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TESI DI LAUREA

PATHOLOGICAL AND MOLECULAR FEATURES OF SPORADIC RECTAL CANCER IN YOUNG ADULTS

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ABSTRACT

Background

Incidence of early-onset sporadic rectal cancer is rising globally but the reasons are still unclear. Cancer among young adults has different features from late-onset cancer, such as an advanced stage at diagnosis, mucinous differentiation, microsatellite stability and a lower degree of lymphocytic infiltrate. Moreover, this subtype of tumor does not exhibit mutations in the oncogenes typically involved in pathogenesis of rectal cancer, as BRAF, KRAS and NRAS. Therefore, the aim of this study was to analyse biological features of sporadic EORC focusing also on the tumoral microenvironment and immune response.

Methods

The pathological and molecular records of a series of consecutive rectal cancer patients operated on from 2015 to 2021 were retrieved for this retrospective study. We defined young adults as patients aged 50 years or younger. Histology for the infiltration of intratumoral lymphomononuclear cells, immunohistochemistry for MLH1, PMS2, MSH2, and MSH6, and mutational analysis of BRAF, KRAS, and NRAS were all performed. Sporadic early-onset rectal cancer and late-onset rectal cancer were compared also considering results from TCGA. Moreover, results from Nonparametric tests were used for small sample size comparison.

Results

94 patients operated on for rectal cancer at the General Surgery 3 Unit of the Azienda Ospedaliera di Padova from 2015 to 2021 were enrolled in the study. We identified 15 patients with sporadic early-onset rectal cancer, and 79 with late-onset cancer, after ruling out patients with hereditary syndromes, IBD, distant metastasis and undergoing neoadjuvant radiotherapy. Microsatellite instability frequency was similar in early-onset and in late-onset rectal cancer (P = 0.19) even if young patients tended to have mutations in MSH1 and PMS2 (P=0.03). Likewise, the mutation frequency of BRAF and KRAS and NRAS was similar in the two groups (P = 0.40, P =0.70 and P=0.72, respectively). Moreover, in our study the lymphocytic infiltration was similar in the two cohorts (P=0.19). On the contrary, TGCA showed more frequent mutations of SACS and MAP3K21 in the young

cohort (P=0.0119 and P=0.0170) and also lower mRNA expression for CD3, CD69 and CD8 beta.

Conclusion

Our study showed no significative differences in oncogenes mutations and microsatellite instability frequency in early-onset and late-onset sporadic rectal cancer. Our data and results from TCGA suggested that in the young cohort an altered immune infiltrate may play a significative role, as predisposing factor or participating in tumoral progression due an improved capacity by tumoral cells escape the immunosurveillance. A deeper investigation of dysregulation in the immune response and tumoral microenvironment is required to analyse pathogenesis of early-onset rectal cancer.

ABSTRACT

Background

L'incidenza dell'early-onset rectal cancer sta aumentando a livello globale ma le ragioni di questo trend non sono ancora chiare. La neoplasia rettale nei pazienti giovani ha caratteristiche differenti rispetto a quelle tipiche dei pazienti anziani, ad esempio uno stadio più avanzato alla diagnosi, l'istotipo mucinoso, la stabilità microsatellitare ed un minor grado di infiltrazione linfocitica. Inoltre, in questo sottotipo di tumore non sono riscontrate mutazioni negli oncogeni tipicamente coinvolti nella patogenesi del tumore del retto, come BRAF, KRAS ed NRAS. Lo scopo di questo studio era perciò quello di analizzare le caratteristiche biologiche del tumore del retto sporadico nei pazienti giovani, concentrandosi soprattutto sul microambiente tumorale e sulla riposta immunitaria.

Materiali e metodi

Per questo studio retrospettivo sono stati valutati i referti istopatologici e molecolari di una serie di pazienti consecutivi operati dal 2015 al 2021 nel reparto di Chirurgia Generale 3 dell'Azienda Ospedaliera di Padova. Sono stati definiti giovani adulti i pazienti di età inferiore o uguale ai 50 anni. Sono state eseguite le analisi istologiche per l'infiltrato linfocitario intra-tumorale, le analisi immunoistochimiche per MLH1, PMS2, MSH2 e MSH6 e l'analisi mutazionale di BRAF, KRAS ed NRAS. Sono state poi confrontate le caratteristiche del tumore del retto sporadico nei pazienti giovani ed anziani, considerando anche i risultati ottenuti dal database TCGA.

Risultati

94 pazienti operati per il tumore del retto nel Reparto di Chirurgia Generale 3 dell'Azienda Ospedaliera di Padova dal 2015 al 2021 sono stati inclusi nello studio. Sono stati identificati 15 pazienti di età inferiore o uguale ai 50 anni e 79 pazienti di età superiore, dopo aver escluso i pazienti affetti da sindromi ereditarie, malattie infiammatore croniche, pazienti con metastasi a distanza e sottoposti a radioterapia neoadiuvante. La frequenza di instabilità dei microsatelliti è risultata simile nelle due coorti (P=0.19) anche se i pazienti giovani tendevano maggiormente ad avere alterazioni dei geni MSH1 e PMS2 (P=0.03). Allo stesso modo, anche la frequenza

di mutazione di BRAF, KRAS ed NRAS era simile nelle due coorti (P=0.40, P=0.70 e P=0.72, rispettivamente). Inoltre, nel nostro studio anche il grado di infiltrazione linfocitica è risultato simile nelle due coorti (P=0.19). Al contrario, il database di TCGA ha mostrato una maggiore frequenza di mutazioni di SACS e MAP3K21 nella coorte giovane (P=0.119 e P=0.0170) ed anche una minore espressione dell'mRNA di CD3, CD69 e CD8 beta.

Conclusioni

Il nostro studio non ha mostrato differenze statisticamente significative nella frequenza né delle mutazioni degli oncogeni né dell'instabilità di microsatelliti tra la coorte dei pazienti giovani e quella dei pazienti anziani. I nostri dati ed i risultati dal database del TCGA hanno suggerito che nella coorte dei giovani un'alterazione dell'infiltrato immunitario potrebbe giocare un ruolo importante sia come fattore predisponente all'esordio della patologia sia come elemento chiave nella progressione della malattia, a causa di un'aumentata capacità delle cellule tumorali di fuggire alla sorveglianza immunitaria. Una più profonda indagine riguardo la de-regolazione del sistema immunitario e del microambiente tumorale è sicuramente necessaria per analizzare la patogenesi dell'early-onset rectal cancer.

INTRODUCTION

Epidemiology

Colorectal cancer (CRC) is the third leading cause of cancer death in both men and women in western countries. It is the third most common cancer in the United States with about 150000 new cases estimated in 2022[1]. A similar scenario can be observed in Europe with 520000 new cases estimated in 2020[2]. The incidence of rectal cancer represents around 35% of the total colorectal cancer incidence with about 125000 new cases per year in Europe and the mortality is 4–10/100 000 people per year[3]. An overall trend of decreased incidence and mortality of colorectal cancer was observed in recent decades, possibly due to the preventive cancer screening[4]. In contrast, recent literature suggests an increasing incidence of colorectal cancer in young patients. Although it was considered a disease that affects the elderly, as the result of this simultaneous decreasing incidence in older patients and increasing incidence in young adults, the typical CRC patient is becoming younger. Parallel to this population shift, a change in tumors localisation is also observed. Indeed, while overall incidence is highest for tumors in the proximal colon, among patients younger than 50 years, rectal tumors are most common (37%), followed by those in the distal colon (25%).[5] Data from the Surveillance, Epidemiology, and End Results registry (SEER, 1973 to 1999) indicate that the incidence rate of colonic adenocarcinoma among patients younger than 40 years increased by 17%, and that of rectal carcinoma rose by 75% during the period of analysis[6]. A retrospective cohort study conducted by Meyer et al.[7] also demonstrated a significative increase in the incidence of rectal cancer without any increment in colon cancer (annual percent change of 2.6% vs −0.2%) in young patients, based on data from SEER, 1973-2005.

