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TESI DI LAUREA

Phenotypic and molecular landscape of colorectal adenosquamous carcinoma

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Riassunto

Presupposti dello studio: Il carcinoma adenosquamoso (ASC) rappresenta meno del 0,1 % delle neoplasie colon rettali. Precedenti studi ne descrivono l'aggressività e la prognosi infausta ma a causa della sua rarità le sue caratteristiche molecolari non sono note.

Scopo dello studio: Descriveere il profilo morfologico e molecolare di una serie di carcinomi adenosquamosi primitivi del colon-retto e delle loro metastasi appaiate.

Materiali e metodi: Dagli archivi di quattro Unità di Patologia sono stati raccolti 29 casi di ASC colon rettale primario, 8 metastasi appaiate e 2 casi di adenoma tubulo-villoso colorettale di alto grado con morule squamoidi. Sono state registrate le variabili demografiche e clinico-patologiche. I campioni sono stati analizzati in un centro di raccolta; sono state valutate le caratteristiche morfologiche e l'espressione delle proteine del mismatch repair (MMR). Infine sui campioni raccolti è stata eseguita un'analisi del profilo molecolare mediante sequenziamento di Next-Generation Sequencing (NGS) mirato.

Risultati: L'età media dei pazienti è pari a 70 anni, e il rapporto tra maschi e femmine è di 2/1. Il tumore, localizzato maggiormente nel colon sinistro (55%), si è manifestato con occlusione e subocclusione nel 59% dei casi, 26 pazienti hanno subito una resezione chirurgica. Tutti i tumori hanno mostrato caratteristiche clinico-patologiche aggressive (il 34% dei pazienti si presentava alla diagnosi in stadio IV); tutti gli ASC hanno mostrato almeno una caratteristica patologica aggressiva quale scarsa differenziazione, invasione vascolare o pattern di crescita infiltrativo. Sedici pazienti (64%) sono morti a causa del tumore con una sopravvivenza media di 10 mesi. Tutti i casi tranne uno hanno mostrato normale espressione delle proteine del MMR. I geni maggiormente mutati nei campioni analizzati sono TP53 (59%), APC (41%), KRAS (37%) e BRAF (14%).

Conclusioni: Questo studio fa luce sul panorama morfologico e molecolare delle ASC del colon-retto. Secondo i nostri dati, gli ASC colorettali hanno un comportamento clinico aggressivo e presentano caratteristiche istopahologiche avverse. Tuttavia, il profilo genomico degli ASC colorettali è simile a quello del carcinoma colorettale convenzionale, con alterazioni genetiche significative trattabili con farmaci. Sono necessari ulteriori studi per comprendere il comportamento clinico più aggressivo di questa neoplasia.

Abstract

Background: Adenosquamous carcinoma (ASC) accounts for less than 0.1% of colorectal cancers (CRC). Previous studies describe its aggressiveness and poor prognosis, but due to its rarity its molecular landscape is still unknown.

Aim of the study: The aim of the study is to describe morphologic and molecular profiles of a multi-institutional series of primary colorectal ASC with paired metastases.

Methods: From the archives of four Pathology Units 29 cases of primary colorectal ASC, 8 matched metastases and 2 cases of colorectal high-grade tubulo-villous adenoma (HG TVA) with squamoid morules were collected. Demographic and clinical-pathologic variables were recorded. All the samples were analyzed in the collection center; morphological features and expression of mismatch repair (MMR) proteins were evaluated. Finally, a molecular profiling analysis was performed using targeted Next-Generation Sequencing (NGS).

Results: The mean age of the patients was 70 years and the male to female ratio was 2/1. The tumor, mostly located in the left colon (55%), manifested with occlusion and subocclusion in 59% of cases, 26 patients underwent surgical resection. All tumors showed aggressive clinicopathological features (34% of patients presented at diagnosis in stage IV); all ASCs showed at least one aggressive pathological feature such as poor differentiation, vascular invasion, or infiltrative growth pattern. Sixteen patients (64%) died of their disease with a median survival of 10 months. All but one case showed proficient MMR profile. The most mutated genes in the analyzed samples were TP53 (59%), APC (41%), KRAS (37%) and BRAF (14%).

Conclusions: This study sheds light on the morphologic and molecular landscape of colorectal ASCs. According to our data, colorectal ASCs have an aggressive clinical behaviour and present adverse histopahologic features. However, the genomic profile of colorectal ASC is similar to that of conventional colorectal carcinoma, with significant druggable genetic alterations. Further studies are required to understand the more aggressive clinical behavior of this neoplasm.

Chapter 1

Introduction

1.1 Epidemiology

Colorectal cancer (CRC), with an estimated 1.9 million new cases and 935.000 deaths worldwide in 2020, is the third cancer in terms of incidence, but second in terms of mortality in the world (1)

The age standardized incidence of CRC varies greatly between countries, this represents a combined effect of multiple factors, including lifestyle, genetics, life expectancy and data quality of cancer registries (2). CRC cases are growing rapidly (globally, incidents have doubled from 1990 to 2020) in low- and middle-income countries due to an increase in the prevalence of modifiable risk factors, such as an unhealthy diet, sedentary behavior, physical activity, alcohol abuse, and increased life expectancy (3). The age-standardized mortality rate of CRC is decreasing in high-income regions, due to the implementation of successful national primary prevention and early detection strategies (3). More than 90% of global cases of CRC occur after the age of 50 and the age adjusted rates are 1,4–1,5 fold higher in men than in women (2).

1.2 Modifiable and inheritable risk factors

The previously mentioned environmental risk factors and genetic factors play a crucial role in the development of CRC (4). Twin studies estimated the heritability of CRC to be 35% (95% CI: 10%-48%; p=0.95) (5). Some CRC cases (25%) have a family history of CRC without any obvious genetic cancer syndrome (6). Only 5% of the cases are attributed to hereditary cancer syndromes such as Lynch syndrome (LS) and Familial adenomatous polyposis (FAP) (6).

1.3 Pathogenesis

Most cancers arise from a polyp and the cell of origin is currently assumed to be a stem cell or stem-cell-like cell (7). The process begins with an aberrant crypt, evolving into a neoplastic precursor lesion (a polyp) and eventually progressing to cancer over an estimated 10–15 years period. The drivers of this process are both genetic and epigenetic alterations of the genes that regulate cell growth and differentiation. CRC can develop through three different molecular pathways: the *chromosomal instability (CIN),* also known as the classic adenoma-carcinoma sequence, *microsatellite instability (MSI)* and *CpG island methylator phenotype (CIMP)* pathways, also known the serrated pathway. (8).

The adenoma–carcinoma sequence leads the development of 70–90% of CRCs. In this pathway, due to chromosomal instability, *APC* gene mutation occurs and results in overactivation of the Wnt/β¬catenin signaling pathway, triggering dysregulated cell proliferation and adenoma development and progression (9). Subsequent mutations of the oncogene *KRAS* promote adenoma growth and ensuing inactivation of *TP53* tumor suppressor gene contributes to the progression to CRC (8).

The serrated pathway (involved in 10–20% of CRC) is associated with *RAS* and *BRAF* mutations, and epigenetic instability (i.e. CpG island methylation) (9).

In some cases, the methylation may involve the promoter of *MLH1* gene, leading to microsatellite instable cancer (10). A small number of microsatellite instable colorectal cancer develop in the context of Lynch Syndrome, which is characterized by with germline mutations in mismatch repair (MMR) genes (11).

