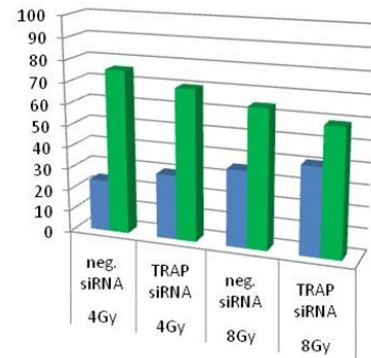


Fig. 36- Percentage of injured and healthy negative and TRAP1 siRNA cells after irradiation.

The role of TRAP1 was verified by clonogenic assays with shTRAP1 clones in HCT116 cells (fig. 37).

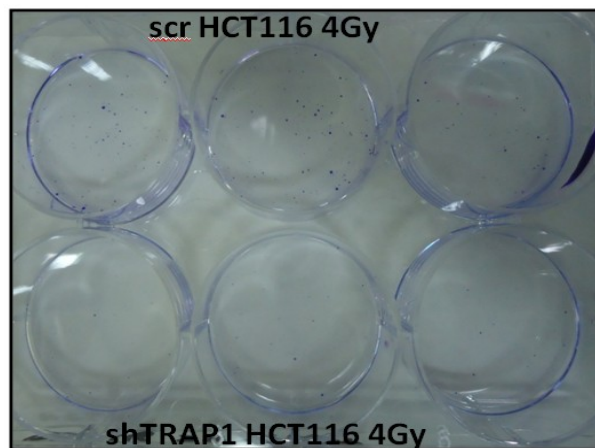


Fig. 37- Clonogenic assay: scramble and shTRAP1 HCT116 colonies after 4Gy irradiation.

The expression of TRAP1 protein was evaluated by Western blot analysis (fig. 38). A statistically significant difference exists between SFs after 4Gy single-dose ($p < 0.05$, Student's t-test) (fig. 39).

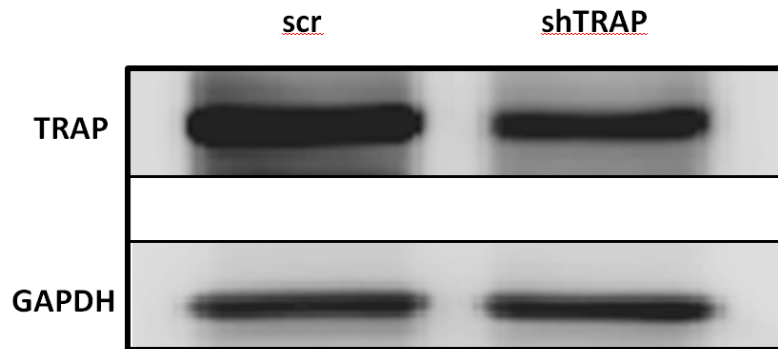


Fig. 38- Western blot. Expression of TRAP1 in scramble and shTRAP1 HCT116 cells.

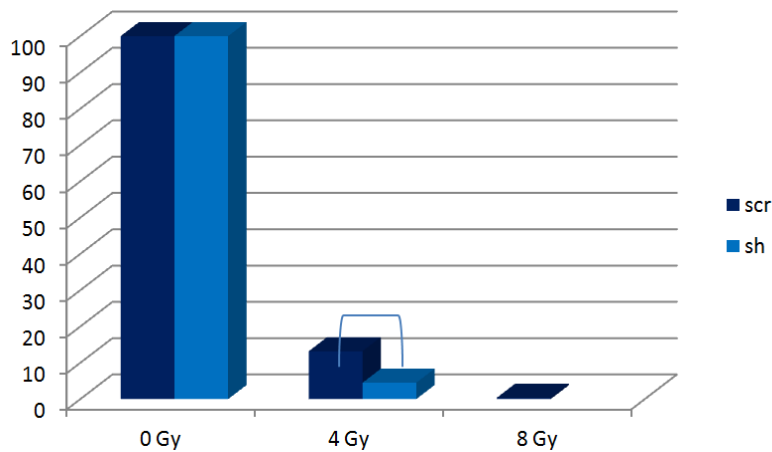


Fig. 39- SFs of scramble and shTRAP1 HCT116 cells after irradiation (4 and 8 Gy). The solid line connecting the columns represents a statistically significant difference ($p < 0.05$).

The effects of radiation in scramble HCT116 cells were compared with the radio-induced effects in shTRAP1 HCT116 cells also using MTT assays (fig. 40). Compared to non-irradiated control, a significant decrease of cell viability in the shTRAP1 cell line was found after each irradiation ($p < 0.05$, Duncan test). However, a statistically significant difference was found between 89% at 2 Gy and 64.2% at 2.5 Gy ($p < 0.05$, Duncan and Tukey tests), earlier than scramble cells. Moreover, the difference between the groups was tested using Student's t-test, for each dose level. Differences became significant at the dose of 2.5 Gy, suggesting that shTRAP1 cells are more sensitive than the scramble ones.

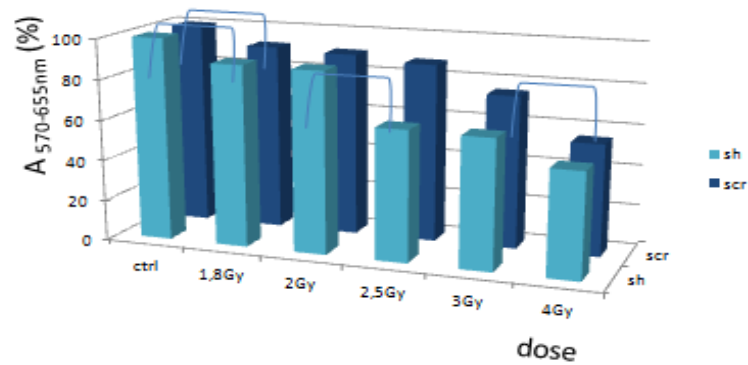


Fig. 40- Adsorbance values obtained from MTT tests in scramble vs shTRAP1 HCT116 cells. Each bar represents the average of three determinations. Solid lines connecting the columns represent statistically significant differences ($p < 0.05$).

Apoptosis assays were carried out to compare scramble and shTRAP1 HCT116 cells as well, in another set of experiments (figs. 41-42).

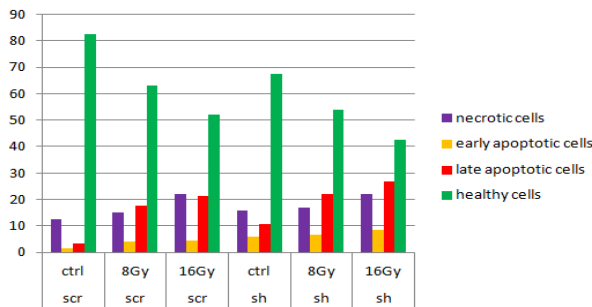


Fig. 41- Clustered bar plots of apoptosis assays in scramble and shTRAP1 HCT116.

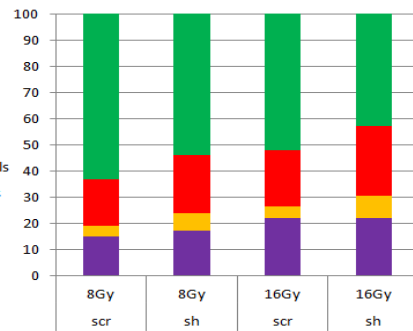


Fig. 42- Stacked bar plots of apoptosis assays in scramble and shTRAP1 HCT116.