Hereditary syndromes

Early-onset rectal cancer should be considered a distinct biological entity characterised by different features and behaviour from both late-onset cancer and hereditary syndromes whose pathogenesis and molecular mechanisms have been identified. Although several hereditary genetic syndromes predispose young adults

to CRC, these diseases account for only a minority of cases, while most of patients have a sporadic cancer. In particular, only 2–5% of all CRCs occur within inherited syndromes, including Lynch syndrome or one of the polyposis syndromes, such as familial adenomatous polyposis (FAP), MUTYH-associated polyposis, Peutz–Jeghers syndrome, juvenile polyposis and Cowden/PTEN hamartoma syndrome[8].

The most common hereditary CRC predisposing syndrome, the Lynch syndrome, is implicated in 2–4% of CRC cases. This disease is characterised by a high penetrance and an increased risk not only of early-onset colorectal cancer but also of extra-intestinal cancers at a young age. Lynch syndrome is defined by a germline mutation in one allele of one of the DNA mismatch repair (MMR) genes which leads to a loss of function in MMR proteins. This results in defects in DNA repair and high DNA microsatellite instability (MSI-High). A microsatellite instability is also observed in the 15% of sporadic CRCs, driven by hypermethylation of the promoter of MLH1[9]. Moreover, BRAF (V600E) mutation is quite common in sporadic MSI-H tumours (63.5%), but it is only rarely present in Lynch syndrome. The second most common inherited CRC syndrome is FAP, accounting for approximately 1% of CRC. This autosomal dominant hereditary cancer syndrome is caused by a germline heterozygous mutation in the adenomatous polyposis coli gene APC. FAP leads to the onset of hundreds to thousands of adenomatous polyps in young age and adenomas inevitably result in CRC if not treated[8].

Clinical presentation and diagnosis

Typical symptoms of late-onset rectal cancer are rectal bleeding, tenesmus, alterations in bowel habits and abdominal pain. Several clinical studies[20,23] revealed young adults have the same symptoms of old patients but both patients and physicians do not consider them as red flags because rectal cancer is thought to be a disease of the elderly.

Diagnosis of rectal cancer is based on a digital rectal examination (DRE) and endoscopy with biopsy for histopathological confirmation[3]. The initial clinical stage of disease plays a critical role in the choice of primary treatment[10]. Imaging is also crucial in preoperative evaluation, including endoscopic rectal ultrasound

(ERUS), chest/abdominal CT and pelvic MRI, both for evaluation of the primary tumor and for detection of distant metastases[11].

Management strategies

Standard approach of treatment for rectal cancer is based on curative surgery. More specifically, for very early cancers local excisional procedures, such as Transanal Endoscopic Microsurgery (TEM), are appropriate as single treatment. Early rectal cancer (cT2N0) can also be adequately managed by curative surgery alone. For locally advanced rectal cancer (LARC) the standard approach implies neoadjuvant chemoradiotherapy (nCRT) followed by Total Mesorectal Excision (TME)[12]. This multimodality treatment reduces local recurrence, but TME is associated with important morbidity, and impairment of bowel function and quality of life. Therefore, patients who show a pathologic complete response (pCR) after nCRT are manageable with a "Watch and Wait strategy" or with a rectum-sparing approach[13]. Indeed, with this strategy, about 80% patients will have their rectum preserved without stoma at long term and survival outcomes appear encouraging[14]. For metastatic rectal cancer the ideal multidisciplinary treatment depends on tumors molecular characteristics and patients performance status. Conventional chemotherapy may be combined with molecular-targeted therapies, immunotherapies and ablative techniques[3].

Several studies evidenced that early onset CRC has more aggressive histopathological features than tumors that typically occur in the elderly. Moreover, younger patients have more advanced stage of disease at diagnosis, rising questions about the need of early screening strategy. Despite the tumor more aggressive behaviour, in younger patients survival outcome is comparable or better than older patients partly due to a more intensive treatment but the gain survival is still controversial[21]. Indeed, some studies indicate a worse prognosis in young patients in reason of the unfavourable histologic and pathologic characteristics while other studies support a comparable or even better prognosis due to a greater fitness translating into the possibility of a more intensive treatment, such as neoadjuvant and adjuvant therapy outside of current guidelines and more often multiagent regimens. On the other hand, some studies evidenced that the survival gain from an aggressive management is minimum compared to older individuals who receive

less treatment, especially considering the risk of unnecessary therapies and potential long-term toxicity[20].

Tumoral immunology

The evidence that immune system is involved in tumor progression and plays an important role in patients outcome led to an increasingly care of tumor immunology. Indeed, over the last decades the concepts of immunosurveillance and immune contexture were developed and explored to the employment of cancer immunotherapies such as immune checkpoint inhibitors (ICIs). The term "immunosurveillance" refers to the physiological capacity by the immune system to recognize transformed cells and kill them. A theory related to immunosurveillance is the "tumor immunoediting" which states that tumor interacts with the immune system in three sequential phases. Firstly, there is the elimination phase, when the immune system kills many tumor cells; after, there is the equilibrium phase, when the tumor is present but contained; and finally, the escape phase, when the tumoral cells attenuate their antigenicity and the immune system is no more able to recognise them. Hence the tumor grows, invades adjacent structures, and metastasizes in distant organs. This interaction between cancer and immune cells greatly influences the prognosis and the outcome of patients. As early as in 1921, MacCarty and Mahle highlighted that intratumoral lymphocytic infiltration confers a survival advantage in patients with cancer. In particular, Galon et al.[15,16,17] underlined that the tumor infiltration by T cells, especially with a Th1 orientation and cytotoxic and memory cells, is correlated with the natural tumoral progression and evolution; consequently, it may influence the strategy treatment and the clinical outcome. Indeed, the presence of high levels of infiltrating memory T cells and cytotoxic cells appear related with a prolonged survival in colorectal cancer in reason of the absence of signs of early metastatic invasion and a less advanced pathological stage. Veritably, immune defences hinder tumor growth at the primary site but challenge also lymphovascular and perineural invasion and migration in the lymph nodes, in the blood stream and finally in distant organs. Moreover, an efficient Th1 response, required for the establishment and maintenance of T cell memory, might also control tumor recurrence. The Th1 immunological polarization seems to activate some mechanisms able to prevent tumor dissemination and development of detectable metastasis. Thus, the

favourable prognostic role of the peritumoral lymphocytic infiltrate appears clear. Furthermore, recent literature emphasizes the need of novel markers capable of driving the decision for the most appropriate treatment. In addition to molecular and genetic analyses, over the last few years many studies have focused on the role of the local immune reaction in the control of cancer. Indeed, Immunoscore was proposed as new component for cancer classification: it is based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO), both in the centre of the tumor and in the invasive margins. This new classification appears to have several advantages as prognostic factor and also for novel therapeutic strategies.

The current challenge of early-onset rectal cancer

The reason of the increasing incidence of rectal cancer in young patients is still unclear and this novel health problem needs to be investigated to improve both prevention and treatment strategies. In addition to adverse pathological and histological features, the lack of awareness and knowledge about this unique disease contributes to a poor prognosis for young patients, even if survival data are still conflicting. Thus, it is clear that this "heterogeneous disease" which predilect rectal localisation is worthy of a deep analysis both of the clinicopathological landscape and of the possible pathogenesis. The absence of a true genetic predisposition implies that other factors may play an important role, in particular the tumoral microenvironment, the inflammatory and lymphocytic response, which appear altered in young patients. Indeed, an attenuated immune response promote tumoral cells proliferation and migration, but also represent a predisposing factor to the cancer onset. Investigating the immune element may encourage the development of screening and prevention programs and also of novel therapeutic strategies.