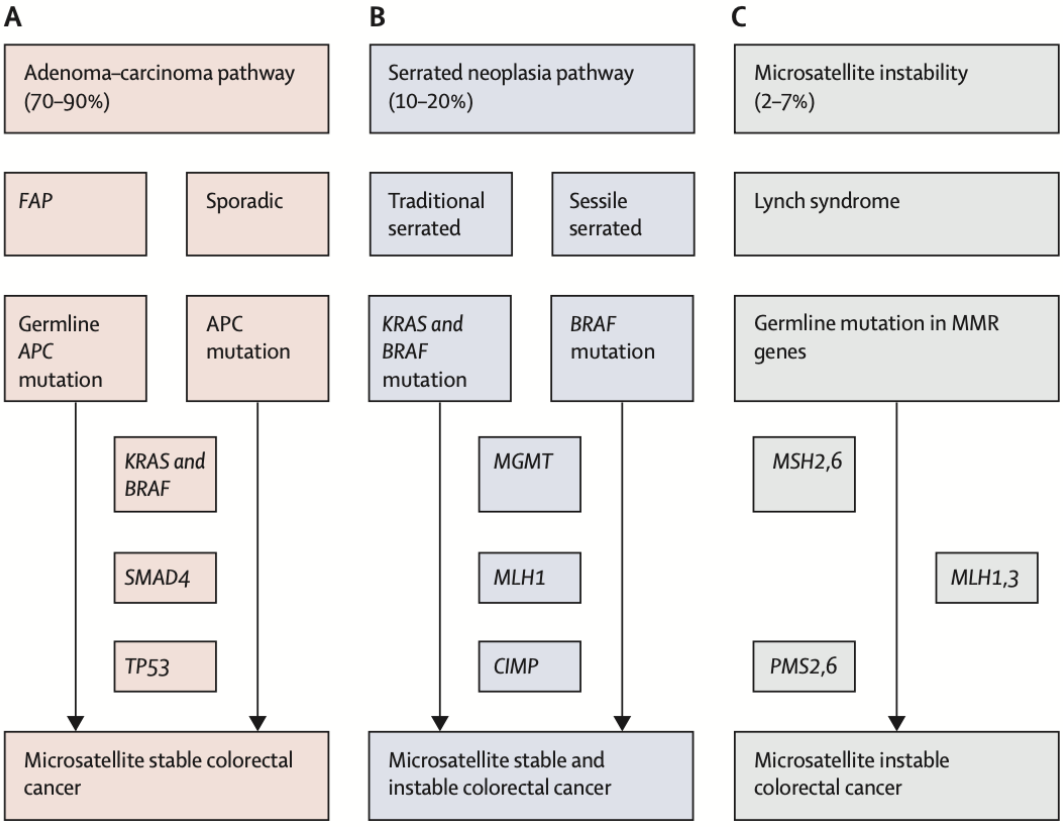


Figure 1: Colorectal cancer development pathways

1.4 Right-sided CRC versus left-sided CRC

CRC may develop anywhere in the large intestine; however, CRCs are formally classified as right/left-sided based on whether they occur before or after the splenic flexure. Molecular, pathological, clinical features and prognostic (especially for metastatic CRC) differences, summarized in *Figure 2,* exist between left and right sided CRCs (12)(13). This distinction has a great impact on treatment choice such as the response to anti-EGFR drugs (14). That said, M. Loree at all suggests that there are not two different entities but a continuum of changes across the bowel (15).

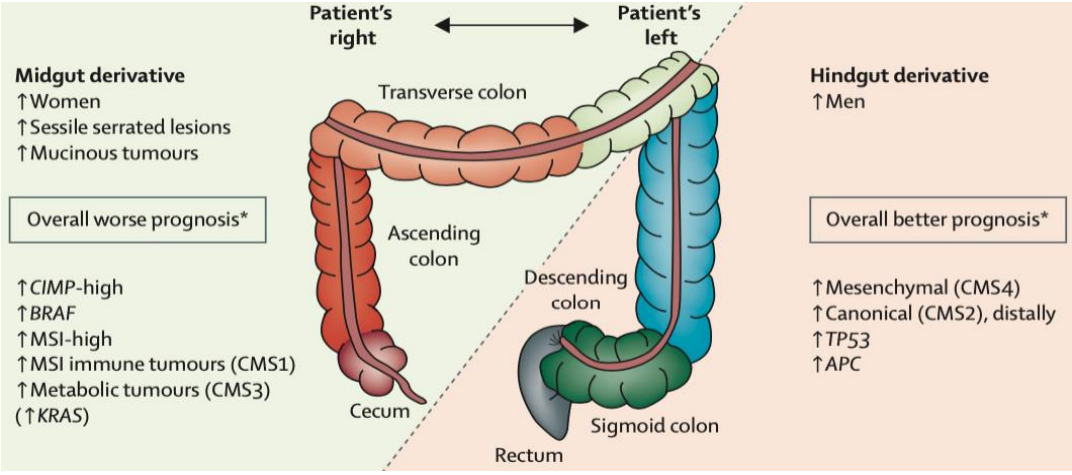


Figure 2: Differences in right-sided versus left-sided colon and rectum

1.5 Staging

The stage of the neoplasm is determined by the depth of infiltration of the intestinal wall and by the distant spread of the disease. CRC can pread by *direct extension* to adjacent bodies, *via* lymphatic or by blood. Regional lymph nodes (LN) constitute the earliest site of metastatic localization. Approximately 50% of CRC patients will develop liver metastasis during the disease (16). Most frequent metastatic CRC (mCRC) site is the liver, followed by the lung and the peritoneum (17).

Currently, the staging systems most used is Tumour Node Metastasis (TNM) classification, 8th edition (18). In this classification Tumor (T) stage describes the size of the tumor. The infiltration by the tumor of the submucosa, of the muscular layer and of the subserosa constitute as many stages, progressively increasing. The Node (N) parameter describes whether the cancer has spread to the LN. Finally, Metastasis (M) describes whether the cancer has spread to a different part of the body (19).

1.6 Histopathology

The vast majority (90%) of all CRCs are adenocarcinomas (ACs). Although most cases of CRC are diagnosed as AC Non-Otherwise Specified (NOS), several histopathological subtypes can be distinguished; many of these could be underdiagnosed, particularly the rarer subtypes (20)(21).

The mucinous adenocarcinoma (MA) is the most common subtype, accounting for 10–15 % of cases of CRC (22). To fulfill the definition of MA as

emphasized by the WHO, more than 50% of the tumor should consist of extracellular mucin (23). If compared to adenocarcinoma NOS, no prognostic difference is shown. MAs are often found in the proximal colon (12)

Medullary carcinoma (MC) accounts for 0,03% of CRC AC (24). It is characterized by sheets of malignant cells with vesicular nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm, exhibiting prominent infiltration by lymphocytes and neutrophilic granulocytes. MCs have not a different clinical outcome respect to other CRCs (21). MCss are frequently characterized by microsatellity instability (MSI) and *BRAF* mutation. (24)

Serrated adenocarcinoma (SA) has an incidence of 10-15 % of all CRC (1). It is defined by morphological similarities with serrated polyps, with glandular serration that can be accompanied by mucinous area (23).

A tumor is designated as signet-ring cell carcinoma (SRC) if > 50% of the tumor cells have prominent intracytoplasmic mucin, typically with displacement and molding of the nucleus (23). The SRC accounts for 1% of AC (20). It has a poor prognosis compared to AC NOS and MA. (25)

Adenosquamous carcinoma (ASC) is extremely rare, with an incidence of 0.05- 0.20 % of all CRC (26) (27). ASC is characterized by two malignant components: a glandular component, with various grades of differentiation, and a squamous cell component, with intercellular bridges and possible keratin pearls. The WHO system states that colorectal ASC should contain at least 20% of each component, even though this cut off is arbitrary. Several pathogenetic hypotheses have been proposed, such as derivation from pluripotent stem cells that exhibit multidirectional differentiation. Other proposed hypotheses support the origin of ASC tumour from squamous metaplasia of the glandular epithelium following chronic irritation or squamous differentiation of adenoma and AC (28). There is no significant difference in sex, age, and ethnicity between patients diagnosed with ASC when compared to those with conventional CRC (29). Most common location of ASC is the right and transverse colon ($p<0,01$) (30). Masoomi et al. found that ASC is associated with a higher rate of metastasis at the time of operation than AC (ASC, 36.56% vs AC NOS, 13.92%, $p<0.001$) and with poorly differentiated tumor grade (ASC, 65.96% vs AC NOS, 19.74%, $p<0.001$). Furthermore, the median overall survival time was significantly greater in the AC group (82.4 months) in comparison with the ASC group (35.3 months). Finally, they found that ASC was

independently associated with increased overall mortality (HR, 1.67; $p < 0.001$) and CRC–specific mortality (HR, 1.69; $p < 0.001$) when compared to AC. No information is available on the molecular landscape of this histotype.

1.7 Prognostic factors

Several histological prognostic factors were proposed by the WHO: 1) *Grading*: it is based on gland formation An high grade (formerly poorly differentiated) is a negative prognostic factor compared with low-grade (formerly well to moderately differentiated) (23); 2) *Histotype*: as already mentioned, some histotypes are associated with a worse prognosis when compared with AC, such as ASC (HR: 1.67; $p < 0.001$) (26)); 3) *Growth patterns:* two types of growth patterns can be distinguished: infiltrative growth and pushing borders, the last one is associated with improved outcome and lower stage. 4) *Perineural invasion*: it is a negative prognostic factor (HR: 2.09) (31); 5) *Lymphatic invasion* (HR: 2.15 (23)): many studies have demonstrated that the lymph node ratio (LNR), ratio between metastatic LNs and the total number of harvested LNs, is a more reliable prognostic factor compared with the absolute number of LNs. The cut-off of LNR ≥ 0.2 is known to be a negative prognostic factor (32) 6) *Intra and extra mural vascular invasion*: they are both negative prognostic factor (HRs are respectively: 2.04 and 3.6) (33)); 7) *Tumor budding*: is described as the presence of isolated carcinoma cells or groups of less than 5 elements in the stroma of the tumor advancement margin. An high degree of tumor is a negative prognostic factor (34) 8) *Immune response:* the presence of an intense intra-tumor, peri-tumor lymphocyte infiltrate or peri-tumor lymphocyte aggregates is indicative of a host immune response and is often associated with MSI and a better prognosis (23); 9) Positive *radial circumferential margins* (neoplastic cells are detected less than 1 mm from radial margins) correlates with a negative prognosis in rectal cancer(HRs: 1.72 (35)) 10) *Response to therapy*: is defined as the set of histopathological changes caused by the effects of neoadjuvant therapy (radiotherapy or radiochemotherapy for rectal cancer and systemic therapy for colon cancer), greater response to therapy correlates with better prognosis (23).