Both for 8 and 16 Gy irradiation, shTRAP1 cells revealed to be more sensitive than scramble HCT116 ($p < 0.05$, Chi-square tests). No significant difference was found between the effects of 8Gy irradiation in shTRAP1 cells and the effects of 16Gy irradiation in scramble cells (fig. 43).

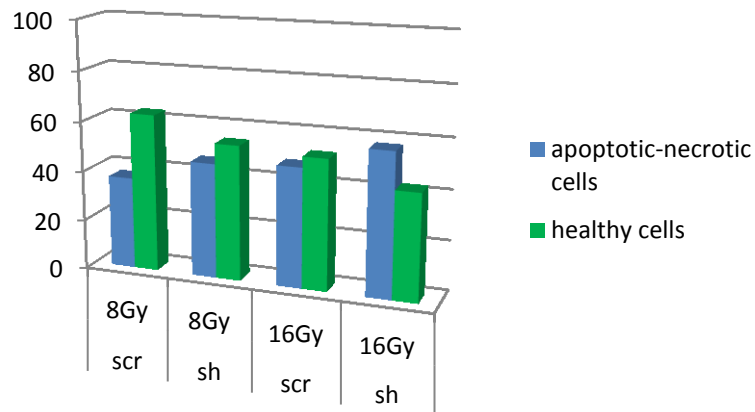


Fig. 43- Percentage of apoptotic/necrotic and vital scramble and shTRAP1 HCT116 cells after irradiation (48hs).

Finally, we tested whether the upregulation of TRAP1 represents an adaptive response of colon cancer cells to survive to radiation. In an experimental design with daily 1Gy fractions for 3 consecutive days, the expression of TRAP1 protein increased, suggesting a possible role for TRAP1 also in the adaptive response to radiation (fig. 44).

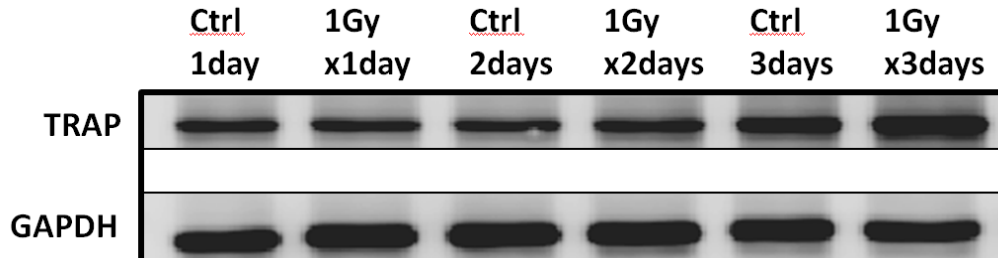


Fig. 44- Western blot. Expression of TRAP1 in HCT116 cells.

		0 Gy	2 Gy	4 Gy	8 Gy	16 Gy
COLO320	scr	100%	35.7%	12.2%	0%	0%
	+5nM FU	100%	25.7%	10.0%	0%	0%
	+15nM FU	100%	24.3%	0%	0%	0%
	+25nM FU	100%	23.5%	0%	0%	0%
	+100nM FU	100%	22.8%	0%	0%	0%
	+500nM FU	100%	14.3%	0%	0%	0%
HCT116	scr	100%	70.8%	13.2%	0.4%	0%
	shTRAP1	100%	n.d.	4.5%	0%	0%
	+5nM FU	100%	66.3%	7.1%	0%	0%
	+15nM FU	100%	55.9%	6.5%	0%	0%
	+25nM FU	100%	50.7%	0%	0%	0%
	+100nM FU	100%	49.4%	0%	0%	0%
HT29	+500nM FU	100%	40.3%	0%	0%	0%
	scr	100%	79.4%	46.5%	9.5%	0%
	+5nM FU	100%	78.0%	39.0%	9.2%	0%
	+15nM FU	100%	52.5%	23.2%	8.5%	0%
	+25nM FU	100%	47.2%	15.7%	0%	0%
	+100nM FU	100%	43.5%	9.0%	0%	0%
	+500nM FU	100%	33.0%	4.5%	0%	0%
	+PLX4720	100%	n.d.	40.3%	5.2%	0%
	BRAFsiRNA	100%	n.d.	24.6%	0%	0%
	neg. siRNA	100%	n.d.	47.3%	3.9%	0%
	+75nM HSP990	100%	20.0%	0%	0%	0%
+150 nM HSP990	100%	15.3%	0%	0%	0%	
TRAPsiRNA	100%	n.d.	32.0%	3.4%	0%	
neg. siRNA	100%	n.d.	42.1%	6.9%	0%	

Tab. 9- Synopsis. Surviving Fractions (SFs) of irradiated cells in all experimental conditions ($n \geq 3$ for each experiment); n.d. = not determined.

6. Discussion

Results of *ex vivo* studies are severely limited by the small number of subjects in each study, the low level of protein expression of any given marker, the timing of tissue collection (i.e., prior to irradiation vs after irradiation), differences in neoadjuvant modalities (i.e., low vs high dose of ionizing radiation), and differences in the cohorts of patients under investigation. With these limitations, it has been difficult to construct a panel of molecules that would accurately predict the response of a tumor to preoperative ionizing radiation¹. Unfortunately, data from *in vitro* studies are substantially more limited because the information derived from cell lines does not provide an indication of the clinical response to ionizing radiation.

Several of the reports addressing cell cycle regulators as predictors for response to ionizing radiation have demonstrated some inconsistencies. Arguments with regard to p53 lean towards an important role of wild-type p53 in pre-irradiated samples to predict a good response to ionizing radiation. The fact that some studies showed negative p53 status in pre-irradiated biopsies as a marker of good response is a reflection of the inability of the antibodies to recognize the wild-type form of the protein as readily compared with the mutant, more stable form assessed by IHC. Positive CDKI status in pre-irradiated samples by IHC also seems to be a reasonable predictor of radiosensitivity in patients with rectal cancers. However, this has been different in preclinical studies where p21-positive cells had a more radioresistant phenotype, which was speculated to be the result of cell cycle arrest². The major problems with clinical studies are the relatively low number of samples tested and the small percentage of positivity of the tissue samples for any given protein such that it is difficult to predict, with certainty, the response to ionizing radiation for those tumors that are not positive.

In vitro and *in vivo* data with regards to the NF- κ B–IAP axis of radioresistance have shown consistently that NF- κ B survival pathway probably in response to direct ionizing radiation activation leads to overexpression of survivin and radioresistance. *Ex vivo* data supports the role of survivin in radioresistance. For instance, the 5-year survival of patients with survivin

¹ Huerta S, Gao X, Saha D. Mechanisms of resistance to ionizing radiation in rectal cancer. *Expert Rev Mol Diagn* 2009;9(5):469-80.

² Tian H, Wittmack EK, Jorgensen TJ. p21WAF1/CIP1 antisense therapy radiosensitizes human colon cancer by converting growth arrest to apoptosis. *Cancer Res* 2000;60:679-84.

positive stage II colon cancer tumors was 41% lower than patients with survivin negative tumors³. The link between NF- κ B and other IAPs (i.e., XIAP and cIAP) and radioresistance remains at large.

The baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), more commonly referred to as survivin, encodes for the smallest and structurally unique member of the inhibitors of apoptosis family of proteins⁴. Survivin is overexpressed in a variety of human tumors, and it plays a prominent role in regulating apoptosis, during cell division, and during adaptation to stress. Following up on the observation that the expression of survivin was inversely correlated with spontaneous and radiation-induced apoptosis⁵, Rödel and colleagues used siRNA-mediated gene silencing to demonstrate that inhibition of survivin sensitizes colorectal cancer cells to radiation therapy, accompanied by increased levels of G2/M phase arrest and increased levels of DNA double-strand breaks after irradiation⁶. Very recently, the same authors could show that survivin rapidly accumulates in the nucleus following irradiation where it subsequently interacts with members of the DNA double-strand break repair machinery in order to regulate the activity of DNA dependent protein kinase⁷. Because survivin inhibitors are currently being investigated in clinical trials, future studies will ultimately demonstrate whether its inhibition represents an effective strategy for (chemo)radiosensitization⁸. In this respect, the potential relevance of survivin for monitoring response to preoperative chemoradiotherapy has recently been confirmed by Sprenger and colleagues who could show that high survivin expression in pretreatment biopsies

³ Rodel F, Frey B, Leitmann W et al. Survivin antisense oligonucleotides effectively radiosensitize colorectal cancer cells in both tissue culture and murine xenograft models. *Int J Radiat Oncol Biol Phys* 2008;71:247-55.

⁴ Altieri DC. Survivin, cancer networks and pathway-directed drug discovery. *Nat Rev Cancer* 2008;8(1):61-70.

⁵ Rodel C, Haas J, Groth A et al. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. *Int J Radiat Oncol Biol Phys* 2003;55(5):1341-47.

⁶ Rodel F, Hoffmann J, Distel L et al. Survivin as a radioresistance factor, and prognostic and therapeutic target for radiotherapy in rectal cancer. *Cancer Res* 2005;65(11):4881-7.

⁷ Capalbo G, Dittmann K, Weiss C et al. Radiation-induced survivin nuclear accumulation is linked to DNA damage repair. *Int J Radiat Oncol Biol Phys* 2010;77(1):226-34.

⁸ Rodel F, Reichert S, Sprenger T et al. The role of survivin for radiation oncology: moving beyond apoptosis inhibition. *Curr Med Chem* 2011;18 (2):191-9.

correlated with advanced post-therapeutical tumor and UICC stage and decreased disease-free survival⁹.

Besides survivin, T cell specific factor 4 (TCF4) represents a prominent and promising predictor of radioresistance. Ghadimi and colleagues reported the identification of a 54-gene signature that differentiated resistant and responsive rectal cancers from patients who had been treated with preoperative chemoradiotherapy, as we already illustrated above¹⁰. Interestingly, within this signature, the transcription factor TCF4 was found to be significantly overexpressed in resistant tumors. TCF4, also known as TCF7L2, represents a key downstream effector that mediates canonical Wnt signaling, a pathway that plays a central role in colorectal tumorigenesis and tumor progression¹¹.

In order to explore the functional relevance of this overexpression for mediating treatment resistance, Kendziorra and colleagues silenced TCF4 in resistant colorectal cancer cell lines and could show that RNAi-mediated inhibition of TCF4 caused a significant radiosensitization of colorectal cancer cells with high TCF reporter activity. Follow-up experiments revealed that the effect of radiosensitization was associated with a G2/M phase arrest, an impaired ability to adequately halt cell cycle progression after irradiation, and a compromised DNA double-strand break repair¹². These data indicate a novel role of the Wnt transcription factor TCF4 in mediating radioresistance and, if further validated, suggest that TCF4 is a promising therapeutic target.

The results of our monoinstitutional study suggest that we were able to treat locally advanced rectal tumours with neoadjuvant chemoradiotherapy and surgery, with good results and acceptable toxicities. Furthermore, nearly 20% of pts had pathological complete response and only 3 in 72 had a positive resection margin. We aimed to quantify response rate by its current gold

⁹ Sprenger T, Rodel F, Beissbarth T et al. Failure of down-regulation of survivin following neoadjuvant radiochemotherapy in rectal cancer is associated with distant metastases and shortened survival. *Clin Cancer Res* 2011;17(6):1623-31.

¹⁰ Ghadimi BM, Grade M, Difilippantonio MJ et al. Effectiveness of gene expression profiling for response prediction of rectal adenocarcinomas to preoperative chemoradiotherapy. *J Clin Oncol* 2005;23(9):1826-38.

¹¹ Moon RT, Kohn AD, De Ferrari GV et al. WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* 2004;5(9):691-701.

Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell* 2006;127(3):469-80.

¹² Kendziorra E, Ahlborn K, Spitzner M et al. Silencing of the Wnt transcription factor TCF4 sensitizes colorectal cancer cells to (chemo-) radiotherapy. *Carcinogenesis* 2011;32(12):1824-31.

standard (that is, pathology), and noticed that it is correlated to the clinical response.

Looking for mechanisms responsible for radioresistance, clonogenic, apoptotic and MTT assays are useful tests for evaluation of radiosensitivity. Their survival curves show the same characteristics: the survival depends on the dose of irradiation and an increase of dose leads to a decrease in survival. Clonogenic and MTT assay show very similar curve progression, while the MTT assay gives slightly higher activity results. This fact may be explained by the presence of cells which are alive and show metabolic activity, but poor or no cell proliferation¹³.

Our results suggest that MTT, apoptotic and clonogenic assays can be alternatives in order to determine survival of irradiated tumour cells. The main disadvantage of MTT assay is that the survival may be overestimated at high radiation in the case of plating before irradiation. In fact, if the plating of cells is done before irradiation, metabolic cell activity during the following assay may vary significantly. Cells with low metabolic activity and slow proliferation or cells which ceased to proliferate, are excluded from the assay by washing and trypsinization, when the plating is done after irradiation. The main advantage of MTT and apoptotic assays is the opportunity to obtain precise survival data for high sample throughput in less time and with less effort than with colony assay.

The first step of our research was to identify a range of radiation doses to which CRC cell lines are sensitive¹⁴. Cell viability assays suggested that a single-dose of about 4 Gy gives a cellular survival of around 50%, roughly considering our three cell lines. We noticed that HT29 (BRAF-V600E mutated) cells are more resistant to radiation than HCT116 (RAS-mutated) cells and COLO320 (RAS/BRAF-wt) cells. Thus, we hypothesized a role of BRAF mutations in radioresistance. In particular, we argued that BRAF-V600E mutation could play a role in protecting cancer cells against radiotherapy. BRAF-V600E cells revealed to be more resistant also to the association of 5-FU and radiation, explaining one possible mechanism of preoperative chemoradiation failure.

¹³ Buch K, Peters T, Nawroth T et al. Determination of cell survival after irradiation via clonogenic assay versus multiple MTT assay- a comparative study. *Radiation Oncology* 2012;7:1.

¹⁴ Djan I, Solajic S, Djan M et al. Radiobiological effects of multiple vs single low-dose pre-irradiation on the HT29 cell line. *Contemp Oncol (Pozn)* 2014;18(4):230-3.