The aim of this study was to analyse features of rectal cancer in a small cohort of young individuals not affected by hereditary syndromes or bowel inflammatory disease and to assess the differences from tumors that typically occur in the elderly. Therefore, our analysis was focused on the histopathological and molecular profile of rectal cancer in young patients, in particular analysing the oncogenes, the microsatellite status and the immune infiltrate. We also compared our series with

data extracted from The Cancer Genome Atlas (TCGA) in order to deeply investigate molecular aberrations of early-onset rectal cancer and evaluate the presence of non-typical alterations which occur in these patients.

MATERIAL AND METHODS

Study design

The pathological and molecular records of a series of consecutive rectal cancer patients operated on at the General Surgery 3 Unit of the Azienda Ospedaliera of Padova from 2015 to 2021 were retrieved for this retrospective and observational study. The study is focused on clinicopathologic and molecular features of early-onset rectal cancer with the aim to identify those features that differentiate tumors in young adults from older patients. Rectum was defined as 15 cm from the anal verge, and we defined young patients as individuals 50 years old or younger. Patients with known familial cancer syndromes (=1) (Familial adenomatous polyposis and Lynch syndrome) as well as patients with inflammatory bowel disease (=1) were excluded to capture only those patients who had a sporadic tumor. Patients with metastatic disease (=37) and patients who underwent neoadjuvant radiotherapy (=169) were also excluded. So, a total of 94 patients constituted the study cohort. Of these, 15 patients were aged $\leq$ 50 years and 79 patients were aged $\geq$ 51 years at the time of surgery. The study design and patient selection are shown in Figure 1.

Histology for the infiltration of intratumoral lymphomononuclear cells, the vascular and perineural invasion, immunohistochemistry for MLH1, PMS2, MSH2, and MSH6 to define MMRd or mismatch repair gene proficiency (MMRp), and mutational analysis of *BRAF* (exon 15), *KRAS* (exons 2, 3, and 4), and *NRAS* (exons 2, 3, and 4) were all performed[18].

The study was performed according to the principles of the Declaration of Helsinki and notified to the Ethical Committee of the Azienda Ospedaliera di Padova that approved it. All participants provided their informed consent.

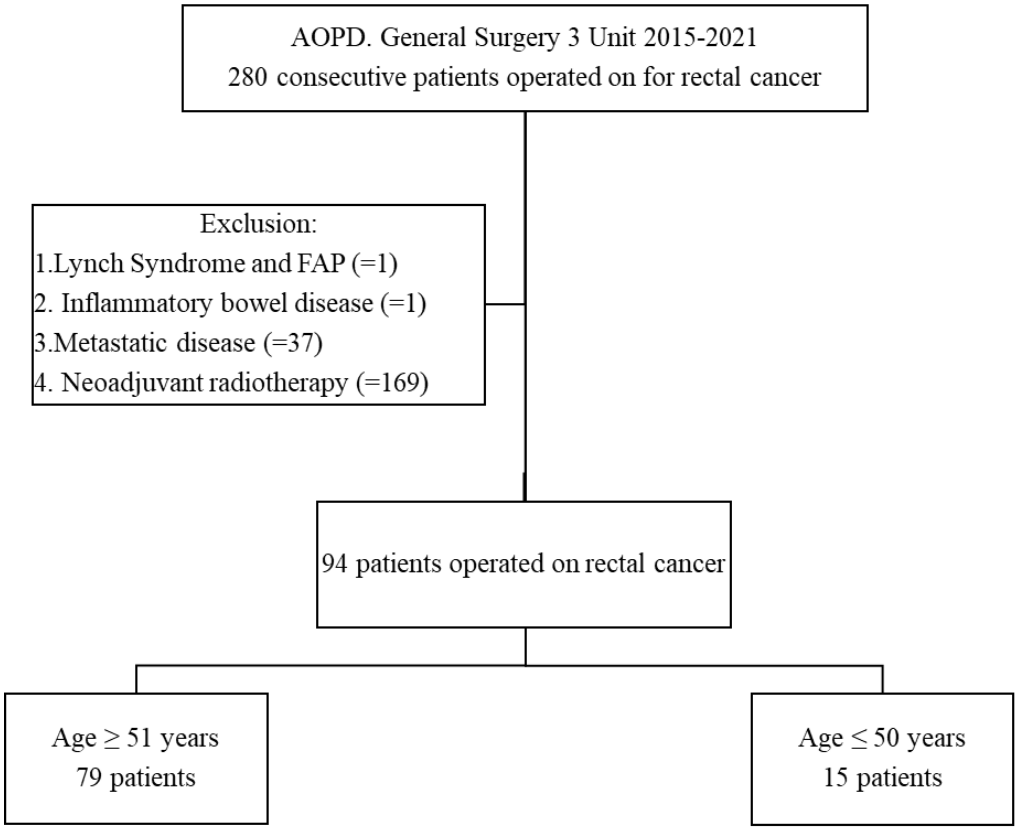


Figure 1: Study design and patients selection.

Histopathology

The histopathological examination of all resected specimens consisted in the evaluation of tumor differentiation and stage, grading, and number of lymph nodes involved. Specimens were fixed in 10% formaldehyde and set in paraffin. The lymph nodes were counted and assessed by a pathologist. The nodal status (N0, N1) was evaluated in accordance with the eighth edition of the TNM classification, but for the purpose of this study, the number of metastatic lymph nodes was also analysed. Furthermore, the infiltration of lymphomononuclear cells was graded as absent, low grade, or high grade. The vascular and perineural invasion were defined as absent, present, or not estimable.

Immunohistochemistry

Immunohistochemical (IHC) analyses were performed using standard procedures, and the resulting sections were evaluated by a single pathologist in a blinded

fashion. Immunocomplexes were detected using the Dako Real Envision System Peroxidase and 3-3'di-aminobenzidine tetrahydrochloride chromogen as a substrate (Dako, Glostrup, Denmark) in formalin-fixed paraffin-embedded sections. IHC staining was performed using monoclonal antibodies for MLH1 (clone ES05, 1:100; Dako, Glostrup, Denmark), PMS2 (clone EP51, 1:100; Dako, Glostrup, Denmark), MSH2 (clone FE11, 1:100; Dako, Glostrup, Denmark), MSH6 (clone EP49, 1:100; Dako, Glostrup, Denmark).

Analysis of MSI

DNA mismatch repair machinery-deficient tumors (MMRd) were defined by the absence of nuclear staining in one of the MLH1/PMS2 or MSH2/MSH6 pairs in tumor cells, as assessed in the colorectal setting. The normal staining pattern of MLH1, PMS2, MSH2, and MSH6 was nuclear and was defined as MMR proficient (MMRp). Infiltrating lymphocytes and stromal cells served as the internal positive controls. Microsatellite instability was studied using the following markers (BAT25, BAT26, D2S123, D17S250, D5S346, NR21, NR24, D18S58, BAT40, *TGFβRII*, TPOX, and TH01) using the Titano kit (Diatech Pharmacogenetics, Jesi, Italy) according to the Bethesda panel proposed by Bocker et al. Extracted DNA (5 μL; ~20 ng) were used in a 50 μL PCR reaction, which contained 10X buffer, MgCl2 (1.5 mmol/L), dNTPS (200 μm), primers (5 μm), and dH2O. The PCR was performed on a GeneAmp PCR system 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) using the following PCR cycling conditions: initial denaturation (8 minutes at 95°C), followed by 10 cycles denaturation (30 seconds at 94°C), annealing (45 seconds at 60°C), and extension (30 seconds at 72°C), followed by 22 cycles denaturation (30 seconds at 92°C), annealing (45 seconds at 55°C), and extension (30 seconds at 72°C). There was a final step of 10 minutes at 72°C and then held at 4°C. The PCR products were analysed on a 10% to 12% nondenaturing polyacrylamide gel and stained by Silver stain (Bio-Rad, San Diego, CA). The samples were run on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and analysed using GeneScan 3.7 software (Applied Biosystems, Foster City, CA). MSI was defined as the presence of additional bands in the PCR amplified product derived from neoplastic lesions in comparison to nonneoplastic tissues from the same patient.