Regarding clinicopathologic prognostic factors, *TNM stage* remains the gold standard of prognostic factors in CRC (36).

Finally, there are many biomarkers that have a prognostic and/or predictive significance. Many of these have been introduced and integrated in histopathological reports to obtain an inclusive morphological and molecular characterization of the biospecimens and to guide prognostication and treatment decision-making.

1) *RAS gene mutations*. The *RAS* gene family is composed of four small cytoplasmic proteins with GTPase activity: H-Ras, K-Ras4a, K-Ras4b, N-Ras. These proteins promote cell growth, differentiation, proliferation, and survival (37). *KRAS* mutations are an early event in colorectal carcinogenesis (38). *KRAS*-mutated CRCs account for 40% of all cases, mostly located in exon 2(39). Being the RAS proteins part of the Epidermal growth factor receptor (EGFR) downstream signaling pathway, mutations in the *RAS* genes are well-recognized biomarkers of resistance to anti-EGFR monoclonal antibodies (40). For these reason patients with CRC being considered for anti-EGFR therapy must be profiled for *RAS* mutational status (41).

2) *BRAF gene mutations.* The *BRAF* gene encodes a serine/threonine protein kinase that regulate cell growth and proliferation (42). Missense somatic mutations in the *BRAF* gene have been found in about 8-15% of metastatic CRCs (43). The most common *BRAF* mutation (> 90%), resulting in a constitutive-active kinase because of an amino acidic substitution from valine to glutamic acid at codon 600 in exon 15 (^{V600E}*BRAF*) (43). *BRAF*-mutated mCRC arise in older patient (> 60 years old), with a higher prevalence in the female gender in comparison to *BRAF*-wild type cases and the proximal colon is the preferential location (37). Moreover, this class of tumors present a unique metastatic pattern, showing high rates of peritoneal metastases, distant LN metastases and low rates of lung metastases (43). *BRAF*-mutated CRCs frequently present mucinous features, poor differentiation, and high stage at diagnosis (43). The presence of *BRAF* mutation is a negative prognostic biomarker in mCRC (41). For this reason, *BRAF* mutational testing should be performed in mCRCs for prognostic stratification. On the other hand, there is insufficient evidence to support its testing as a predictive molecular biomarker for response to anti-EGFR inhibitors (44). The BEACON CRC trial set a new standard of care in patients with *BRAF*mt progressive cancers, consisting of the combination of the BRAF inhibitor encorafenib plus the anti-epidermal growth factor receptor (EGFR) monoclonal antibody cetuximab (45). ^{V600E}*BRAF* mutation

is strongly associated (60%) with the somatic inactivation of the DNA mismatch repair (MMR) genes, absent in LS (46). For this reason, somatic *BRAF* mutation testing has been included into the LS screening algorithm (37).

3) Defective DNA mismatch repair complex (dMMR) and microsatellite instability (MSI). MMR is a highly conserved protein complex that recognizes and repairs short insertions, short deletions and single base mismatches that can arise during DNA replication and recombination, after escaping proofreading process. The four genes that play a critical role in this process include *MLH1, MSH2, MSH6, PMS2*. The inactivation of these genes can occur due to germline (such as in LS) and/or somatic mutations or epigenetic silencing. Epigenetic silencing is usually represented by a monoallelic *MLH1* gene promoter hypermethylation (47). Microsatellites are repetitive DNA sequences that are distributed along the genome of both coding and non-coding regions and are particularly sensitive to DNA mismatching errors. The identification of microsatellite instability is, therefore, indirect evidence of a dMMR (48). Evidence suggests that dMMR/MSI CRCs, which account for approximately 17% of all cases, are associated with a favorable prognosis in comparison to proficient MMR (pMMR) (49). MSI CRC are preferentially located in the right colon and most of them show distinctive histopathological features, such as presence of Tumor Infiltrating Lymphocytes (TIL) and mucinous growth (21,37). Due to high concordance rate between immunohistochemistry (IHC) for the four MMR proteins and MSI molecular testing based on PCR, IHC analysis is usually preferred to investigate the presence or absence of dMMR/MSI status (50). The use of IHC to assess the loss of expression of MMR proteins has been recommended to all patients with CRC as a workup test to evaluate for possible LS (44). In colorectal AC *MLH1/PMS2* negative tumors should be tested for ^{V600E}*BRAF* since this mutation is frequently observed in sporadic cases. Another option to identify a *MLH1/PMS2* negative tumor as sporadic is the evaluation of *MLH1* promoter methylation (37). Moreover, patients with locally advanced cancers should be tested for dMMR/MSI because they do not seem to benefit from 5-fluorouracil adjuvant therapy (51). Finally, in the metastatic setting, first-line immunotherapy with checkpoint inhibitors has been approved in dMMR/MSI tumors (52).

4) Amplification of the HER2 gene. Amplification and/or overexpression of human epidermal growth factor receptor 2 (HER2) characterizes around 5% of

KRAS/NRAS/BRAF wild type colorectal AC in the metastatic setting (53). Activation of the HER2 pathway as a bypass signalling pathway has been identified as a mechanism of resistance for anti EGFR antibody therapy (54). Although the role of HER2 as a biomarker for prognosis in CRC remains uncertain (55). HER2 assessment in CRC is assessed by IHC analysis and, according to the HERACLES diagnostic criteria, 2+/3+ HER2-IHC in ≥ 50% tumor cells confirmed by Fluorescence in situ hybridization (FISH) (56).

1.8 Clinical symptoms and signs

Although many screening programs are increasing the number of asymptomatic CRC cases detected, there are several symptoms and signs that CRC can manifest with. However, they are associated with relatively large tumors and/or advanced disease stages and may not be specific for CRC (57). Of note, many differences exist between the symptoms and signs of proximal and distal CRCs. Left CRCs are more likely to present with rectal bleeding and alteration in bowel habits, while right CRCs present more frequently with subtle symptoms such as iron deficiency anemia and weight loss which are not easily detectable until advanced stage (58).

1.9 Diagnosis

A complete work-up should be carried out to achieve an accurate histological diagnosis of the primary tumor, assess the baseline characteristics of the patient and determine the extent of the disease (59).

European society for medical oncology (ESMO) guidelines suggest that a total colonoscopy is recommended for diagnostic confirmation of colon cancer. There are many advantages of endoscopy including determination and marking of the exact tumor location and biopsy of the lesion and detection and removal of (further) synchronous precancerous or cancerous lesions (60). After CRC diagnosis, clinical examination and laboratory tests must be carried out to provide a correct assessment of patient status and characteristics before deciding the definitive treatment approach. Preoperative assessment of tumor extension is required to determine whether the patient should be referred for primary tumor resection or, in the presence of unresectable distant metastases, systemic therapy. Computed tomography (CT) of the thoracic, abdominal, and pelvic cavities with endovenous

contrast administration is the gold standard for the evaluation of the extent of CRC. Contrast-enhanced Magnetic resonance imaging (MRI) constitutes the reference test for evaluation of the relationship of locally advanced tumors with surrounding structures or in defining ambiguous liver lesions (59).

ESMO guidelines for rectal cancer is similar to colon cancer about the definition of functional status and presence of metastases. But for the evaluation of the localized rectal tumor, rigid rectoscopy and preoperative colonoscopy to the cecal pole are required, or, in the case of obstruction, virtual colonoscopy to exclude synchronous colonic tumors. Endoscopic rectal ultrasound (ERUS) may define treatment for the earliest tumors. Finally pelvic magnetic resonance imaging (MRI) is the most accurate test to define locoregional clinical staging. (61).

1.10 Screening

European and american evidence-based guideline suggest the use of invasive tests and non-invasive tests for CRC screening (62,63).

ESMO guidelines recommended the use of colonoscopic techniques in average-risk men and women based on higher sensitivity and specificity. The optimal age range for testing is 50-74 years with an optimal repetition interval for a negative test of 10 years. Flexible sigmoidoscopy (FS), carried out every 5-10 years, may be an alternative for those who refuse colonoscopy, it could be combined with a yearly faecal occult blood test (FOBT).

Non-invasive tests are recommended in average-risk men and women from the age of 50 not already taking part in colonoscopic screening programmes. The optimal frequency of testing is every year and no later than every three years. A colonoscopy must be carried out at the earliest convenience when the test results are positive.

Individuals with a medical history of adenoma, colon cancer, inflammatory bowel disease or with a significant family history of CRC or adenoma; or with an inherited cancer syndrome are considered at high risk of CRC and must be actively screened depend on their condition. For example, surveillance colonoscopy every 1-2 years in asymptomatic individuals with LS is recommended, for these patients the onset of colonoscopy surveillance is recommended at the age of 25-35 years (64).

