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# Molecular characterization of "sessile serrated" adenoma to carcinoma transition in six early colorectal cancers

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ABSTRACT

Colorectal cancer (CRC) is a heterogeneous group of diseases both from the morphological and molecular point of view. The sessile serrated adenoma/polyp (SSA/P) has been proposed as the precursor lesion of CRCs characterized by CpG island methylator phenotype (CIMP), DNA mismatch repair (MMR) system deficiency, and *BRAF* gene mutations. However, no study so far investigated the molecular landscape of "sessile serrated" adenoma to carcinoma transition in early CRCs. Six formalin-fixed paraffin-embedded CRCs developed within SSA/P were profiled for the immunohistochemical expression of MMR proteins (MLH1, MSH2, MSH6, PMS2, and Ep-CAM), p16, and β-catenin. DNA was extracted from the two components of each sample, after microdissection, and characterized for CIMP status and by applying a custom hotspot multigene mutational profiling of 164 hotspot regions of eleven CRC-associated genes (*AKT1, APC, BRAF, CTNNB1, KIT, KRAS, NRAS, PDGFRA, PIK3CA, PTEN*, and *TP53*). Five out of the six CRCs shared the same molecular profile (*i.e.* CIMP positive, MSI status, and *BRAF* mutation) with their SSA/P components. One out of five CRCs was also *APC* mutated, whereas another one showed an additional *TP53* mutation. The remaining case was CIMP negative and MMR proficient in both the components, harbored a *BRAF* mutation in the SSA/P counterpart, whereas the CRC one was *APC* and *TP53* mutated and showed p16 and β-catenin dysregulation. This study provides the molecular evidence that SSA/P, even without cytological dysplasia, is a precursor lesion of CRC and that conventional CRC might arise from mixed polyp.

## 1. Introduction

Colorectal cancer (CRC) ranks third in men and second in women as the most common cancer worldwide, with an overall estimated incidence of about 1,361,000 new cases per year and a mortality of about 694,000 patients per year [1]. Screening programs based on fecal occult blood testing (FOBT) followed by colonoscopy demonstrated to be effective in controlling CRC burden in the population [2]. The timely detection and subsequent endoscopic resection of early CRCs and premalignant lesions, allows to treat the disease when it is still confined or, otherwise, to properly set the follow up of the patients according to their own risk of cancer development. The latter is founded on the Fearon and Vogelstein paradigm which states that most CRCs derives from the morphological adenoma to carcinoma cascade [3], molecularly depicted by the occurrence of chromosomal instability and of the *APC - KRAS - TP53* mutation pathway [4–6]. However, it is now clear that, from both a morphological and molecular point of view, CRC is a heterogeneous group of tumors presumed to arise from distinct precursor lesions [5]. Indeed, up to 25% of sporadic CRCs seems to follow the so called serrated neoplasia pathway characterized by CpG island methylator phenotype (CIMP), DNA mismatch repair-deficiency (MMRd), and *BRAF* gene mutation [5–10]. Among the other colorectal

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preneoplastic lesions, the sessile serrated adenoma/polyp (SSA/P) has been proposed as the precursor lesion of this CRC molecular subtype [11–16]. SSA/P is histologically characterized by abnormal crypt architecture without needing of cytological dysplasia, and many pathologists did not sustain its neoplastic potential supporting the use of the term "polyp" instead of "adenoma" [11,17]. Providing indisputable evidence for the neoplastic potential of SSA/P has been not possible as yet due to the unsuccessful attempts to generate cellular and animal models for the study of the serrated neoplasia pathway [5,18–21]. Moreover, longitudinal and epidemiological studies are few and limited by bias [22–27]. Apart the demonstration of a common molecular landscape between series of SSA/P and of CRCs, most supporting data on the "sessile serrated" adenoma to carcinoma transition are derived from case reports describing the development of CRCs in the context of SSA/Ps [28,29], or from a retrospective study analyzing the morphological features of the CRC arising in SSA/P or in tubular adenoma [30]. However, no study, so far, have profiled the molecular alterations in different components within the same early lesions.

The aim of this study was to molecularly characterize the malignant and premalignant components of a series of adenocarcinomas infiltrating the submucosa (pT1) showing a morphological continuity with a SSA/P without cytological dysplasia, thus suggesting that the invasive cancer might be originated from the SSA/P.