BRAF, *KRAS*, and *NRAS* molecular profiling

The *BRAF*, *KRAS,* and *NRAS* status was obtained from routine diagnostic surveys (Sequenom MassArray and Sanger sequencing). DNA was extracted from the formalin-fixed paraffin-embedded samples after enrichment of neoplastic cellularity (i.e., at least 25% of neoplastic cells in the sample). Serially cut 5-µm-thin sections were set on uncharged slides, deparaffinized, and lightly counterstained with haematoxylin. Microdissection was manually performed (under a light microscope) using a sterile injection needle. DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen, Milan, Italy).

Analysis of TCGA data

Study design

Data on the mutational profile of rectal adenocarcinoma were extracted from The Cancer Genome Atlas (TCGA) deposited public database available at https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations). This dataset contains mutation, gene expression profiles of rectal cancer in different settings. The PanCancer study encompassed 11,286 tumor samples from 33 cancer types, for which molecular data were available from at least one of the five assay platforms. Of these, 9,759 had complete data for 4 platforms: aneuploidy and mutations, DNA methylation, mRNA, and miRNA. RPPA protein data were available for a subset of samples (7,858). We selected the dataset including patients with rectum adenocarcinoma [READ], who did not undergo nCRT and did not present distant metastasis. We obtained a database of 121 patients, of which 18 were 50 years old or younger and 103 older than 50 years at diagnosis. Original data were plotted on a novel database and analysed to detect different molecular alterations between young and old patients. Supposing an important involvement of the immune system in the pathogenesis of EORC, we analysed the degree of mRNA expression for T-cells population markers, in particular CD3 (marker for T-cells), CD69 (early activation marker expressed by T-cells) and CD8 beta (part of the heterodimer expressed by cytotoxic T-cells activated).

Mutational analysis

TCGA used single nucleotide polymorphism (SNP) arrays (Affymetrix) and low pass (3-5X coverage) whole genome sequencing (Illumina HiSeq 2000) to detect chromosome and subchromosomal copy number changes and translocations, microarray (Agilent) and RNA-Seq (Illumina) for mRNA expression profiling, Illumina Infinium HumanMethylation27 arrays to profile DNA methylation at gene promoters, miRNA quantification via Illumina sequencing and whole exome sequencing using both the Illumina and Solid platforms to detect coding mutations. For mutation detection BAM files generated from alignment of Illumina sequencing reads were pre-processed using GATK. Mutations in Illumina data were discovered by the MuTect algorithm2 (see also http://www.broadinstitute.org/cancer/cga/MuTect). Mutations in BAM files generated from SOLiD reads were detected as follows: SamTools Pileup was run to list all variants found in multiple reads at a single locus. The variants were further filtered to remove all those observed fewer than 5 times or were present in less than 0.10 of the reads. At least one variant had to be Q30 or better, and the variant had to lie in the central portion of the read, 15% from the 5' end of the read and 20% from the 3' end. In addition, reads harboring the variant must have been observed in both forward and reverse orientations. Finally, the variant base was not observed in the normal tissue. Insertion or deletion variants ("indels") were discovered by similar processing except indels must have been observed in 0.25 of the reads[33].

mRNA expression

Total RNA for each sample was converted into a library of template molecules for sequencing on the Illumina Cluster Station and Genome Analyzer according to the protocol for the Illumina mRNA Sample preparation kit (Part#1004898, Rev A: Illumina, San Diego, CA). Briefly, poly-A mRNA was purified from total RNA (2 μg) using poly-T oligo-attached magnetic beads. The mRNA was then fragmented, and the first strand of cDNA was synthesized from the cleaved RNA fragments using reverse transcriptase and random primers. Following the synthesis of the second strand of cDNA, end repair was performed on overhangs using T4 DNA polymerase and Klenow DNA polymerase, followed by ligation of sequencing Adapters to the ends of the DNA fragments. The cDNA fragments were purified using a gel run at 80 V for approximately 3 hours until the Orange G dye band

reached the bottom of the gel. The gel was stained with SYBR green to visualize the DNA band. A band at 350 – 450 bp was excised vertically from the gel, which was then dissolved at room temperature using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified cDNA templates were enriched for 15 cycles of PCR amplification and validated using a BioAnalyzer to assess size, purity, and concentration of the purified cDNA libraries. The cDNA libraries were placed on an Illumina Cluster Station for single end cluster generation according to the protocol outlined in the Illumina Genome Analysis User Guide (Part# 11251649, RevA). The template cDNA libraries (1.5 μg) were hybridized to a flow cell, amplified, and linearized and denatured to create a flow cell with ssDNA ready for sequencing. Each flow cell was sequenced on an Illumina GAIIX Genome Analyzer. Each sample underwent a single lane of sequencing using single end sequencing for 76 cycles according to the protocol outlined in the Illumina Genome Analysis User Guide (Part# 11251649, RevA). After completion of the 76-cycle sequencing run, the raw sequence data entered the UNC RNAseq Workflow[33].

Statistics

Continuous data were expressed as median and interquartile range, and categorical data as number and percentage. Several pathological and molecular features were compared between the younger and older groups of patients. The χ^2 or Fisher's test was used to analyse the categorical data. All tests were 2-sided. Statistical analysis was performed using R 3.3 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Patients characteristics

In the study, 94 consecutive patients operated on for rectal cancer at the General Surgery 3 Unit of the Azienda Ospedaliera of Padova from 2015 to 2021 were selected. As explained earlier, we identified 15 patients 50 years old or younger with mean age at diagnosis of 45.6 years and 79 patients older than 50 years with mean age 72.6 years. Patient characteristics and controls are outlined in Table I.

Table I. Study patient characteristics

Features	**Age ≥ 51 years**	**Age ≤ 50 years**
Total patients	79	15
Sex, n (%)	46 (58) males 33 (42) females	4 (27) males 11 (73) females
Age, mean (range) *y*	72.6 (52-87)	45.6 (30-50)
Median BMI (range) kg/m^2	23.7 (17.6-41.4)	23.6 (16.4-36.6)
Cancer stage, n (%)	0)8 (10) I)25 (32) II)17 (21) III)29 (37)	0)0 I)6 (40) II)5 (33) III)4 (27)
Histological type, n (%)	7 (9) Adenocarcinoma in situ 70 (89) adenocarcinoma 2 (2) mucinous carcinoma	0 adenocarcinoma in situ 14 (93) adenocarcinoma 1 (7) mucinous carcinoma
Vascular invasion, n (%)	47 (59) present 22 (28) not present 10 (13) not estimable	7 (47) present 7 (47) not present 1 (7) not estimable
Perineural invasion, n (%)	35 (44) present 32 (41) not present 12 (15) not estimable	7 (47) present 7 (47) not present 1 (7) not estimable

Oncogene mutational status

The *BRAF*, *KRAS,* and *NRAS* mutational status was analysed to define the prevalence of these mutations in early-onset rectal cancer. *BRAF* mutation was observed in 10% of young adults not receiving neoadjuvant radiotherapy and in 5% of old patients (P=0.40). *KRAS* mutation was detected in 25% of young adults and in 40% of the elderly (P=0.70) and *NRAS* mutation in 5% of old patients whereas this mutation did not occur in the young cohort (P=0.72). Therefore, our analysis did not show a difference statistically significative between young and old cohort in the perspective of the oncogenes alterations. Oncogene mutational status is shown in figure 2.