1.11 Treatment options

Depending on the stage of the disease, different treatment options are available. They are different for colon and rectal cancer.

Endoscopic resection is sufficient for hyperplastic or adenomatous polyps and intramucosal (pTis) AC of both colon and rectum (65) (61).

For colon invasive carcinomas (T1), the management it could be endoscopic resection with proper follow-up or surgical resection with complete lesion resection including LN removal for optimal risk assessment. The type of approach is determined by the operative risk of the patient, the polyp morphology and the presence of histological features associated with adverse outcome such as lymphatic or venous invasion; grade 3 differentiation; significant (grade >1) tumor budding (66).

Locally infiltrative colon cancers (stage I-III) require the resection of the involved bowel segment and its lymphatic drainage. The extent of the colonic resection (at least 5 cm on either side of the tumor) is determined by the blood supply and distribution of regional LNs. En-bloc colonic and mesentery resection is recommended to clearly define stage II versus stage III and to identify and eradicate potential LN metastases; at least 12 LNs should be resected when feasible (67). Laparoscopic colectomy compared to open surgical treatment has a similar oncological outcome but reduce the morbidity and improved the tolerance, for this reason it can be safely carried out for colon cancer when technical expertise is available in the absence of contraindications (68). ESMO guidelines suggest that adjuvant therapy options should be evaluated, taking into consideration tumor risk of recurrence, expected benefit from chemotherapy and risk of complications. Combinations of fluoropyrimidines, 5-Fluorouracil (5-FU) or capecitabine, and oxaliplatin constitute the bases for stage III and high-risk stage II colon cancer adjuvant treatment. Finally for patients with intermediate risk 6 months of fluoropyrimidines should be recommended. (66)

For very early rectal cancer (T1N0) with no adverse features, guidelines suggest a local excision of the tumor with transanal endoscopic microsurgery (TEM) (61). Local radiotherapy (RT) may be used as an alternative to local surgery (69).

More advanced rectal tumors up to and including cT2c/T3a/b should be treated by total mesorectal excision (TME). All mesorectal fat and including all LNs should be meticulously excised (70).

For patients with intermediate or locally advance rectal cancer (LARC), treatment decisions regarding neoadjuvant therapy with chemoradiotherapy (CRT) or short-course preoperative radiotherapy (SCPRT) should be based on preoperative, MRI-predicted circumferential resection margin (CRM) (<1 mm), extramural vascular invasion (EMVI) and more advanced T3 substages (T3c/T3d), which define the risk of both local recurrence and/or synchronous and subsequent metastatic disease (71).

Finally preoperative CRT followed by surgery or preoperative SCPRT plus FOLFOX and delay to surgery is recommended for advance tumor (T3 with any mesorectal fascia (MRF) involved, any T4a/b, lateral node+) (61).

Postoperative CRT is recommended for all patients with T3-4 or N+ rectal tumors, but the routine use of CRT to reduce local recurrence can be questioned if a good-quality TME can be assured (61).

A specialized and dedicated multidisciplinary team (MDT) should attend regular meetings and discuss all patients (72). MDT play a crucial role to define the initial diagnostic workup and then the treatment focus for patients with mCRC (41).

Generally, oligometastatic disease (OMD) may be characterized by the existence of metastases at up to 2-3 sites and visceral and/or five lymph nodal lesions. For patients with OMD systemic therapy should be considered as the initial part of every treatment strategy (41). Exceptions are made for patients with single/few liver or lung resectable lesions for whom a complete ablation of all tumor masses, using surgical R0 resection and/or localized interventions (LAT) is recommended (41).

For non resectable mCRC the definition of a (potential) treatment target and strategy is important for both the upfront integration of a multimodal treatment approach and for the choice of a systemic treatment strategy (first-line and later-line) as part of a ‘continuum of care’ (41). For these patients, knowledge of the RAS and BRAF mutational status of their disease is used to further refine treatment strategies (73). Targeted agents are indicated in the first-line treatment of most patients unless contraindicated. To date, there is no unequivocal evidence for the superiority of one class of biological over another (anti-VEGF versus anti-EGFR

therapies). Each one of these antibodies should be used in combination with other agents including FOLFOX, CAPOX, FOLFIRI. (41,72). Subsequent lines of therapy depend on the characteristics of the patient, the organ function, and the characteristics of the first-line therapy choice (41).

Chapter 2

Aim of the study

In the present study we analyze the morphologic and molecular profiles of a multi-institutional series of primary colorectal ASC with paired metastases. The goal is to describe the features of this rare hystotipe of CRC that could have a potential diagnostic and clinical impact in the oncological practice.

Specifically, the objectives of our study are to 1) assess the clinicopathological features of our case series; 2) evaluate the MMR status; 3) detect the frequencies in colorectal ASC of mutations and copy number variations (CNVs) in a set of genes that are frequently mutated in solid tumors; 4) correlate the histopathologic findings with molecular alterations; 5) compare ASC's profile to that of conventional colorectal adenocarcinoma.

Chapter 3

Materials and methods

3.1.1 Case selection

We retrospectively collected 29 cases of primary colorectal ASC 9 matched distant metastasis (4 synchronous and 5 metachronous) and 2 cases of colorectal high-grade tubulo-villous adenoma (HG TVA) with squamoid morules examined between 1992-2020 from the pathology archives of the following centers: Unit of Pathology, Fondazione IRCCS Ospedale Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; Unit of Pathology of University of Genova, IRCCS Ospedale Policlinico San Martino, Genova, Italy; Unit of Pathology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; Unit of Pathology of University of Padova, Azienda Ospedale-Università Padova, Padova, Italy,.

Selection was based on one of the following diagnoses or microscopic descriptions in colorectal samples: adenosquamous carcinoma, adenocarcinoma with squamous or squamoid differentiation, mixed adenocarcinoma, and squamous carcinoma. Cases with bland squamous morules were excluded. Finally, only cases showing at least 20% of both components of both adenoma and squamous components, as specified in the WHO classification 2019 (23), were enrolled in the study.

All information regarding human tissue was managed using anonymous numerical codes, and all samples were handled in compliance with the Helsinki Declaration (74)

3.2 Clinico-pathologic evaluation

The following data were collected: 1) *demographic and clinical data*: age at diagnosis, gender, associated neoplasms, clinical symptoms, 2) *site of tumor*: right-sided colon (ileocecaljunction, cecum, ascending colon, hepatic flexure and

transverse colon), left-sided colon (splenic flexure, descending colon and sigmoid colon) or rectum; 3) *type of specimen* (surgical resection *vs* biopsy *vs* polypectomy), 4) *gross findings* (size and morphologic features), 5) *tumor staging* according to the TNM staging system of the American Joint Committee on Cancer (AJCC) (8th edition, 2017) 6) *local/distant metastatic sites*.

Morphology of the collected tumor samples was revised, initially by the submitting center and subsequently by the central collection center (San Giovanni Rotondo), and new sections microtome cut and stained with Haematoxylin and Eosin (H&E) when necessary.

The following morphologic parameters were evaluated: 1) *percentage of glandular and squamous components*, 2) *grade of differentiation of glandular component*, 3) *vascular (blood vessel and lymphatic) invasion*, 4) *perineural invasion*, 5) *growth patterns at the leading edge (expansile vs infiltrative)*, 6) *intra and/or peritumoral lymphocytic infiltrate*, 7) *number of isolated LNs*, 8) *number of metastatic lymph nodes*, 9) *lymph node ratio* (LNR of $<$ or $\geq$ 0.2, (75) 10) *other associated histologic subtypes,* e.g. mucinous, signet ring, neuroendocrine neoplasm, according to WHO classification 2019, 10) *response to therapy* for those who received neoadjuvant therapy (3 cases).

Finally, *survival data* for patients was obtained through patient charts at the various submitting institutions, both with regards to follow up time and disease specific survival (in months).

3.3 Immunohistochemical analysis of MMR proteins

Immunohistochemical (IHC) staining of representative tumor sections of the primary tumors, metastatic LNs and distant metastases were performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle upon Tyne, UK) in the BOND-MAX system (Leica Biosystems), following appropriate staining protocols as per manufacturer's instructions.

IHC staining was performed using 4 μm thick unstained sections of formalin-fixed paraffin- embedded (FFPE) tissue, using MLH1 (clone ES05 diluted 1:50, Dako), PMS2 (clone EP51 diluted 1:40, Dako), MSH2 (clone FE11 diluted 1:50, Dako), MSH6 (clone EP49 diluted 1:50, Dako) antibodies with appropriate negative and positive controls.

MMR protein nuclear expression was evaluated following the AIFEG-SIAPeC criteria (76). It was interpreted as: 1) *Retained*, when a moderate to strong expression (similar to what is observed in the stromal cells as internal control) was present in ≥10% tumor cells; 2) *Lost*, in case of complete loss of nuclear expression in cancer cells; 3) *Indeterminate*, if IHC staining intensity in tumor cells was lower than the internal control or the tumor is positive in <10% (77).