## 2. Materials and methods

### 2.1. Case selection

A search was performed in the files of the Surgical Pathological Unit of the University of Padua to identify all the patients from the Padua CRC screening program with a diagnosis of pT1 CCR during the period 1st January 2009 to 31st December 2016. Overall, 105 patients with an endoscopically resected CRC were found and the relative original sections stained with hematoxylin and eosin and also the corresponding formalin-fixed and paraffin-embedded (FFPE) samples were retrieved from the archives. All the cases were reviewed by two pathologists (R.C. and M.L.M.) and 6 lesions, where the CRC was morphologically in continuity with a component comprising at least three crypts of ordinary SSA/P without cytological dysplasia, were selected for this study (Fig. 1). All the cases showing a component of conventional adenoma, SSA/P with cytological dysplasia, or traditional serrated adenoma were excluded to ensure to represent a homogenous group. None of the patients had a serrated polyposis syndrome. Clinicopathological features of the patients are reported in Table 1.

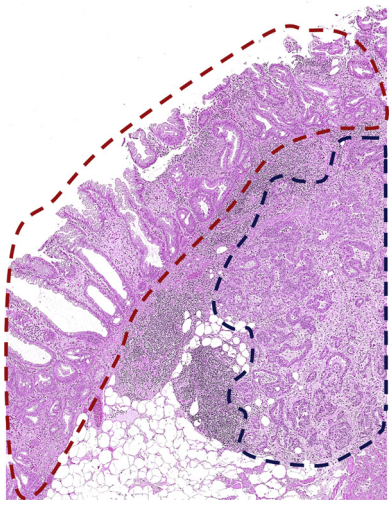

**Fig. 1.** Photomicrograph of one of the six lesions included in this study. On the left, the sessile serrated adenoma/polyp (SSA/P) component is characterized by crypt distortion with dilatation towards the base and L-shape features and by serrated epithelium without cytological dysplasia in more than three crypts. On the right, there is the colorectal cancer (CRC) infiltrating the submucosa. Dashed lines encase the two components that were separately microdissected for molecular analyses. Original magnification 200 × .

### 2.2. Immunohistochemistry

Immunohistochemistry was performed automatically by using the Bond Polymer Refine Detection kit (Leica Biosystems, Newcastle upon Tyne, UK) in the BOND-MAX system (Leica Biosystems) on 4 μm-thick consecutive sections from each FFPE sample with the primary antibodies listed in Table 2. Sections were counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently. All the immunostained slides were jointly assessed by two pathologists (R.C. and M.F.). Nuclear immunostaining for MLH1, MSH2, MSH6, and PMS2 and membranous immunostaining for Ep-CAM were evaluated following the criteria stated by Shia et al. to identify MMRd and mismatch repair-proficiency (MMRp) in both the SSA/P and CRC components of the lesions [31,32]. Both nuclear and cytoplasmic immunostaining for p16, and only nuclear immunostaining for β-catenin, were evaluated as present or absent in both the pre-invasive and invasive parts of the lesions.

### 2.3. Microdissection and DNA extraction

To avoid cross-contamination, a new sterile microtome blade was used to cut five consecutive sections of 10-μm of thickness from each FFPE sample. The sections were slightly stained with hematoxylin. The cells of the SSA/P component were manually microdissected from these sections under a light microscope by a pathologist (R.C.) using a new sterile needle for each sample and collected in si x 1.5 mL tubes. As for the CRC component, the same procedure was followed recovering the material in six different 1.5 mL tubes. DNA extraction was performed with the QIAmp DNA FFPE Tissue kit (Qiagen, Milan, Italy) and qualified as previously described by an investigator (G.M.) who, to avoid a possible source of bias, was unaware of the clinicopathological information of the samples [33].

### 2.4. CpG island methylator phenotype

DNA was treated with the EpiTect Bisulfite Kit (Qiagen) and subsequently analysed using the quantitative real-time PCR method EpiTect MethyLight (Qiagen) on a Rotor-Gene Q (Qiagen) to evaluate the methylation levels of a panel of genes including *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1,* in both the microdissected components of each of the six colorectal lesions (primers reported in Table 3) [34]. Universal methylated DNA served as a methylated reference, while an Alu-based normalization control reaction (*ALU-C4*) was used to measure the levels of input DNA. All reactions were performed in triplicate. An Alu representative calculated concentration greater than 1000 and a Ct value less than 23 were the validity requirements [35]. The CIMP status was considered positive when a percentage of methylation reference greater than 10 was present in at least three genes [34].