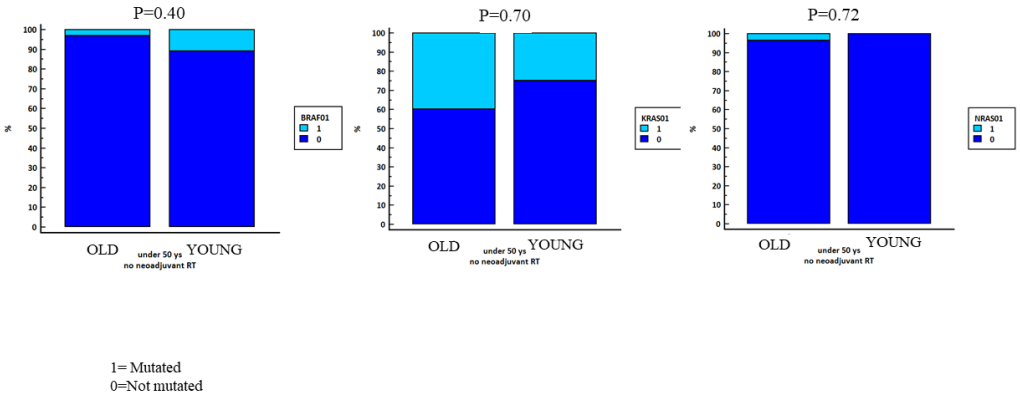


Figure 2: Oncogene mutation in untreated rectal cancer according to their age

MMR gene expression, MSI status

MMR gene immunohistochemistry and MSI detection were performed to analyse the possible role of a mismatch repair gene deficiency. After having excluded patients with Lynch syndrome, we noticed that mismatch repair gene deficiencies and MSI frequency occurred in 15% of early-onset cancer and about 2% of late-onset (P=0.19). In particular, young adults tended to have a higher frequency of mutations in MLH1 and PMS2 than old patients (P=0.03).

Microsatellite status is shown in Figure 3.

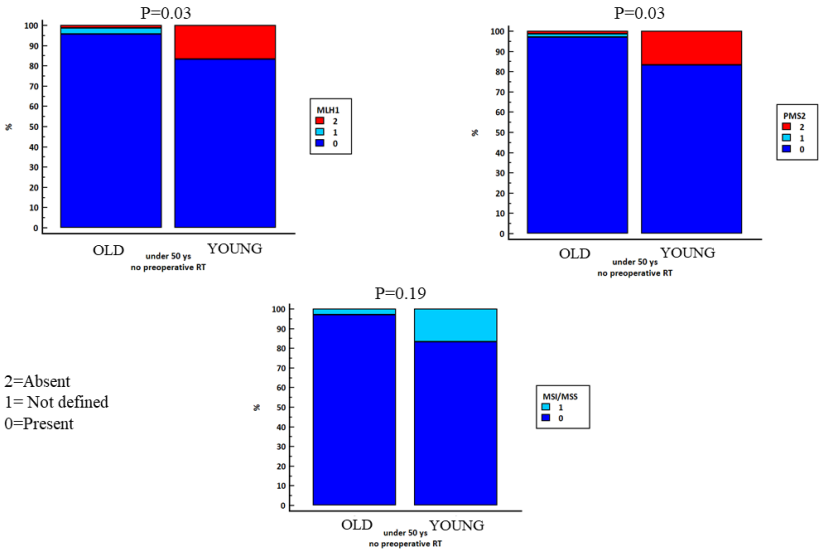


Figure 3: MMR gene expression and MSI status

Lymphomonuclear infiltration within the tumor

Histopathologic examination was performed to assess the lymphomonuclear infiltration within the tumor and investigate the role of the tumor microenvironment in the occurrence of rectal cancer in young individuals. Immune infiltration was present at low grade in 85% of young adults and absent in the remaining 15%. By contrast, immune infiltrate was identified as high-grade in 13% of controls, low grade in the 70% and absent in the 17% (P=0.19). However, a significative difference between the two age groups was not noticed. Immune infiltration within the tumor is shown in Figure 4.

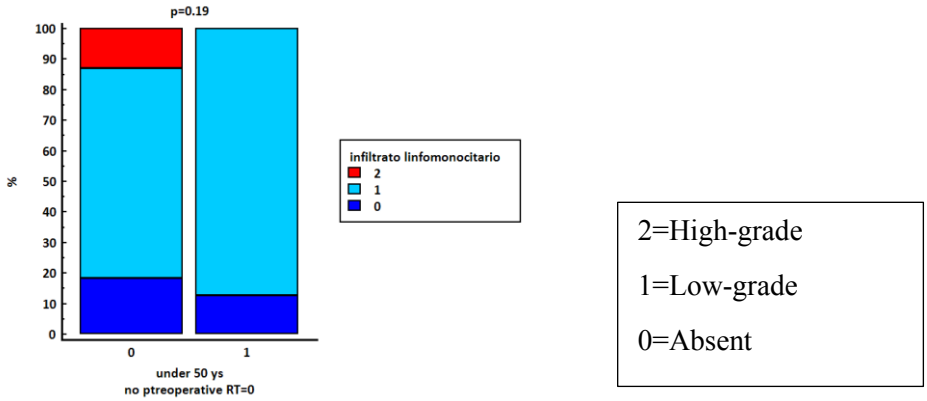


Figure 4: lymphomonuclear infiltration within the tumor

TCGA panCancer Atlas (2018)

We analysed molecular alteration in a cohort of 121 patients of which 18 were 50 years old or younger and 103 older than 50 years at diagnosis. We found no difference in in term of APC, TP53, TTN, KRAS, MUC16, SYNE1, CSMD1, SMAD4, FLG, RYR2, LRB1B, MACROD2, ASXL1, PTPRT, RBFOX1, PIK3CA, DMNT3B, and DNAH11 while mutations of MAP3K21 (27.78% vs 6.80%, P=0.0170) and SACS (38.89% vs 12.62%, P=0.0119) are more frequent in patients under 50 years old. SACS encodes for a co-chaperone which acts as a regulator of the Hsp70 chaperone machinery, besides the involvement in the processing of other ataxia-linked proteins, while MAP3K21 is a negative regulator of TLR4 signalling. Moreover, we found that other genes were mutated in young patients and wild type in the elderly. Of these genes, 7 are involved in the immune response (CD5L, PYHIN1, NCKAPL1, DNAJB9, ITPKB, MEP1B, ADAM15), and 2 are implicated in epigenetic modifications (ASXL2, ZNF304). Alteration event frequency is

shown in Figure 5 and Figure 6. Genes function is shown in Table II and Table III. Then, we detected lower mRNA expression of CD69 (P=0.0319), CD3 (P=0.0492) and CD8 beta (0.0265) in young patients than the older cohort. mRNA expression is shown in Figure 7.

These results underlined that alterations in immune system and tumoral microenvironment probably play a major role in pathogenesis of rectal cancer in young adults compared to typical molecular alterations involved in pathogenetic pathways in old patients.