Diffuse and homogeneous expression of all four MMR proteins, identified a pMMR. Conversely, the loss of expression of one or more of these proteins indicated a dMMR.

3.4 Genetic analysis

Introduction to the Next Generation Sequencing technique

The term Next Generation Sequencing (NGS) is a massively parallel sequencing technology that offers ultra-high throughput, scalability, speed, and relatively low cost compared to be other sequencing modalities. The technology is used to determine the order of nucleotides in entire genomes or targeted regions of DNA or RNA. Various commercial platforms have been developed, which differ in their use of different sequencing technologies, but all NGS platforms perform sequencing millions of DNA (or RNA) fragments in parallel.

NGS has recently moved into clinical practice, allowing the detection of alterations with diagnostic, prognostic, and predictive value (78). CRC is one of the most interesting fields of NGS application (79). According to ESMO recommendations, NGS could use in clinical practice to study mCRC (41).

Specifically, all DNA-based NGS are characterized by three basic steps. *1) Preparation of the sequencing library*. NGS can be performed on nucleic acid isolated from any source, however the most widely used template in clinical practice is formalin-fixed paraffin-embedded (FFPE) tissue (78). The DNA sample to be analyzed is prepared by a random fragmentation process. To the DNA fragments predefined sequences (known as "adaptors") are added to anchor and immobilize the fragments to the substrate on which sequencing will subsequently take place. 2) *Amplification*. DNA fragments subjected to an amplification by PCR. 3) *Sequencing*. It is carried out through several rounds of sequencing that consist of grappling the immobilized DNA with a solution containing a nucleotide (which, if

complementary to the sequence, is incorporated), a subsequent washing and finally the evaluation of the event (80).

Targeted Next-Generation Sequencing by Archer®VariantPlex®Solid Tumor

The NGS analysis subsequently explained was performed on the 29 primary tumors, 2 matched local metastases, 9 matched distant metastasis and 2 cases of colorectal high-grade tubulo-villous adenoma (HG TVA) with squamoid morules.

Two experienced pathologists (M.F. and P.P.) carefully marked a representative area for every tumor sample, to ensure that each area contained >50% of neoplastic cells. Five consecutive 10-μm-thick sections from each FFPE sample were obtained. The previously marked areas were manually (i.e., scalpel blade-assisted) micro dissected from adjacent tissue.

The QIAmp FFPE tissue Kit (Qiagen) was used to isolate DNA from the dissected material, according to the manufacturer's instructions. The concentration and the purity of DNA sample were evaluated by Qubit® 3.0 fluorometer and the Qubit® DNA BR Assay kit (Thermo Fisher Scientific).

The Archer® VariantPlex® Solid Tumor Panel is based on a targeted enrichment method called anchored multiplex PCR (AMP). The panel allows the detection of single nucleotide variant (SNV) of 63 target genes, and the analysis of copy number variation (CNV) for 44 genes, frequently associated with cancer.
Only samples with good DNA quality, assessed using the Archer PreSeq DNA QC assay, have been used to create the libraries, accordingly to the manufacturer's instructions (ArcherDX). For each patient, 50-200 ng of total DNA have been fragmented and amplified using specific primers provided by the manufacturer. Libraries have been quantified using the KAPA library quantification kit (Roche) and pooled to equimolar concentration.

The NGS was performed on a NextSeq-550 Platform (Illumina) and results have been analyzed using the Archer® Analysis v6.0 software.

SNVs were considered pathogenic based on previous interpretations of exonuclease domains (ClinVar). However, for variants of uncertain significance, VarSome was used to determine potential pathogenicity(81). Only pathogenetic and likely pathogenetic variants are being discussed

3.5 Statistical analysis

Descriptive statistics were used to summarize patient characteristics and demographics, specifics of the tumor disease and outcomes. Continuous covariates are summarized as mean and respective range, categorical covariates as absolute and percentage frequencies.

Chapter 4

Results

4.1 Clinico-pathologic findings

Median patient age was 70 years (range 42-94) and male to female ratio was 2/1 (20 males, 9 females). Most patients presented with occlusion/sub-occlusion symptoms (17 cases, 58.62%) or rectal bleeding (6 cases, 20.69%). Other symptoms include anemia, constipation, and clinical deterioration. Two patients presented with multiple hepatic metastases. One patient was diagnosed during follow up for multiple adenomas while one patient was identified during colorectal cancer screening.

As for the tumor location, 13 (44.83%) tumors were located on right-sided colon while 16 (55.17%) tumors were located on left-sided colon. Twenty-six out of 29 cases were surgical resections, 2 were endoscopic biopsies and 1 was an endoscopic polypectomy. Of the 26 surgical resections, 12 patients underwent right hemicolectomy, 1 an anterior resection and 6 patients underwent left hemicolectomy; 4 cases underwent abdomino-perineal resections according to Miles and one patient underwent a low anterior resection - three of these patients were treated with neoadjuvant chemoradiotherapy. Two cases underwent total colectomy. Of the 3 non-surgical cases, 1 case was an endoscopic polypectomy with synchronous hepatic metastases diagnosed at liver biopsy and 2 cases were endoscopic biopsies, one of which with synchronous liver metastases diagnosed at liver biopsy.

At onset, 10 patients (34.48%) presented with distant metastases (stage IV disease), 4 of which were histologically proven by liver biopsy (2 patients), skin biopsy (1 patient) and peritoneal/omental sampling (1 patient). The remaining patients were staged as follows: 10 patients in stage III (34.48%) and 8 patients in stage II (27.59%). For 1 patient, stage was not available as the patient was treated elsewhere and only the diagnostic biopsy was available for study. Three patients

had associated adenomas while 2 had synchronous colorectal conventional type ACs.

At endoscopy (in biopsy samples) and at gross description (in surgical specimens), tumors presented as polypoid-mass forming (14 cases, 48.28%) and ulcerating tumors (14 cases, 48.28%), sometimes with substenotic features. Mean tumor size was 6 cm (ranging from 0.6 cm to a maximum of 11 cm). Interestingly, the only case presenting as a 0.6 cm sessile polyp (case 21P) was associated with multiple synchronous, histologically documented, hepatic metastases.

Follow up was available for 25 patients (three were lost to follow up and one patient died following surgery); median follow up was 16 months (range 1 to 168 months). Sixteen patients (64.00%) died of their disease with median survival of 10 months (range 1 to 72 months); 8 patients were alive at last follow up, one with disease.

4.2 Histologic findings

ASC showed a ‘combined’ pattern with glandular components intimately admixed with squamous areas, in all cases of colorectal primary. Mean percentage of squamous component in ASCs was 38% (range 20%-80%). Five cases also showed tertiary features with variable extension (but <30% of the whole area) including mucinous areas (cases 22P and 25P), undifferentiated component (in case 7P) and neuroendocrine features (case 20P and 3P).

Out of 26 neoplasms which did not undergo neoadjuvant treatment, 17 cases (65.38%) were high grade by WHO 2019 classification (23) and 9 (34.62%) were low grade. The squamous component was poorly differentiated (G3) in 15 cases (51.72%). The three cases which had been treated with neoadjuvant chemoradiation had minimal regression (case 20P, case 10P, and case 18P).

An infiltrative growth pattern was more frequently seen compared to an expansile growth pattern (65.48% versus 24.46%). Vascular (blood vessel and/or lymphatic) invasion was present in 27/29 (93.10%) cases while peri-intraneural invasion was seen in 14 cases (48.28%). One tumor showed a dense peri and intra-tumoral lymphocytic infiltrate, both in the primary tumor and at metastatic sites including liver and skin metastases (case 5P).

Nodal metastases: Of the 26 surgically resected cases, 15 (57.69%) showed loco-regional node metastases. Median nodal count was 16.5 (range between 0 and 34); 10 patients presented LNR $\geq$ 0.2 which is known to be a negative prognostic factor (32). Out of the 15 patients with nodal metastases, 9 cases presented metastases with both glandular and squamous morphology, 1 case had only the squamous component and 4 cases showed only the glandular component in their metastases (one case slides of nodal metastases were not available for the study).

Distant metastases: Four patients with biopsy proven synchronous metastases (cases 3, 5, 10, 21) showed both glandular and squamous components; metachronous metastases to the skin (cases 5; 9) and to the liver (case 5) showed glandular/squamous component. Omental metachronous metastases (case 9, 11) showed an isolated glandular component.