BRAF is one of the top 12 mutant genes in human malignancies, with the substitution at position 600 from a valine to a glutamic acid (BRAF-V600E) the most common¹⁵. Human BRAF-driven tumours, mostly melanomas, and thyroid and colorectal carcinomas, are biologically and clinically aggressive malignancies, frequently resistant to conventional anticancer therapies¹⁶. Indeed, the oncogenic activation of BRAF drives the inappropriate activation of ERK signaling and the deregulation of cell proliferation¹⁷, and is responsible for the inhibition of the mitochondrial apoptotic pathway¹⁸, the latter being consistent with the apoptosis-resistant phenotype of BRAF-driven cancer cells. In this perspective, BRAF translocation to mitochondria represents a prerequisite for enabling resistance to apoptosis and this results in inhibition of cytochrome c release and inactivation of the caspase cascade¹⁹, although the molecular mechanisms of BRAF antiapoptotic responses in mitochondria are not fully elucidated. From a clinical perspective, BRAF-mutated colorectal carcinomas CRCs are frequently addicted to this mitochondrial survival pathway, resistant to apoptosis and poorly responsive to standard chemotherapeutics and EGFR monoclonals²⁰. Thus, the molecular

¹⁵ Dienstmann R, Tabernero J. BRAF as a target for cancer therapy. Anti-cancer agents in medicinal chemistry. 2011;11:285-95.

Garnett MJ, Marais R. Guilty as charged: B-RAF is a human oncogene. Cancer cell 2004;6:313-9.

¹⁶Pritchard C, Carragher L, Aldridge V et al. Mouse models for BRAF-induced cancers. Biochemical Society transactions. 2007;35:1329-33.

Cantwell-Dorris ER, O'Leary JJ, Sheils OM. BRAFV600E: implications for carcinogenesis and molecular therapy. Molecular cancer therapeutics 2011;10:385-94.

¹⁷ Santarpia L, Lippman SM, El-Naggar AK. Targeting the MAPK-RAS-RAF signaling pathway in cancer therapy. Expert opinion on therapeutic targets 2012;16:103-19.

¹⁸ Burgeiro A, Gajate C, Dakir el H et al. Involvement of mitochondrial and B-RAF/ERK signaling pathways in berberine-induced apoptosis in human melanoma cells. Anti-cancer drugs 2011;22:507-18.

Berger A, Quast SA, Plotz M et al. RAF inhibition overcomes resistance to TRAIL-induced apoptosis in melanoma cells. The Journal of investigative dermatology 2014;134:430-40.

¹⁹ Lee MH, Lee SE, Kim DW et al. Mitochondrial localization and regulation of BRAFV600E in thyroid cancer: a clinically used RAF inhibitor is unable to block the mitochondrial activities of BRAFV600E. The Journal of clinical endocrinology and metabolism 2011;96:19-30.

²⁰ Cui D, Cao D, Yang Y et al. Effect of BRAF V600E mutation on tumor response of anti-EGFR monoclonal antibodies for first-line metastatic colorectal cancer treatment: a meta-analysis of randomized studies. Molecular biology reports 2014;41:1291-8.

characterization of BRAF-dependent antiapoptotic mechanisms is the prerequisite for targeting the BRAF survival pathway, thus representing a major clinical need, based on the lack of appropriate and effective treatments for these tumours²¹. Since it is well known that human BRAF-addicted CRCs are characterized by reduced responsiveness to chemotherapeutics, we evaluated the radio-sensitivity of BRAF-mutated compared to BRAF-wild type (wt) human CRC cell lines. Indeed, BRAF-V600E HT29 showed poor sensitivity to radiation compared to BRAF-wt COLO320 cells. In such a perspective, we observed that BRAF has a role also in radioresistance and that the inhibition of B-Raf protein with PLX4720 has a moderate but not significant sensitizing effect, while BRAF siRNA silenced cells resulted significantly more sensitive than control and negative transfected cells to an extent similar to TRAP1 inhibition/silencing. TRAP1 silencing was evaluated based on the evidence that BRAF is a client protein of TRAP1. Consistently with this premise, transfection of resistant HT29 cells with TRAP1 siRNAs increases cancer cell killing as well. The role of TRAP1 in radioresistance is confirmed in stable clones of HCT116 cells in which TRAP1 is silenced. Lastly, with daily irradiation the expression of TRAP1 increases, suggesting a role also in the adaptive response to radiation.

Our group already studied the role of TRAP1 (TNF receptor-associated protein 1), a mitochondrial chaperone (Hsp75), in the adaptation to mild conditions of oxidative stress and demonstrated that this gene may be responsible for protecting from ROS-induced DNA damage and cisplatin-triggered apoptosis²². Since several mechanisms involved in ROS-adaptive responses have also been described as mechanisms responsible for resistance to chemotherapeutic agents in tumour cells²³, at a preclinical level, the role of TRAP1 in inducing a chemoresistant phenotype was explored in human colorectal carcinoma. In fact, TRAP1 protein levels are increased in HT-29 colorectal carcinoma cells resistant to 5-fluorouracil (FU), oxaliplatin (l-OHP),

Roepman P, Schlicker A, Tabernero J et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *International Journal of cancer* 2014;134:552-62.

²¹ Asghar U, Hawkes E, Cunningham D. Predictive and prognostic biomarkers for targeted therapy in metastatic colorectal cancer. *Clinical colorectal cancer* 2010;9:274-81.

²² Montesano Gesualdi N, Chirico G, Pirozzi G et al. Tumor necrosis factor-associated protein 1 (TRAP1) protects cells from oxidative stress and apoptosis, *Stress* 2007;10(4):342-50.

²³ Pennington JD, Wang TJ, Nguyen P et al. Redox-sensitive signaling factors as a novel molecular targets for cancer therapy, *Drug Resist Updat* 2005;8(5):322-30.

and irinotecan (IRI), and in the majority of human colorectal cancers. Furthermore, HT-29 colorectal carcinoma and Saos-2 osteosarcoma cells transfected with TRAP1 exhibited a phenotype resistant to FU-, I-OHP-, and IRI-induced apoptosis, whereas a TRAP1 dominant negative deletion mutant sensitized tumour cells to apoptotic cell death²⁴.

TRAP1 expression is up-regulated in about 60% of human colorectal cancers and TRAP1 up-regulation induces a multi-drug resistant phenotype in colon carcinoma cells. However, ROS-induced damage is also the mechanism of radiation effects and these are the first attempts to study TRAP1 in relation with radioresistance mechanisms. Starting from these preliminary observations, we evaluated the role of TRAP1 in favoring a radioresistant phenotype in vitro.

Several lines of evidence support a role for molecular chaperones in driving cell transformation and resistance to apoptosis. It has been proposed that, because of its restricted repertoire of client proteins, mainly kinases and signaling molecules, Hsp90 chaperones occupy a critical role in cellular homeostasis²⁵. Hsp90 chaperones are, indeed, required for the activity of several key regulators of apoptosis and through these associations may confer survival advantages to tumour cells²⁶.

Heat shock protein 90 (Hsp90) is an evolutionary conserved molecular chaperone which under physiological conditions participates in protein folding, intracellular transport, maintenance and degradation of proteins. Proteins, which are activated and stabilized by Hsp90, are referred to as “clients”. There is a growing list of Hsp90 client proteins, now including several hundred proteins. A lot of them are crucial for constitutive cell signaling and adaptive responses to stress²⁷. However, there are multiple differences between Hsp90 isoforms in cell differentiation and embryonic development in various organisms²⁸.

²⁴ Landriscina M, Lauriero G, Maddalena F et al. Mitochondrial chaperone Trap1 and the calcium binding protein Sorcin interact and protect cells against apoptosis induced by antiproliferative agents. *Cancer Res* 2010;70(16):6577-86.