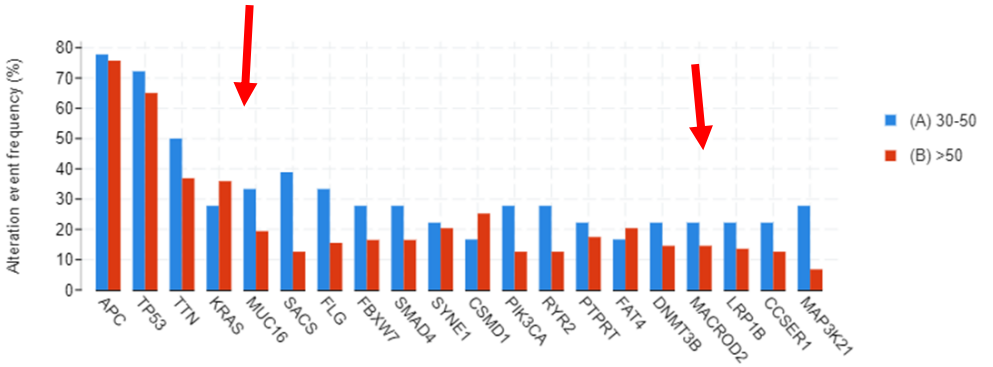


Figure 5: Genes with highest average frequency

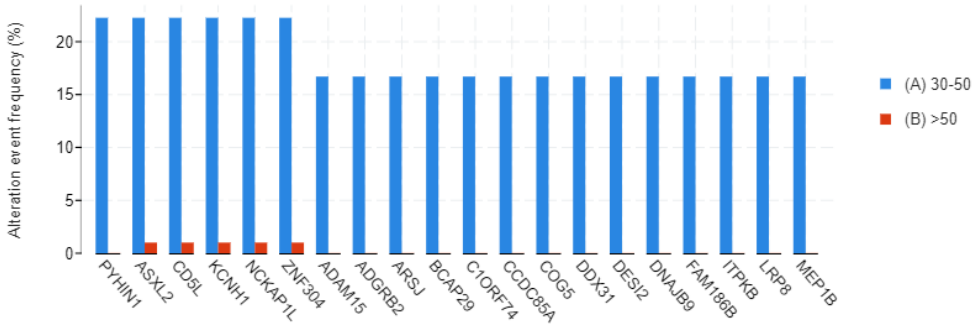


Figure 6: Genes with most significant p-value

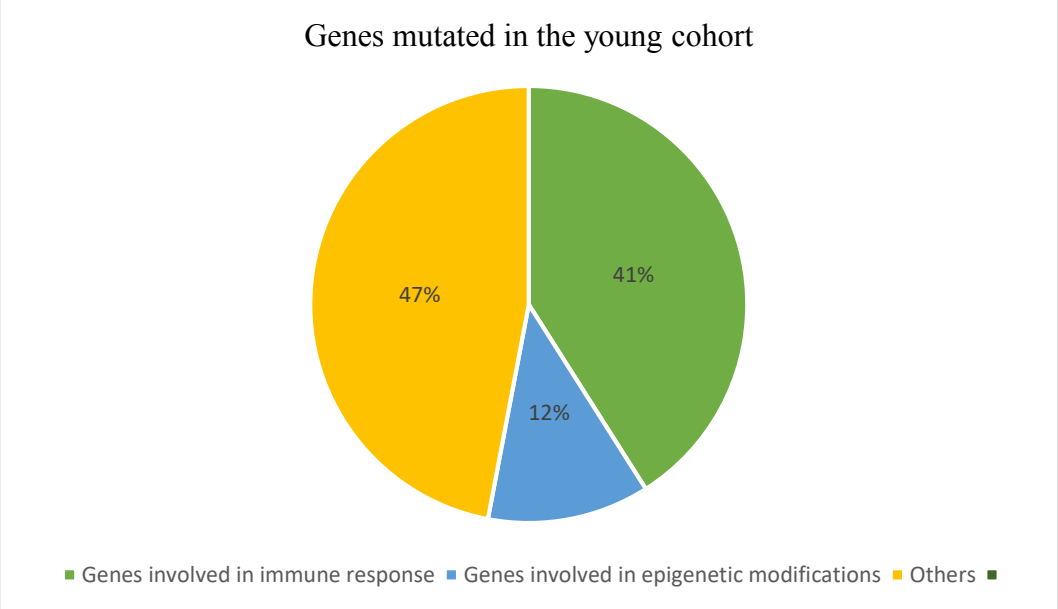
Table II: Function of gene involved in immune response altered in EORC[34]

GENE	PROTEIN	FUNCTION
CD5L	CD5 Molecule Like	Expressed by macrophages in lymphoid and inflamed tissues regulates mechanisms in inflammatory responses; CD5L-induced lipolysis participates in obesity-associated inflammation. Key regulator of metabolic switch in T-helper Th17 cells. Promotes macrophage survival from the apoptotic effects of oxidized lipids. Involved in early response to microbial infection acting as a pattern recognition receptor and promoting autophagy.
PYHIN1	Pyrin And HIN Domain Family Member 1	Interferon-inducible protein acts as tumor suppressor by promoting ubiquitination and degradation of MDM2, which leads to stabilization of p53/TP53.
NCKAP1L	NCK Associated Protein 1 Like	Controls lymphocyte development, activation, proliferation and homeostasis, erythrocyte membrane stability, phagocytosis and migration by neutrophils and macrophages. Required for efficient T-lymphocyte and neutrophil migration. In T-cells, required for mTORC2-dependent AKT phosphorylation, cell proliferation and cytokine secretion, including of IL2 and TNF.
DNAJB9	DNA J Heat Shock Protein Family (Hsp40) Member B9	Co-chaperone for Hsp70 protein HSPA5/BiP that acts as a key repressor of the ERN1/IRE1-mediated unfolded protein response. Required for survival of B-cell progenitors and normal antibody production.
ITPKB	Inositol-Trisphosphate 3-Kinase B	Catalyses the phosphorylation of InsP3. Participates to the regulation of calcium homeostasis. Regulates immune cell function and is required for T and B cell development.
MEP1B	Meprin A Subunit Beta	Membrane metallopeptidase sheds many membrane-bound proteins, implicated in inflammation. Substrates include: FGF19, VGFA, IL1B, IL18, procollagen I and III, E-cadherin, ADAM10. Involved in tissue remodelling due to its capability to degrade extracellular matrix components.
ADAM15	ADAM Metallopeptidase Domain 15	Member of the ADAM (disintegrin and metalloproteinase) protein family involved in cell adhesion and proteolytic processing of cytokines and adhesion molecules.

Table III: Function of gene involved in epigenetic regulation and cell proliferation altered in EORC

GENE	PROTEIN	FUNCTION
DDX31	DEAD-Box Helicase 31	ATP-dependent RNA helicase participates in ribosome biogenesis and TP53/p53 regulation through its interaction with NPM1

ASXL2	ASXL Transcriptional Regulator 2	Epigenetic regulator binds involved in the assembly of transcription factors at specific genomic loci. Has a role in neurodevelopment, cardiac function, adipogenesis, and osteoclast genesis
ZNF304	Zinc Finger Protein 304	Transcriptional repressor stimulates promoter hypermethylation and transcriptional silencing of target genes. It may promote cancer cell survival, growth, and invasion
KCNH1	Potassium Voltage-Gated Channel Subfamily H Member 1	Subunit of voltage-gated potassium channels expression in the adult CNS. It is activated at the onset of myoblast differentiation. Overexpression may confer a growth advantage to cancer cells and favour tumor cell proliferation
ADGRB2	Adhesion G Protein-Coupled Receptor B2	Transmembrane member of the secretin receptor family, brain-specific inhibitor of angiogenesis
ARSJ	Arylsulfatase Family Member J	Sulfatase involved in hormone biosynthesis, modulation of cell signalling, and degradation of macromolecules
BCAP29	B-cell receptor-associated protein 29	Endoplasmic reticulum (ER) protein functions as a molecular chaperone in processing and trafficking of P-glycoprotein to the cell surface.
COG5	Component Of Oligomeric Golgi Complex 5	One of eight proteins (Cog1-8) which form a Golgi-localized complex (COG) required for normal Golgi morphology and function
DESI2	Desumoylating Isopeptidase 2	Deubiquitinating activity
LRP8	LDL receptor related protein 8	Member of the low-density lipoprotein receptor (LDLR) family. Has a role in the migration of neurons during development, and functions as a receptor for apolipoprotein E.