Case	Age at diagnosis	Gender	Site	Specimen	Clinical features	Size (cm)	Stage	T	N	M	Follow up (months)
1	90	F	R	RH	subocclusion	2.5	III	pT3	N2a	M0	lost to follow up
2	49	M	R	RH	subocclusion	9	II	pT3	N0	M0	AFD (168)
3	76	M	R	RH	multiple liver mets	8	IV	pT4a	N2a	pM1c (peritoneal, biopsy)	post operative death
4	78	F	L	LH	subocclusion	11	III	pT3	N1a	M0	AFD (57)
5	70	M	L	LH	subocclusion	6	IV	pT4a	N2a	pM1a (skin, biopsy)	DOD (24)
6	77	M	R	RH	subocclusion	10	III	pT4b	N1b	M0	lost to follow up
7	94	F	L	LH	subocclusion	10	IV	pT4a	N2a	cM1a (liver, imaging)	DOD (6)
8	78	M	L	LH	subocclusion, rectal bleeding	3.5	III	pT2	N1b	M0	DOD (45)
9	76	M	R	RH	subocclusion	6	III	pT4a	N2b	M0	DOD (12)
10	58	F	L	Endoscopic biopsy	multiple liver mets	6	IV	pTx	Nx	pM1a (liver, biopsy)	DOD (5)
11	87	M	L	LH	occlusion, perforation	5	IV	pT4a	N1a	cM1c (peritoneal biopsy; liver imaging)	DOD (2)
12	76	M	R	RH	subocclusion	4	II	pT4a	N0	M0	AFD (42)
13	73	M	L	Total colectomy	rectal bleeding	4	III	pT3	N2b	M0	DOD (1)
14	69	F	R	RH	screening	1.8	II	pT3	N0	M0	AFD (22)
15	59	M	L	Endoscopic biopsy	rectal bleeding	6	na	pTx	Nx	M0	lost to follow up
16	77	F	R	RH	subocclusion	8.5	III	pT3	N1c	M0	DOD (8)

17	69	M	L	Miles	subocclusion	4	II	pT3	N0	M0	DOD (72)
18	65	M	L	Miles*	anemia	4	IV	(y)pT4b	N1a	cM1a (liver, imaging)	DOD (21)
19	69	M	L	AR	occlusion, acute abdomen	4.5	II	pT3	N0	M0	DOD (65)
20	61	M	L	Miles*	rectal bleeding	4	III	(y)pT4a	N2a	M0	DOD (5)
21	59	M	L	polypectomy	multiple adenoma	0.6	IV	pT1	Nx	pM1a (liver, biopsy)	AWD (12)
22	42	M	R	RH	subocclusion	5	IV	pT4b	N2a	cM1a (liver, imaging)	DOD (6)
23	70	F	L	LAR*	rectal bleeding	6	II	(y)pT3	N0	M0	DOD (72)
24	61	M	R	RH	rectal bleeding	6	IV	p(m)T3	N0	cM1a (liver, imaging)	DOD (11)
25	85	M	L	LH	anemia	2.8	II	pT3	N0	M0	AFD (24)
26	44	M	R	RH	weight loss	6	III	pT3	N1c	M0	AFD (12)
27	73	M	L	Miles	subocclusion	4	IV	pT3	N2a	cM1a (liver, imaging)	DOD (9)
28	67	F	R	RH	subocclusion	9	III	pT4a	N1a	M0	AFD (16)
29	92	F	R	Total colectomy	occlusion	7	II	pT4a	N0	M0	AFD (24)

F – female; M – male; R-firght; L-left; LH – left hemicolectomy; RH – right hemicolectomy; * - treated with neoadjuvant chemoradiotherapy; LAR – low anterior resection; AR – anterior resection; p – pathological stage; c – clinical stage; y – neoadjuvant treatment; DOD – died of disease; AWD – alive with disease; AFD – alive free of disease.

Table I: Clinico-pathologic characteristics of colorectal adenosquamous carcinomas.

4.3 Immunohistochemical findings

All cases of primary tumor and available metastatic deposits were immunostained for MMR protein, except for one case of needle biopsy from a liver metastasis (due to insufficient tissue - case 23P).

About MMR profile, 27 cases showed proficient MMR expression (primary and metastases); only one case (case 7P) showed loss of MLH1 and PMS2, in both the primary and in nodal metastatic deposits.

4.4 Genomic profiling

Due to the low DNA yeald or low DNA quality, seven cases of primary ASCs (case 24-29) and four cases of metastasis (synchronous metastases of cases 3, 5, 10, 21) were discarded from the analysis .

A comprehensive and summarizing representation of all detected alterations (single nucleotide variants [SNVs] and copy number variations [CNVs]) of the 22 primary colorectal ASCs and the two HG TVA with squamoid morules is provided in *Figure4*.

Across all the samples analyzed, a total of 22 out of 63 (34.92%) cancer-related genes were found to harbor SNVs, either missense, frameshift, stop gained (nonsense) or splice variants. In 20 of 22 (90.91%) primary ASC samples and in one of the two (50%) HG TVAs with squamoid morules at least one SNV was detected.

Across all the samples analyzed, a total of 11 out of 44 (25.00%) cancer-related genes were found to harbor CNVs. In 4 of 22 (18.18%) primary colorectal ASC samples and in one of the two (50%) HG TVA with squamoid morules at least one CNV was detected.

Among the primary ASCs and TVAs, the median number of genetic alterations (SNVs and CNVs) per sample was 2 and ranged from 0 to 9, with 9 of 24 (37.50%) samples harboring four or more alterations.

TP53 SNVs were the most frequent genetic alterations observed in the series, occurring in 13 of 22 (59.09%) of primary ASC samples. In three cases the *TP53* gene harbored a double SNV (shown as “multi hits” in the *Figure 3*). The

most common variants were p.Arg248Trp and p.His179Leu, with four and three cases respectively.

APC SNVs were observed in 9 of 22 (40.91%) of primary ASC samples. In two cases the *APC* gene harbored a double SNV (shown as "multi hits" in the Figure). The most common variants were p.Thr1556AsnfsTer3 (two cases) and p.Trp699Ter (two cases).

KRAS SNVs were observed in 8 of 22 (36.36%) of primary ASC samples. The most common variant was p.Gly12Asp (three cases).

BRAF SNVs were observed in 3 of 22 (13.64%) of primary ASC samples and in in none of HG TVAs. The only variants detected was p.Val600Glu.

Among primary ASCs, SNVs in other genes were detected at lower frequencies: *GNAS* (2/22, 9.09%), *CDH1* (2/22, 9.09%), *NRAS* (2/22, 9.09%), *VHL* (2/22, 9.09%), *PTEN* (1/22, 4.55%), *PIK3CA* (1/22, 4.55%), *CDKN2A* (1/22, 4.55%), *ATM* (1/22, 4.55%), *ERBB4* (1/22, 4.55%), *CTNNB1*(1/22, 4.55%), *FBXW7* (1/22, 4.55%), *SMAD4* (1/22, 4.55%), *DDR2* (1/22, 4.55%), *FOXL2* (1/22, 4.55%), *MET* (1/22, 4.55%), *ROS1* (1/22, 4.55%), KIT (1/22, 4.55%), PIK3R1 (1/22, 4.55%).

Among primary ASCs, the following CNVs were observed: *ERBB2* loss (2/22, 9.09%), *KRAS* partial loss (1/22, 4.55%), *PIK3CA* loss (1/22, 4.55%), *CDKN2A* gain (1/22, 4.55%), *ATM* partial loss (1/22, 4.55%), *ERBB4* loss (1/22, 4.55%), *SMAD4* partial loss, *NOTCH1* gain (1/22, 4.55%), *RB1* loss (1/22, 4.55%), *STK11* gain (1/22, 4.55%).

The seven local or distant metastatic lesions analyzed were distributed as follows: one metachronous cutaneous and one metachronous liver metastasis matched with primary 5P; one synchronous lymph node metastasis and two metachronous metastases (cutaneous and omental) matched with primary 9P; one synchronous omental metastasis matched with primary 11P; one synchronous lymph node metastasis matched with primary 20P.

In case 5, the primary harbors the following alterations: *KRAS, CTNNB1* and *GNAS* mutations and *ERBB2* loss; while both the metastases' harbors only *KRAS, CTNNB1* and *GNAS* mutations. In case 9P the lymph node metastasis and cutaneous metastasis show the same genomic alterations of the primary, while the omental metachronous metastasis shows an additional splice variant of *SMAD4*. In case 11 the primary harbors the following alterations: *TP53, APC*, *VHL and KIT* SNVs, while the synchronous omental metastasis harbors *TP53, APC* and *PTEN*

SNVs. In case 20 the metastasis harbors an additional *MYC* gain. The genomic alterations evidenced by the primary tumor and related metastasis are shown in