²⁵ Young JC, Moarefi I, Hartl FU. Hsp90: a specialized but essential protein-folding tool. *J Cell Biol* 2001;154(2):267-73.

²⁶ Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nat Rev Cancer* 2005;5(10):761-72.

²⁷ Wandinger SK, Richter K, Buchner J. The Hsp90 chaperone machinery. *J Biol Chem* 2008;283:18473-7.

²⁸ Sreedhar AM, Kalmar E, Csermely P et al. Hsp90 isoforms: functions, expression and clinical importance. *FEBS Letters* 2004;562:11-5.

There are two major cytoplasmic isoforms of Hsp90, Hsp90 α (inducible form/major form) and Hsp90 β (constitutive form/minor form). Additional Hsp90 analogues include Grp94 in the endoplasmic reticulum and Hsp75/TRAP1 in the mitochondrial matrix. The genomic locations of human hsp90 α at 14q32-33, hsp90 β at 6p21 and hsp75 at 16p13.3 are recognized as functional. Hsp90 is mainly a constitutive dimer ($\alpha\alpha$ or $\beta\beta$), however, monomers (α or β), heterodimers ($\alpha\beta$) and higher oligomers of both isoforms also exist. The dimerization potential resides mainly at the carboxy-terminal 190 amino acids of Hsp90 (fig. 45)

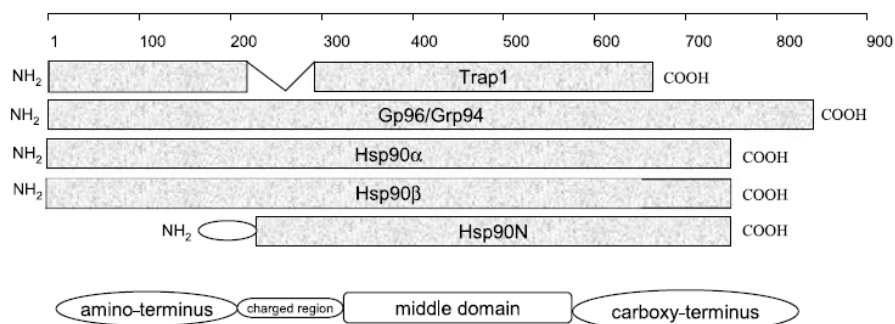


Fig. 45- Schematic representation of various Hsp90 isoforms. The numbering 1 through 900 refers to the amino acid sequence. The functional significance of the domains are illustrated.

Hsp90 is expressed at 2–10-fold higher levels in tumour tissue than in normal tissue²⁹. Its most important function is to protect mutated and overexpressed oncoproteins from misfolding and degradation. It has been recognized to be essential for the stability and function of a wide variety of kinases involved in cell cycle regulation, survival and oncogenic signaling³⁰. These proteins play also critical roles in the regulation of radiosensitivity³¹. Thus, the inhibition of Hsp90 may represent an attractive therapeutic strategy not only reducing basal survival of tumour cells but also increasing their radiosensitivity. NVP-HSP990 is a novel, highly potent orally available 2-

²⁹ Gooljarsingh LT, Fernandes C, Yan K et al. A biochemical rationale for the anticancer effects of Hsp90 inhibitors: slow, tight binding inhibition by geldanamycin and its analogues. Proc Natl Acad Sci USA 2006;103:7625-30.

³⁰ Trepel J, Mollapour M, Giaccone G et al. Targeting the dynamic HSP90 complex in cancer. Nat Rev Cancer 2010;10:537-49.

³¹ Milanovic D, Firat E, Grosu AL et al. Increased radiosensitivity and radiothermosensitivity of human pancreatic MIA PaCa-2 and U251 glioblastoma cell lines treated with the novel Hsp90 inhibitor NVP-HSP990. Radiation Oncology 2013;8:42.

aminothienopyrimidine class, non-geldanamycin based Hsp90 inhibitor³². In our experience, HSP990 significantly increases the radiosensitivity of BRAF-mutated cells, consistently with the chaperoning activity of HSP90 chaperones toward BRAF. HSP990 is also a TRAP1 inhibitor.

TRAP1 has been reported in mitochondria (fig. 46). Consistent with this, an antibody to TRAP1 detected an abundant ~75 kDa immunoreactive band in purified mitochondria from various tumour cells. Conversely, TRAP1 was expressed at very low levels in mitochondria isolated from normal mouse tissues and was absent in the cytosol of tumour or normal cells. TRAP1 expression was also studied in primary tumour specimens and their matched normal tissues *in vivo*. By immunohistochemistry, TRAP1 was intensely expressed in tumour cells of adenocarcinoma of the pancreas, breast, colon, and lung. Conversely, normal matched epithelia contained very low levels of TRAP1³³.

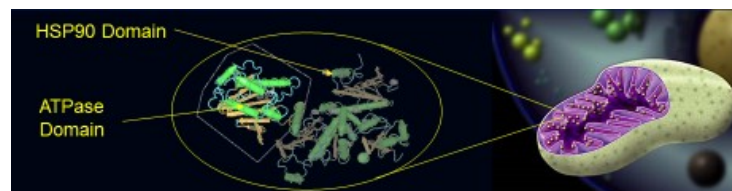


Fig. 46- Mitochondrial localization of TRAP1. Its principal domains are illustrated.

Mitochondria play a critical role in cell survival and cell death. Once in mitochondria, Hsp90 chaperones form a physical complex with cyclophilin D (CypD), an immunophilin component of the organelle permeability transition pore, or at least a pivotal regulator of it³⁴. A detailed structure-function relationship of CypD chaperone complex(es) is still missing, but there is initial evidence that mitochondrial Hsp90, TRAP-1, and Hsp60 may simultaneously bind CypD through non-overlapping recognition sites. In turn, the multichaperone-CypD complex antagonizes the opening of the mitochondrial permeability transition pore, potentially by protein (re)folding, shutting off the

³² Massey AJ, Schoepfer J, Brough PA et al. Preclinical antitumor activity of the orally available heat shock protein 90 inhibitor NVPBEP800. *Mol Cancer Ther* 2010;9:906-19.

³³ Kang BH, Plescia J, Dohi T et al. Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. *Cell* 2007;131:257-70.

³⁴ Rasola A, Sciacovelli M, Pantic B et al. Signal transduction to the permeability transition pore. *FEBS Lett* 2010;584:1989-96.

initiation of apoptosis in tumours³⁵. Mechanistically, this pathway appears ideally suited to globally elevate the anti-apoptotic threshold in transformed cells, favoring the acquisition of additional malignant traits, including adaptation to unfavorable, i.e. hypoxic, environments, and resistance to conventional or targeted therapy. Inhibition of mitochondrial Hsp90 chaperones with a novel class of mitochondria-directed ATPase antagonists causes sudden loss of mitochondrial membrane potential, release of cytochrome c, and massive death of tumour but not normal cells.

TRAP1 has not only a mitochondrial localization: it is in endoplasmic reticulum (ER) as well. The ER is the entry site for proteins destined to the endo/exocytotic pathway, and provides an optimal and unique environment for protein folding, assembly, and disulfide bond formation prior to exposure to the extracellular space. The concentration of proteins within the ER lumen is extremely high, approximately 100 mg/ml.