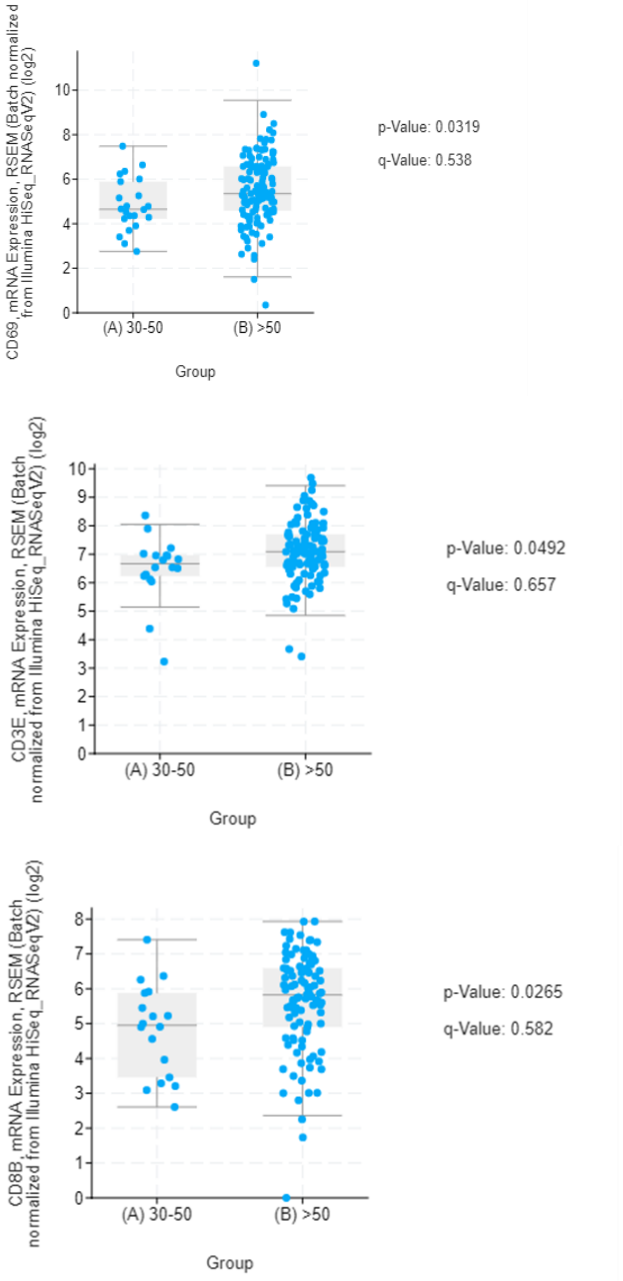


Figure 7: CD69, CD3, CD8 beta mRNA expression

DISCUSSION

The overall rectal cancer incidence has generally been declining since at least two decades probably due the improving prevention and screening programs. On contrast, incidence of rectal cancer in young adults is increasing and, although early-onset rectal cancer has an important familial component, most patients have a sporadic cancer.

The review by REACCT Collaborative[32] reported as one reason for this rising incidence trend the increasing influence of the exposome, that is the totality of

exposure to environmental factors, such as obesity, physical inactivity, and the western diet, which consists primarily of processed meats, red meat, fast food, and low levels of vegetables and fruits. In our study no difference in BMI between younger and older patients was encountered and median BMI was below the threshold of overweight for both groups of patients. This finding is in line with literature which underlines a strong association between obesity and colon cancer whereas no association is observed with rectal cancer. Concerning the other known risk factors, both the western diet and the excessive use of antibiotics have been proved to promote dysbiosis and transform the healthy gut microbiome into a proinflammatory and procarcinogen environment. Furthermore, the western diet favours carcinogenesis also generating advanced glycation end products and through food additives, such as monosodium glutamate and titanium dioxide, both of which promote tumorigenesis in animal models.

Rapid decline in CRC incidence among old individuals over the past decades is attributed to an improving adherence to the screening program recommended for the average-risk population between 50 and 74 years and for younger individual at high risk of developing cancer. On the other hand, the concerning trend of increasing incidence of early-onset sporadic rectal cancer could not be ignored and the U.S. Multi-Society Task Force suggested average-risk CRC screening begin at age 45[21]. Bearing in mind emerging data on rectal cancer epidemiology, this disease should no longer be perceived as a condition which affects only the elderly. Indeed, nowadays it is reasonable not to exclude the hypothesis of rectal cancer only because of the young age. This observation translates into the importance of a prompt evaluation for young patients who experience typical symptoms of the disease, as obstructive symptoms, rectal bleeding, and abdominal pain. Awareness of these symptoms may contribute to increase clinical suspicion and hence ensure that young adults undergo timely sigmoidoscopy or full colonoscopy[22].
A higher knowledge and understanding of this “heterogeneous disease”[20] is becoming increasingly necessary, since it is characterised by distinct biological and molecular alterations from late-onset cancer.

In terms of clinicopathological features, in young patients, cancer is more commonly diagnosed at an advanced stage, in particular III and IV, while in older

patients diagnosis is more frequently formulated at II or III stage. Earlier detection in old patients may be explained by population-based screening while diagnosis in young adults is often formulated with a delay of about 6 months after the symptoms onset. Moreover, literature suggests that young-onset tumors often display adverse histopathological features such us poor differentiation, venous and perineural invasion and mucinous or signet ring morphology. Our findings about venous and perineural invasion did not show a meaningful difference between young and old patients, but this is probably attributable to the limited number of young individuals enrolled. On the other side, we detected a not negligible percentage of tumors with mucinous differentiation among young individuals, higher than that among old patients. A meta-analysis by McCawley at al.[25] underlined that mucinous differentiation represents a negative prognostic indicator, associated with poorer response to neoadjuvant chemoradiotherapy, attributable to a reduced susceptibility or resistance to standard therapies.

On a molecular perspective, MAPK pathway mutations are common in CRC, including BRAF, KRAS, NRAS, hence mutational analysis is routinely performed in metastatic colorectal tumours to predict resistance to anti-epidermal growth factor receptor (EGFR) monoclonal antibodies and prognosis. In our series, BRAF mutations were detected in a minimum proportion of both young and old patients confirming that this alteration is not extremely relevant in rectal cancer, as reported in a study conducted by Sclafani et al.[26] which underlined that mutations of the BRAF gene were detected only in a small percentage of patients with rectal cancer. Moreover, non-V600 BRAF mutations, which account for 22% of all BRAF mutations, occur more frequently in rectal cancers and are associated with more favourable clinicopathological features and better outcomes than canonical V600 BRAF mutation. Regarding the other oncogenes, in our analysis we noticed the presence of KRAS mutations in a moderate percentage of young individuals while NRAS resulted altered only in a negligible proportion of patients. Even if not remarkable, the percentage of KRAS mutation was lower in the young group compared to the old one, and this is in line with previous studies. Indeed, literature highlights that the rate of KRAS mutations is lower in young individuals than the elderly also giving indication that the conventional carcinogenesis sequence adenoma-carcinoma, which typically depends on early KRAS mutation,

may not be applied to the young patient population. Data from TCGA also did not show any significative difference in the mutational status of KRAS comparing the young and the old cohort. The absence of typical oncogenes mutations in young adults implies that pathogenesis of early-onset cancer involves other alterations, but it also underlines the need to research novel possible prognostic factors and therapeutic targets.