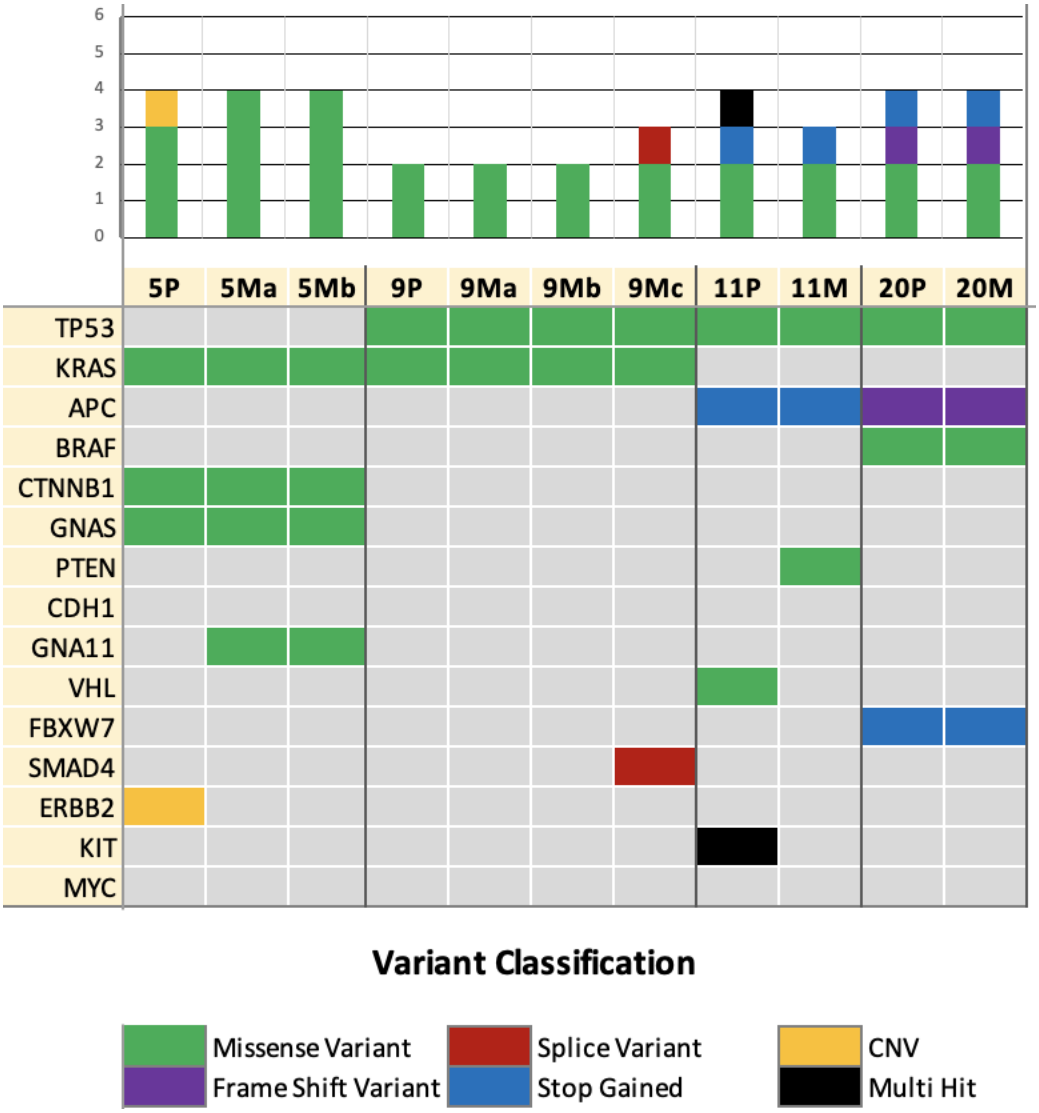
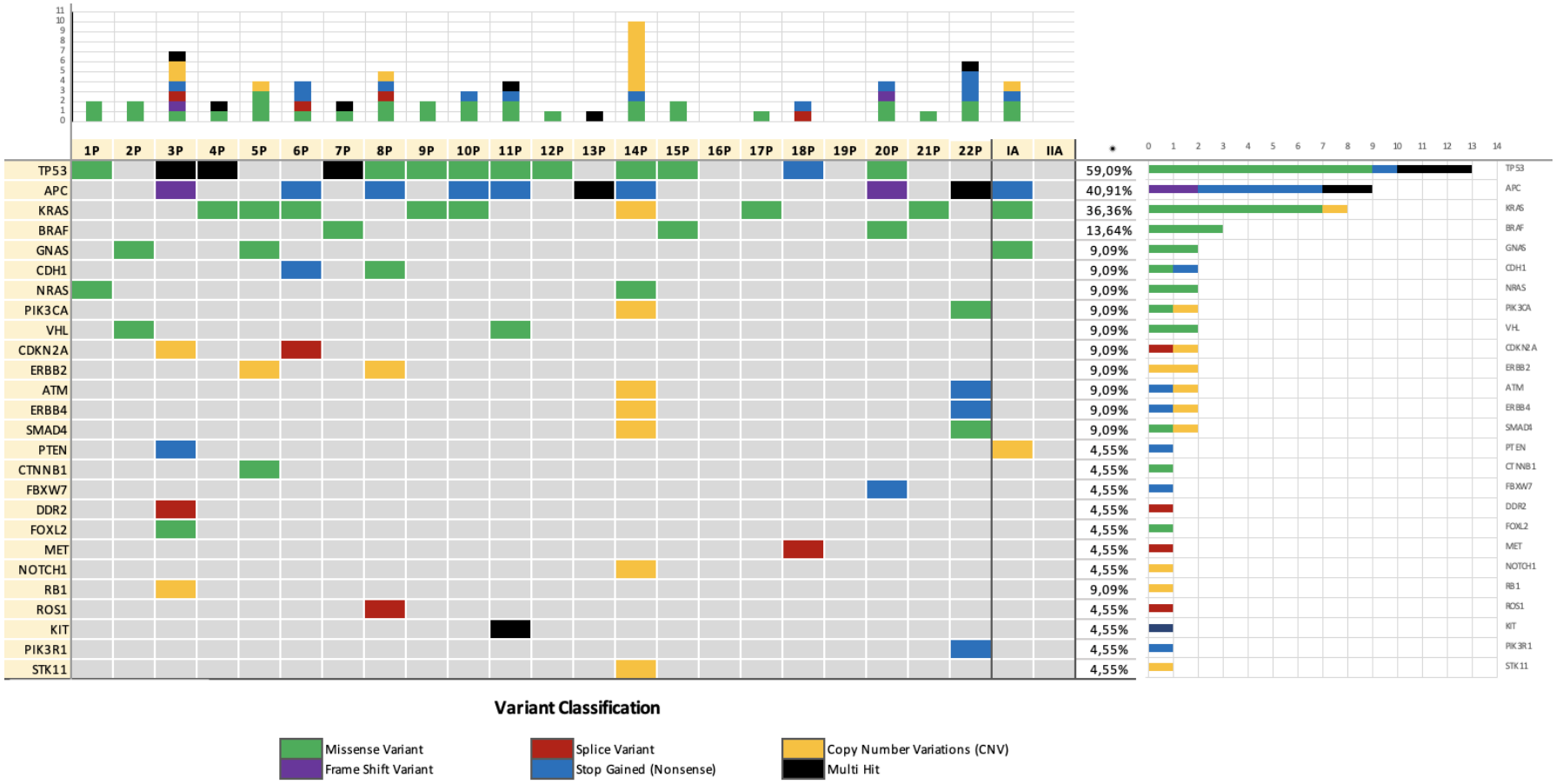


Figure 3.

P – Primary tumor; M - Metastases

Figure 3: Oncoplot depicting genomic alterations in primary tumors and related metastases.

As regards HG TVAs with squamoid morules, one of the two lesions analyzed harbors no alterations (either SNVs or CNVs). While the other harbors the following genomic alterations: *KRAS*, *APC* and *GNAS* SNVs and *PTEN* loss.



P – Primary tumor

Figure 4: Oncoplot depicting genomic alterations in primary tumor ordered by decreasing frequency

* The percentages and the graph on the side refer only to adenosquamous carcinomas, not to adenomas.

Chapter 5

Discussion and conclusions

Colorectal ASC is a rare histotype. While a few case series are present in the literature describing morphology and prognosis in these cancers, no study has yet investigated their specific morphology, immunoprofile and molecular feature. We are the first to provide a mutational analysis of colorectal ASC using an extended set of cancer-related genes panel.

Earlier studies indicated that colorectal ASC typically arises in the right colon (56.3%), similarly to Conventional Adenocarcinoma (CA) of the colon and rectum (26). By contrast, our results show that ASC could be frequently found in left-sided colon (55.17%). From previous studies, no difference has been documented that the proportion of males and females in ASC and CA but in our study a higher ratio was found in ASC (2.2:1 in ASC vs 1,4:1 in CA). Most studies have reported the occurrence of ASC in patients' sixth and seventh decade of life, similar to CA (26,82). In accordance with this statement, our study reported a mean age of 70 years old.

The clinical and morphologic revision of our cases documented a tumor with aggressive clinical characteristics and an aggressive pathologic phenotype. A more advanced TNM stage at the time of diagnosis (34.43% of cases per stage IV at diagnosis), compared with CA, was confirmed by our study, in line with previous investigations (26,83). A predominance of cases with poorly differentiated (G3) glandular component (65.38%) were seen. Moreover, a poorly differentiated (G3) squamous component was documented in about half of ASC (51.72%) (2 of which were admixed with a low-grade glandular component).