Homeostasis within the ER lumen is meticulously monitored and elegantly maintained. A broad spectrum of insults can lead to the activation of a coordinated adaptive program called the unfolded protein response (UPR). In response to the accumulation of unfolded proteins in the ER, the rate of general translation initiation is attenuated, the expression of ER resident protein chaperones and protein foldases is induced, the ER compartment proliferates, and ER-associated degradation (ERAD) is activated to eliminate the irreparably misfolded proteins. When the prosurvival efforts are exhausted, ER-stress related apoptosis commences. A number of insults lead to protein misfolding in the ER. These include nutrient deprivation, alterations in the oxidation–reduction balance, changes in calcium concentration, failure of post-translational modifications, or simply increases in secretory protein synthesis³⁶.

Evidences by our group suggest that TRAP1 is responsible for the translational regulation of BRAF synthesis/ubiquitination in CRC cells³⁷. Indeed, TRAP1 is a molecular chaperone, a member of the HSP90 chaperone family, involved in the maintenance of mitochondrial integrity and regulation of

³⁵ Kang BH, Altieri DC. Compartmentalized cancer drug discovery targeting mitochondrial Hsp90 chaperones. *Oncogene* 2009;28:3681-8.

³⁶ Wu J, Kaufman RJ. From acute ER stress to physiological roles of the Unfolded Protein Response. *Cell Death and Differentiation* 2006;13:374-84.

³⁷ Condelli V, Piscazzi A, Sisinni L et al. TRAP1 is involved in BRAF regulation and downstream attenuation of ERK phosphorylation and cell cycle progression: a novel target for BRAF-mutated colorectal tumors. *Cancer research* 2014;74:6693-704.

mitochondrial transition pore (MTP)³⁸. Several lines of evidence suggest that TRAP1 is responsible for dual control on mitochondrial apoptotic pathway: 1) folding/ stability regulation on cyclophilin D and, likely, other client proteins critical for MTP opening within mitochondria and regulation of cellular energy production, especially in tumours³⁹, and 2) quality control regulation on specific client proteins in the endoplasmic reticulum (ER), most of which are extremely important regulators of mitochondrial apoptosis⁴⁰. In this context, our group has previously demonstrated that TRAP1 a) interacts with the proteasome regulatory protein particle TBP7 in the ER, 2) is involved in extra-mitochondrial quality control of nuclear-encoded proteins through co-translational regulation of their ubiquitination/synthesis, and 3) induces parallel activation of a cytoprotective UPR and consequent protection from apoptosis⁴¹ (fig. 47).

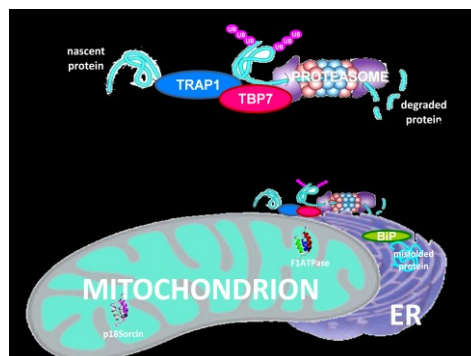


Fig. 47- TRAP1 interacts with the proteasome regulatory protein particle TBP7 in the ER and is involved in translational regulation of proteins.

³⁸ Kang BH, Plescia J, Dohi T et al. Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network. *Cell* 2007;131:257-270.

³⁹ Chae YC, Caino MC, Lisanti S et al. Control of tumor bioenergetics and survival stress signalling by mitochondrial HSP90s. *Cancer Cell* 2012;22:331-44.

⁴⁰ Maddalena F, Sisinni L, Lettini G et al. Resistance to paclitxel in breast carcinoma cells requires a quality control of mitochondrial antiapoptotic proteins by TRAP1. *Molecular oncology* 2013;7:895-906.

Landriscina M, Laudiero G, Maddalena F et al. Mitochondrial chaperone Trap1 and the calcium binding protein Sorcin interact and protect cells against apoptosis induced by antitublastic agents. *Cancer research* 2010;70:6577-86.

Suarez J, McDonough PM, Scott BT et al. Sorcin modulates mitochondrial Ca(2+) handling and reduces apoptosis in neonatal rat cardiac myocytes. *American journal of physiology-Cell physiology* 2013;304:248-56.

⁴¹ Amoroso MR, Matassa DS, Laudiero G et al. TRAP1 and the proteasome regulatory particle TBP7/Rpt3 interact in the endoplasmic reticulum and control cellular ubiquitination of specific mitochondrial proteins. *Cell death and differentiation* 2012;19:592-604.

In this context, BRAF synthesis/ ubiquitination is tightly regulated by ER-associated TRAP1, as an additional and non redundant mechanism respect to HSP90 control of BRAF stability⁴². It is worth noting that this regulation is conserved in human malignancies, since the two proteins are significantly co-expressed in human CRCs (fig. 48), thus representing a potential therapeutic window for tumour-selective targeting of BRAF-driven colorectal malignancies⁴³.

	BRAF-positive	BRAF-negative
TRAP1-positive	14	7
TRAP1-negative	5	15

Fig. 48- Co-expression of TRAP1 and BRAF in a sample of 41 CRCs.

Based on this well-characterized TRAP1 cytoprotective network and the knowledge that the RAS-RAF-ERK axis drives extracellular survival stimuli to mitochondria⁴⁴, our group evaluated the relationship between TRAP1 regulation of MTP and BRAF signaling in mitochondria, with a study reporting that TRAP1 is a downstream effector of the BRAF cytoprotective pathway⁴⁵. BRAF induces a cell phenotype resistant to apoptosis by inhibiting the mitochondrial apoptotic pathway.

The antiapoptotic function of BRAF is TRAP1-dependent. It has been suggested that BRAF signaling alters cell responses to apoptotic stimuli upon traslocation to mitochondria⁴⁶ and that TRAP1 regulates BRAF

⁴² Grbovic OM, Basso AD, Sawai A et al. V600E B-Raf requires the Hsp90 chaperone for stability and is degraded in response to Hsp90 inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 2006;103:57-62.

⁴³ Condelli V, Piscazzi A, Sisinni L et al. TRAP1 is involved in BRAF regulation and downstream attenuation of ERK phosphorylation and cell cycle progression: a novel target for BRAF-mutated colorectal tumors. *Cancer research* 2014;74:6693-704.

⁴⁴ Cantwell-Dorris ER, O'Leary JJ, Sheils OM. BRAFV600E: implications for carcinogenesis and molecular therapy. *Molecular cancer therapeutics* 2011;10:385-94.

⁴⁵ Condelli V, Maddalena F, Sisinni L et al. Targeting TRAP1 as a downstream effector of BRAF cytoprotective pathway: a novel strategy for human BRAF-driven colorectal carcinoma. *Oncotarget* 2015;6(26):22298-309.

⁴⁶ Lee MH, Lee SE, Kim DW et al. Mitochondrial localization and regulation of BRAFV600E in thyroid cancer: a clinically used RAF inhibitor is unable to block the mitochondrial activities of BRAFV600E. *The Journal of clinical endocrinology and metabolism* 2011;96:19-30.

expression/ubiquitination at the translational level⁴⁷. Indeed, TRAP1 silencing resulted in the downregulation of endogenous BRAF in both cytosolic and mitochondrial fractions. In addition to TRAP1 regulation on BRAF synthesis/ubiquitination in the ER, further control exists since TRAP1 represents a downstream effector of BRAF cytoprotective pathway in mitochondria. BRAF signaling activation results in induction of TRAP1 serine phosphorylation, which likely enables TRAP1 antiapoptotic function through inhibition of the MTP opening. Indeed, BRAF interacts with TRAP1 and favors its serine phosphorylation so BRAF silencing/inhibition results in reduced TRAP1 antiapoptotic activity.