We find that the analysis for microsatellite instability (MSI) was performed more often than oncogenes status, especially in young patients, in order to investigate a hereditary condition as Lynch syndrome. Effectively, MSI-tumors in young population are most commonly caused by germline mutations in one of MMR genes within the Lynch syndrome even if microsatellite instability may be detected also in sporadic tumors due mainly to epigenetic silencing of genes promoters. In our study, after ruling out all cases with hereditary disease to remove a possible bias, the greater percentage of young individuals showed microsatellite stability. Anyway, the rate of MSI-tumors observed was slightly higher in the young cohort than the old group. Taking into consideration sporadic MSI-tumors, we noticed a higher rate of alterations in MLH1/PMS2 expression in young patients and a lower frequency in the elderly. By contrast, a study conducted by Yiu et al.[27] evidenced that MSI tumors in the older age were associated with MLH1 inactivation or promoter methylation whereas tumors in the young group were especially associated with MSH2 inactivation. Anyway, the largest proportion of both early and late-onset sporadic cancer showed microsatellite stability (MSS), but they are characterised by different alterations in gene expression and pathways involved, as observed in the study conducted by Kirzin et al[28]. An over-activation of beta catenin (CTNNB1) and an up-regulation of the pathways related to adhesion/motility and inflammation/apoptosis were identified in early-onset cancer compared to late-onset patients. Deregulation of pathways linked to TNF-RI signalling appears related to the role of inflammation or the immune response in sporadic early-onset cancer. The evidence that increased production of inflammatory mediators may promote early-onset tumor is a significant clue that immunity plays a key role in the pathogenesis of this subtype of cancer. At last, the higher rate of MSS-tumors may contribute to the poor prognosis often observed in young patients. Notably, microsatellite instability confers a considerable survival advantage in reason of an

improved therapeutic effect of DNA-damaging chemotherapeutic agents, which means a major percentage of complete response to nCRT, and of an enhanced anti-tumour immune response, promoted by upregulation of immunogenic heat shock proteins which induce release of cytokines such as IL-18 and IFN-γ[29]. This kind of tumoral microenvironment leads to an increased efficacity of immunotherapy which is reflected in a better prognosis compared to MSS-tumors. Finally, although the rate of MSI is higher in young people related to the Lynch syndrome, sporadic cancer is more often characterised by microsatellite stability and deregulation of inflammatory response.

Results from our cohort and from TCGA database confirmed data reported in literature, that the onset of sporadic rectal cancer in young adults does not depend on typical genetic abnormalities and its natural history is not based on one of the three pathogenetic pathways defined for late onset cancer. The first pathway is the chromosomal instability (CIN) which is responsible for the 70% of sporadic CRC, associated with activation of the proto-oncogenes KRAS and C-MYC and inactivation of tumor suppressor genes APC and p53. The second pathway is the microsatellite instability (MSI) pathway, caused by a dysfunction in DNA MMR genes present in 15% of sporadic CRC cases, as a result of MMR genes silencing by promoter hypermethylation. The third pathway, the CpG island methylator phenotype (CIMP) pathway, is characterized by high levels of promoter methylation of CpG islands and it exhibits mutations in BRAF, and rarely KRAS mutations. A subset of tumors identified most commonly in younger patients is the so-called microsatellite and chromosome stable CRC (MACS), characterised by absence both microsatellite and chromosomal instability. The knowledge regarding molecular profile is limited but it seems associated with CIMP-law and LINE-1 hypomethylation and rare BRAF mutation. Moreover, an attenuation of the lymphocyte response and cytokine release was identified, and this suggested that the immune response may be inhibited in this tumour sub-group. All these unfavourable factors lead to an early disease recurrence and lower survival than patients with MSI or CIN[24].

The role of immune reaction in the pathogenesis and evolution of cancer has been debated for decades resulting in the confirm that a pronounced lymphocytic

infiltrate confers a survival advantage in neoplastic patients. In particular, a study carried out by Jass JR[30] observed a relation between a strong immune infiltrate and a prolonged survival in patients with rectal cancer and evaluated the high lymphocyte density as prognostic factor independent of the TNM classification. This observation was explained by two hypothesis. On one side, the lymphocytic infiltrate accounts for a specific response by the organism against the tumor. On the other, the peritumoral infiltrate often observed at the advancing front of the tumour may reflect the persistence of normal epithelial-stromal interaction and hence a high level of functional differentiation and a low grade of tumoral growth.

In our analysis, we noticed the presence of the lymphocytic infiltrate in the largest proportion of tumors both in young and old patients. Interestingly, a difference in degree of the infiltrate was encountered between the two cohorts: indeed, in a fair proportion of old individuals a high-grade infiltrate was detected while in young adults we found only low-grade infiltrate. This result, even if not significative, may be related to the molecular carcinogenesis of early-onset tumors characterised by chromosomal and microsatellite stability among young adults. Notably, MACS phenotype is associated with an attenuated lymphocytic reaction and a reduced cytokine release which suggests a probable inhibition of the immune system in this tumor subtype. TCGA also confirms an attenuation of the immune response in the young cohort, who exhibits lower mRNA expression for lymphocytic markers CD3, CD69 and CD8 beta. This evidence is a sign of a low presence of activated T-cells in the peritumoral site, translating a greater capacity by tumoral cells to escape the immune system and hence to grow, invade adjacent structures, and metastasize in distant organs. Moreover, data from TCGA showed that several genes required for lymphocytes development and functions and also for inflammatory response and cytokines realising were altered only in the young cohort. Hence, we deduced that abnormalities in the immune response and in the immunosurveillance may contribute to explain the pathogenesis of tumors among young individuals. A study conducted by Pagès et al.[16] underlined that presence of high levels of infiltrating memory T cells and cytotoxic cells were related with a prolonged survival in colorectal cancer in reason of the absence of signs of early metastatic invasion and a less advanced pathological stage. Therefore, a deregulation of the immune response may represent a predisposing factor to the onset of cancer in young adults

and participate with microsatellite and chromosomal stability to the aggressive progression of the tumor. Moreover, as reported in a study by El Sissy et al.[31] the heterogeneity of functional orientation and density of the immune infiltration within tumoral site in the centre of the tumor and in the invasive margin is also a strong prognostic factor for tumor dissemination and survival of patients, regardless of the stage. Consequently, the Immunoscore (IS) and biopsies-adapted IS (IS_B) biomarkers, based on quantification of CD3+ and CD8+ cytotoxic T cells in the CT and IM regions, may be a valid indicator of prognosis and contribute to the management strategy of young patients. In particular, Immunoscore is able to predict the degree of response to neoadjuvant chemoradiotherapy in patient with LARC, hence it represents a helpful tool to identify those patients eligible for the "Watch and Wait" strategy.

LIMITS OF THE STUDY

The purpose of this study was to describe pathological and molecular characteristics of sporadic rectal cancer in a cohort of young adults compared to typical cancer that occurs in the old cohort. This analysis was limited by the retrospective and observational design of the study and the restricted number of patient enrolled. Moreover, molecular analysis was not performed for all the tissue collected from biopsies and surgery or it was often incomplete. A follow-up analysis would be also required in order to evaluate the efficacy of therapies and prognosis of young adults in comparison with the elderly.

CONCLUSIONS

Over the few last decades, sporadic early-onset rectal cancer has become a global healthy challenge for whom a greater understanding is increasingly required. In addition to the proposal of bring forward the starting age for the screening, it is necessary to investigate accurately risk factors both environmental and individual. This unique disease is characterised by distinctive pathological features which include mucinous differentiation, advanced stage at diagnosis and limited degree of immunogenicity resulting in a low-grade peritumoral lymphocytic infiltrate. From a molecular standpoint, sporadic rectal cancer does not exhibit the typical alterations detected in late-onset cancer involving the oncogenes BRAF, KRAS and

NRAS and mismatch repair genes (MMR). The carcinogenesis includes unusual pathways resulting in a rare phenotype of chromosomal and microsatellite stability (MACS). It appears mandatory a deep investigation of molecular features of this disease, searching for not canonical abnormalities, for example non-V600E BRAF mutations. In addition to genetic mutations, also epigenetic alterations, such as LINE-1 hypomethylation, may contribute to explain the distinctive nature of tumors among young individuals. Finally, tumoral microenvironment and deregulation of the immune response, in particular of the lymphocytic infiltrate, needs to be investigated both as predisposing factor and key element in the evolution and progression of cancer. This is also underlined by detection of a higher frequency of mutations in genes involved in the immune response and of a lower mRNA expression for lymphocytic markers CD3, CD69 and CD8 beta in young patients. Hence, the analysis of these two factors may improve the knowledge about the disease and contribute to the development of personalised management strategies more appropriate for younger patients. Indeed, it appears necessary to explore novel therapeutic algorithms tailored to a different patient and a different disease which exhibits distinctive possible targets with particular attention to the immune substrate.

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