In summary, more than three quarters of ASC (76.92%) showed a poorly differentiated component, squamous or glandular or both. Furthermore, nearly to all cases (93.10%) showed vascular invasion and an infiltrative growth pattern was identified in about two-thirds of cases (65.38%), including the only case with low grade glandular and squamous component without vascular invasion.

In conclusion, all 29 cases had at least one aggressive morphologic prognostic factor: *high grade glandular* and/or *squamous component* and/or *vascular invasion* and/or *infiltrative growth pattern*. These morphologic findings are consistent with the aggressive behavior underlined by our case series; indeed 64.00% of patients succumbed to their disease, 10 patients within 1 year from onset and this finding is in keeping with other reports from the literature (26,29,83).

CRC patients with dMMR/microsatellite instable (MSI) tumors are more frequently identified at lower pathological stage, they are more often characterized by a prolonged overall survival in comparison to pMMR/microsatellite stable (MSS) cases. MSI is predictive of resistance to 5-Fluoro-Uracile therapy (making adjuvant therapy less indicated in this setting) and of response to immune checkpoint blockade (84). Only one case of colorectal ASC with dMMR/MSI profile has been described in the literature, in the context of germline mutation of MLH1, configuring Lynch syndrome-related ASC (85). In our series, all but one case had pMMR profile, in both the primary and metastases; only one case (case 7) showed a dMMR/MSI profile but was *BRAF* mutated, suggesting a sporadic origin.

In our series, *TP53* (59.09%) is the most mutated gene in colorectal ASC, followed by *APC* (40.91%) and *KRAS* (36.36%). Several studies provided genomic data on conventional CRC and identified these three genes as the most frequently mutated in this cancer type (86). However, the reported mutation rates of *TP53, APC* and *KRAS* are variable, possibly due to the different platforms and assays used in previous studies and to the different ethnicities of the populations. According to The Cancer GenomeAtlas data, *APC* mutation (71%), followed by *TP53* (54%) and *KRAS* (42%) mutations are the most frequent in conventional CRC (87). By using targeted NGS, Lee and colleagues showed that *TP53* (67%) is the most mutated gene in CRC, followed by *APC* (60%) and *KRAS* (47%) (88). In a recent Chinese study, 32 CRC were investigated by whole exome sequencing (WES), revealing a mutation rate of 59.38% in *APC*, of 50% in *TP53* and 28.13% in *KRAS* (86).

TP53 is a stress-inducible transcription factor, which regulates many diverse downstream genes to exert regulative function in multiple signaling processes. *TP53* mutations correlate with the site (left) and biologic behavior of CRC, by contrast not significant prognostic value of *TP53* mutation was found. Russo et al. found that *TP53* mutated CRC occurs in distant colon and left tumor was associated with lymphatic invasion (89). Our data confirm these results: 61.54% of *TP53*

mutated ASC was a distal carcinoma; moreover, all but two (77.78%), for which it could not be defined, of left *TP53* mutated *ASC* presents lymphatic invasion (vs 60% of right site).

Activation of the Wnt pathway plays a central role in colorectal tumorigenesis and is often the result of truncating mutations of the *APC* gene, a negative regulator WNT signaling, which cannot facilitate the proteolysis of b-catenin. *APC* results mutated in approximately 70%, by contrast in our study *APC* is mutated in 40.91%. Suggesting the possibility that a lower percentage of tumors in the colon rectal ASC group than in the conventional CRC group follow a classical pathogenesis. However, in absence of *APC* mutations, mutations in the β-catenin gene (*CTNNB1*) can be responsible for the activation of the Wnt pathway. (89–91) In our results *CTNB1,* mutation rate is 4.55 %, with no significant difference from TCGA data (5% of CRCs) (87).

Because *KRAS* mutations are predictors of resistance to anti-EGFR antibodies (44), their prevalence in CRC has been extensively investigated and it is estimated to be approximately 40% (39), similarly to our results (36.36%). However, *KRAS* prognostic value remains controversial. While some studies showed no prognostic role of *KRAS* mutations, others demonstrated an association with a shorter disease-free survival (DSF) and overall survival (OS) and with liver metastases. (44).

^{V600E}*BRAF* mutation is an established negative prognostic marker and has also relevant therapeutic implications (90). In our series the prevalence of ^{V600E}*BRAF* mutations is higher than in conventional CRC (13.64% vs 8-15% (43)), possibly due to the enrichment of metastatic cases in our case series. Among CRCs, the rate of *BRAF* mutations is significantly higher in the metastatic setting (stage IV) rather than in stage II-III (15–20% vs 10–5%) (43).

Activating *GNAS* mutations are common in mucinous neoplasms such us intraductal papillary mucinous neoplasms (IPMNs) and low-grade appendiceal mucinous neoplasms (LAMNs) and several studies have showed that *GNAS* mutant CRCs often contain a mucinous component (91). With the limitation posed by the small sample size, our data suggest that *GNAS* mutation rate is higher in colorectal ASC than conventional CRC. Previous studies reported values raging from 0 to 3.1% among CRC and raging from 2.9% to 15% among TVAs (92)(93) . In our series, *GNAS* mutations were detected in 9.09% of colorectal ASCs was and in one

of two (50%) HG TVA with squamoid morules. Only two SNVs involving codon 201 of the *GNAS* gene were identified in our series (*i.e.* p.Arg201His and p.Arg201Cys*)*, which have been described in previous works as two most common *GNAS* activating mutations (92).

CDH1 is a tumor suppressor gene located on chromosome 16q22.1. The mutation of the *CDH1* gene and the loss of its related protein, E-cadherin, leads to an epithelial-mesenchymal transition (EMT) process, which in turn causes the loss of cell-cell adhesion and a series of events that can promote tumor occurrence (94). *CDH1* germline mutation is the hall mark of Hereditary Diffuse Gastric Cancer syndrome, which accounts for 1-3% of all gastric cancers (88). The of loss E-cadherin in CRC is a marker of poor prognosis and has been associated with tumor differentiation, invasion depth, lymph node metastasis and tumor stage (94).

In CRC the prevalence of *NRAS* mutations is 5–9% (95) similarly to that of our case series (9.09%). *NRAS* mutations are a late event tumor progression and drive resistance to anti-EGFR therapy in *KRAS* wild-type tumors. Kuhn et al. conclude that the NRAS mutation could be responsible for the inflammatory phenotype in CRC (95).

PI3K is one of the crucial kinases in the PI3K/AKT1/MTOR pathway, playing a role in the cellular growth, proliferation, and survival of multiple solid tumors. Approximately 15%–20% of colorectal cancers (CRCs) harbor activating mutations in *PIK3CA* (96). In comparison only two of our samples reported a *PIK3CA* alterations (one SNV and one CNV). A systematic review and meta-analysis that investigate the prognostic significance of *PIK3CA* mutations in CRC concluded that *PIK3CA* mutation has no established prognostic effects on CRC overall survival (OS) and progression-free survival (PFS) (97). Huang et al. found no significant association between mutations of *PIK3CA* and CRC metastasis (98).

As regards CNVs, interestingly, *ERBB4* loss and *ERBB2* loss are extremely rare in conventional CRC. *ERBB4* loss is most frequently found in invasive breast carcinoma and prostate adenocarcinoma, while *ERBB2* loss is found in ovarian high-grade serous carcinoma and invasive breast carcinoma. RB loss is an infrequent event in CRC, however it is a common molecular event in colorectal neuroendocrine carcinoma.

NOTCH1 amplifications are present in 0.08% of all colon carcinoma patients; *NOTCH1* alterations are thought to drive progression and metastatic seeding by TGF-beta signaling (99).

As expected, the mutational status of the driver genes (*TP53, APC, KRAS, BRAF*) was preserved between the primary tumor and metastasis. However, in all the four cases of primary ASCs with metastases, differences in SNVs and/or CNVs in other genes were identified, suggesting that the metastatic lesion originated from a subclone of the bulk of the primary tumor, which possibly developed further molecular alterations within the process of metastatic seeding and growth.

For what concerns the two HG-TVAs with squamoid morules, in one case four genetic alterations (three SNVs and one CNV) have identified, encompassing *APC* and *KRAS* driver mutations. This molecular profile is compatible with the CIN pathway of colorectal cancer tumorigenesis.

In conclusion, colorectal ASC is characterized by an aggressive clinical behavior and adverse histopathologic features. Despite being limited by the small sample size due to the rarity of this histotype, our study showed that the genomic profile of colorectal ASC is similar to that of conventional CRC, with an overlapping prevalence of driver mutations, such as *TP53*, *APC*, *KRAS* and *BRAF*. Notably, an enrichment of certain SNVs such as *GNAS* and *CDH1*, a lower prevalence of *PIK3CA* mutations and the presence of infrequent CNVs in conventional CRC were observed.

The optimize the therapeutic approaches, the collection of more pathologic data and a more in-depth knowledge of the molecular events which lead to ASC, may be important.

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