In other words, a dual and reciprocal regulation exists between TRAP1 antiapoptotic network and BRAF signaling. The regulation of TRAP1 function by BRAF likely contributes to the enhancement of the apoptotic threshold of cancer cells and induces resistance in human BRAF-driven malignancies with TRAP1 upregulation, through the downstream inhibition of the mitochondrial apoptotic pathway. At the same time, TRAP1 overexpression likely represents a mechanism to enhance BRAF synthesis, reduce its ubiquitination and activate its downstream signaling through the ER quality control function. On the other hand, TRAP1 silencing has an inhibitor effect on BRAF (fig. 49).

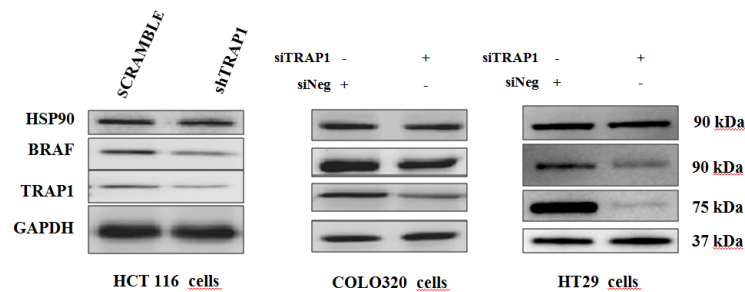


Fig. 49- Western blots suggesting TRAP1 modulation on BRAF.

Based on our recent observation that BRAF is a client protein of TRAP1, we evaluated the molecular mechanisms responsible for radioresistance to apoptosis induced by BRAF activation in human CRCs and found that HSP90 chaperone inhibition significantly reduced viability of BRAF-V600E HT29.

⁴⁷ Condelli V, Piscazzi A, Sisinni L et al. TRAP1 is involved in BRAF regulation and downstream attenuation of ERK phosphorylation and cell cycle progression: a novel target for BRAF-mutated colorectal tumors. *Cancer research* 2014;74:6693-704.

BRAF-addicted colorectal carcinoma cells are in fact highly sensitive to HSP90 chaperones inhibition (figs. 50-51).

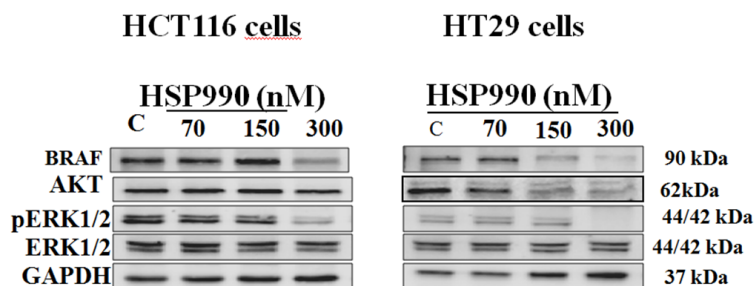


Fig. 50- Hsp90 inhibitors cause an important cytostatic effect in BRAF-driven CRC cells.

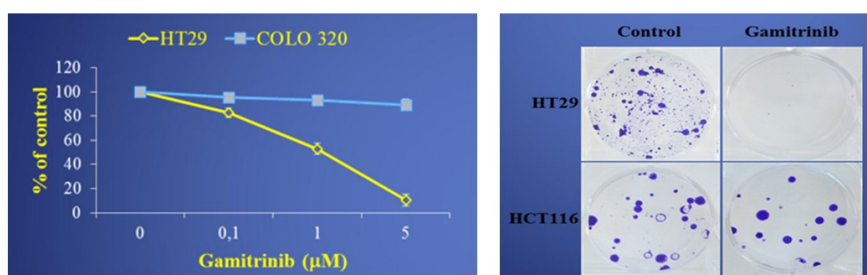


Fig. 51- BRAF-mutated CRC cell lines are highly sensitive to Hsp90 inhibitors.

These data provide new evidence regarding the reciprocal regulation between TRAP1 chaperoning functions and the BRAF signaling pathway and suggest that HSP90 chaperones targeting may represent a potential therapeutic strategy in BRAF-addicted CRC cell lines. Hsp90 inhibition provides a recently developed, important pharmacological platform for anticancer therapy. With the inhibition of this pleiotropic chaperone, many survival and signaling pathways can be inhibited simultaneously. For this reason, mitochondrial Hsp90 chaperones provide attractive targets for cancer therapeutics (fig. 52).

Embodying the concept of subcellularly-targeted therapy for human diseases, a novel class of small molecule Hsp90 inhibitors was recently engineered to target the chaperone pool selectively in mitochondria. Hence, it is important to design and test more specific inhibitors⁴⁸.

⁴⁸ Altieri DC. Mitochondrial compartmentalized protein folding and tumor cell survival. *Oncotarget* 2011;2:347-51.

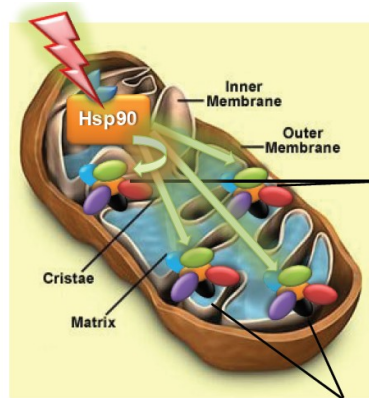


Fig. 52- Treatment of tumour cells with non-cytotoxic concentrations of mitochondrially-targeted Hsp90 inhibitor induces a proteotoxic response within accumulation of unfolded proteins (arrows) within the organelle and resulting in activation of autophagy and a stress response gene expression signature.

The study has several important limitations. It was a single-group study with a small sample size, a short follow-up and unknown mutational status. Furthermore, the *in vitro* work was performed in cells derived from the colon and not the rectum, even if we do not expect substantial differences. Moreover, the cells we used were derived from real patients, whose clinical response to ionizing radiation is uncertain if they had been treated with this modality for the management of their primary tumor.

Establishment of cell lines derived from preirradiated tumor biopsies with a clinical correlation to the pathological response of a tumor will permit corroboration of the response of cells by external beam irradiation. This system will allow the study of phenotypic molecular differences between radioresistant and radiosensitive cells. Construction of tissue microarrays from preirradiated tumor tissues followed by IHC with several molecules known to participate in radioresistance could potentially allow detection of radioresistant tumors which may need different interventions (i.e., earlier surgical intervention or administration of different radiosensitizing agents).

Overall, this study shows that rates of recurrence and survival in pts with locally advanced rectal tumours treated with chemoradiotherapy and surgery are similar to the results of other studies. Most patients treated with neoadjuvant chemoradiotherapy and surgery preserve their anorectal function and a good quality of life. Anyway, to overcome radioresistance in BRAF-mutated CRCs, our observations support that the TRAP1/Hsp90 pathway may be regarded as a

novel molecular target in humans and that BRAF-addicted CRCs are a suitable and attractive tumour cell model to evaluate this novel therapeutic strategy. Indeed, although recently the combination of standard chemotherapy with bevacizumab has been proposed as the best therapeutic option for BRAF-mutated advanced CRCs⁴⁹, the prognosis of these patients is still dismal compared to other molecular subtypes of colon cancers⁵⁰. Thus, the development of novel effective therapies represents a clinical need in BRAF-mutant CRCs and, seen in this light, our data provide a strong rationale to design novel specific TRAP1 inhibitors and evaluate BRAF mutational status as a potential biomarker in the selection of tumours suitable for TRAP1 targeting therapy.

Certainly, further studies are required to evaluate whether the up-regulation of TRAP1 expression may be predictive of resistance to currently used radiotherapy regimens in human colorectal tumours and whether the expression of TRAP1 may represent a decision tool for selecting the appropriate chemoradiotherapy schedule in patients. A blend of new techniques and novel approaches to elucidate pathways leading to radioresistance will lead to a further understanding of molecular predictors to individualize treatment in patients affected by rectal cancer.

⁴⁹ Loupakis F, Cremolini C, Salvatore L et al. FOLFOXIRI plus bevacizumab as first-line treatment in BRAF mutant metastatic colorectal cancer. *Eur J Cancer* 2014;50:57-63.

⁵⁰ Roepman P, Schlicker A, Tabernero J et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *International journal of cancer* 2014;134:552-62.

Morris V, Overman MJ, Jiang ZQ et al. Progression-free survival remains poor over sequential lines of systemic therapy in patients with BRAF-mutated colorectal cancer. *Clinical colorectal cancer* 2014;13:164-71.

7. Conclusion

Rectal cancer represents a prominent example on how to individualize multimodal treatment regimens. Our study confirms that it is possible to treat locally advanced rectal tumours with neoadjuvant chemoradiotherapy and surgery, with good clinical and pathological responses and acceptable toxicities. However, the genetic diversity of rectal cancer is associated with varying responses to chemoradiotherapy, and varying toxicity rates. This offers a wide range of options to pretherapeutically assess both response and toxicity for the individual patient. Consequently, a plethora of potential biomarkers has already been evaluated using whole-genome and singlemarker or multimarker analyses.

Unfortunately, there are several drawbacks of these findings that still impede transition to routine clinical practice. First, conflicting results were obtained by different investigators. Second, virtually most biomarkers described to date have been identified in retrospective studies and lack independent validation in a prospective setting using standardized analytical procedures.

Thus, there is a clinical need to establish molecular biomarkers that differentiate responsive and resistant tumours because such biomarkers could be used pretherapeutically to predict the response of an individual patient's tumour to multimodal treatment (diagnostic approach). In addition, genes that are differentially expressed between resistant and responsive tumours could be used to identify novel therapeutic targets and thereby assist in implementing novel therapeutic strategies (therapeutic approach).

In our experience, clonogenic, apoptotic and MTT assays were useful tests for evaluation of radiosensitivity. The main advantage of MTT and apoptotic assays is the opportunity to obtain precise survival data for high sample throughput in less time and with less effort than with colony assay. Clonogenic assay remains the simplest method, giving clear results without any need for specific technological devices. When MTT assay is chosen, plating should be done after irradiation.

BRAF and TRAP1 may be responsible for protecting from ROS-induced DNA damage and apoptosis. Since several mechanisms involved in ROS responses have also been described as mechanisms of radiotherapy, we explored, at a preclinical level, the role of BRAF and TRAP1 in inducing a radioresistant phenotype in human colorectal carcinoma.

We noticed that HT29 (BRAF-V600E mutated) cells are more resistant to radiation than HCT116 (RAS-mutated) cells and COLO320 (RAS/BRAF-wt)

cells. So we hypothesized a role of BRAF mutations in radioresistance. In particular, we argued that BRAF-V600E mutation could play a role in protecting cancer cells against radiotherapy. BRAF-V600E cells revealed to be resistant also to the association of 5-FU and radiation, explaining one possible mechanism of preoperative chemoradiation failure. BRAF siRNA silenced cells resulted significantly more sensitive than control and negative transfected cells.

HSP90 significantly increased the radiosensitivity of BRAF-mutated cells, consistently with the chaperoning activity of HSP90 chaperones toward BRAF. Transfection of resistant HT29 cells with TRAP1 siRNAs increased cancer cell killing as well. The role of TRAP1 in radioresistance was confirmed in stable clones of HCT116 cells in which TRAP1 is silenced. Thus, our study sheds light on the role of BRAF and TRAP1 in radioresistance, confirming the dual and reciprocal regulation existing between TRAP1 antiapoptotic network and BRAF signaling.

The present work has at least two important limitations. It was a single-group study with a small sample size and a short follow-up. Moreover, mutational status of irradiated pts was unknown and we could not operate any correlation between gene status and response in our group of pts.

We strongly believe that molecular biomarkers will be implemented into clinical decision-making in the near future. In a potential scenario, pretherapeutic patient material from both tumour and normal tissue will be ascertained at the initial diagnosis and subjected to multilayer genomic analyses to predict both response and toxicity. Based on the results of these analyses, the individual patient will be stratified into different alternative treatments, in a concept of personalized medicine.

In this setting, patients with a responder profile will be subjected to the standard preoperative regimen. In contrast, a more aggressive approach is needed in patients with a biomarker profile indicating “nonresponder to standard treatment”. For instance, an intensified regimen could be pursued, including the application of more effective systemic agents such as oxaliplatin or the association of a sensitizer. An induction combination chemotherapy (preoperative chemoradiotherapy followed by chemotherapy with sufficient dose and intensity prior to surgery) would be another interesting option because many patients do not receive adjuvant chemotherapy after preoperative chemoradiotherapy and surgical resection, either due to surgical complications or refusal.

For patients predicted to be “nonresponder to standard treatment” and to develop high acute organ toxicity, primary surgery may be an option. With respect to novel therapeutic target genes, we showed examples of molecular targets that have the potential to be incorporated into treatment concepts. This holds considerable promise to improve the outcome of patients with rectal cancer, although extensive validation and testing are required.

Overall, our study shows that rates of recurrence and survival in pts with locally advanced rectal tumours treated with chemoradiotherapy and surgery are similar to the results of other studies. Most patients treated with neoadjuvant chemoradiotherapy and surgery preserve their anorectal function and a good quality of life. However, to overcome radioresistance in BRAF-mutated CRCs and improve results, our observations support that TRAP1/Hsp90 pathway may be regarded as a novel molecular target.

TNF receptor-associated protein 1 (TRAP1) encodes for a mitochondrial heat-shock protein homologous to Hsp90 family members. It has been recently proposed that TRAP1 and Hsp90 are components of a mitochondrial survival pathway which is selectively activated in tumour cells and is responsible for antagonizing the proapoptotic activity of cyclophilin D and thus favoring mitochondria integrity and cell survival.

Interestingly, strategies aimed at inhibiting TRAP1 function, based on novel TRAP1 ATPase antagonists, induce sudden collapse of mitochondrial function and apoptosis, thus improving the efficacy of anticancer treatments. In such a perspective, TRAP1/Hsp90 chaperone may represent a novel molecular target for overcoming radioresistance.

The development of novel effective therapies represents a clinical need in BRAF-mutant CRCs and, seen in this light, our data provide a strong rationale to design novel specific TRAP1 inhibitors and to evaluate BRAF mutational status as a potential biomarker in the selection of tumours suitable for TRAP1 targeting therapy. Certainly, further studies are required to evaluate whether the mutational status of BRAF and the up-regulation of TRAP1 may be predictive of resistance to currently used radiotherapy in human CRCs and whether their expression may represent a decision tool for selecting the appropriate chemoradiation schedule in patients.

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