### 2.5. Mutational analysis

A multigene custom panel (Diatech Pharmacogenetics, Jesi, Italy; primers and protocol available upon request) was specifically designed to cover 164 hot-spot regions in the most commonly mutated genes in CRC, namely *AKT1*, *APC*, *BRAF*, *CTNNB1*, *KIT*, *KRAS*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN* and *TP53*. Mutational analysis of the DNA extracted from both the SSA/P and CRC components of each of the six lesions was performed using a MassARRAY Dx Analyzer 4 (Agena Bioscience, Hamburg, Germany). The identified somatic mutations were further confirmed by Sanger sequencing (Applied Biosystems 3130xl Genetic

**Table 1**
Clinicopathological characteristics of the six colorectal cancer patients.

| Case | Gender | Age (yr) | CCR familiarity | Lesion location* | Lesion size (cm) | Growth pattern | Concurrent colorectal lesions | Grading |
|---|---|---|---|---|---|---|---|---|
| **#1** | ♀ | 62 | No | Right colon | 0.9 | Polypoid, sessile | No | 2 |
| **#2** | ♀ | 68 | No | Right colon | 0.9 | Polypoid, sessile | No | 2 |
| **#3** | ♀ | 67 | Yes | Right colon | 1.5 | Polypoid, sessile | Yes (2 SSA/Ps) | 2 |
| **#4** | ♀ | 62 | No | Right colon | 1.5 | Non-polypoid, flat | Yes (1 conventional adenoma and 2 SSA/Ps) | 2 |
| **#5** | ♂ | 63 | No | Right colon | 1.5 | Polypoid, sessile | No | 3 |
| **#6** | ♂ | 66 | No | Right colon | 2.5 | Polypoid, sessile | Yes (1 conventional adenoma) | 2 |

CCR = colorectal cancer. * Location was divided into right colon (proximal to the liver flexure), transverse colon (from the liver to the splenic flexure), and left colon (from splenic flexure to the rectum).

**Table 2**
Primary antibodies used for immunohistochemical analysis.

| Antigen | Clone | Source | Company | Dilution |
|---|---|---|---|---|
| MLH1 | ES05 | Mouse | Agilent Technologies, Santa Clara, USA | 1:25 |
| MSH2 | FE11 | Mouse | Agilent Technologies, Santa Clara, USA | 1:25 |
| MSH6 | EP49 | Mouse | Agilent Technologies, Santa Clara, USA | 1:25 |
| PMS2 | EP51 | Mouse | Agilent Technologies, Santa Clara, USA | 1:20 |
| Ep-CAM | Ber-EP4 | Mouse | Merck KgaA, Darmstadt, Germany | 1:150 |
| p16 | E6H4 | Mouse | Roche Diagnostics, Mannheim, Germany | Prediluted |
| β-catenin | 17C2 | Mouse | Leica Biosystems, Newcastle upon Tyne, UK | 1:150 |

Analyser; ThermoFisher Scientific, Waltham, USA).

## 3. Results

### 3.1. Immunohistochemistry

Four CRCs retained the nuclear expression of MLH1 and PMS2, while two others showed loss of these markers (Fig. 2 and Table 4) and were hence considered as MMRd. One of these two lesions lacked MLH1 and PMS2 nuclear immunoreaction also in the SSA/P component. MSH2, MSH6, and Ep-CAM were normally expressed in all tested samples. Except for an adenocarcinoma component, p16 and nuclear β-catenin immunoreactions were always negative (Table 4).

### 3.2. CpG island methylator phenotype

In all cases except one, the CIMP status was considered positive in both the pre-invasive and invasive parts of the lesions (Table 4). The promoters of *CACNA1G*, *IGF2*, *NEUROG1*, and *RUNX3* were always methylated in the CIMP positive lesions. As for *SOCS1* promoter, it was methylated in two SSA/P and in four adenocarcinoma components (Table 4). None of the five markers was methylated in the CIMP negative lesion (Table 4).

### 3.3. Mutational profiling

In all instances the SSA/P component harbored the *BRAF* c.1799 T > A (p.V600E) mutation (Table 4). This mutation was present also in five out of six invasive components (Table 4). Two CRCs showed also an additional mutation, namely *TP53* c.743 G > A (p.R248Q/L) and *APC* c.4348C > T (p.R1450) (Table 4). The *BRAF* wild-type adenocarcinoma component was characterized by the *APC* c.4057 G > T (p.E1353*) and *TP53* c.741-742CC > TT (p.R248 W) mutations (Table 4).

## 4. Discussion

Sessile serrated pathway is thought to account approximately for 25% of all CRCs and the putative precursor lesion of this subtype of adenocarcinomas is SSA/P [5–11,17]. Despite the huge amount of literature data supporting this association, the premalignant nature of SSA/P is still debated. Some pathologists argue that architectural distortion *per se* is not enough to categorize a colorectal lesion as dysplastic, and that an association with cytological dysplasia is required. Our results clearly prove that the SSA/P component without cytological dysplasia in continuity with the CRC infiltrating the submucosa already harbors the driver molecular alterations that are present in the invasive counterpart. The detection of a mostly shared immunohistochemical and molecular profile in both the components strongly supports the morphological hypothesis that the adenocarcinoma is derived from the SSA/P. Moreover, the discovery of additional mutations in the malignant part of the lesion demonstrates its further evolution towards progressive accumulation of molecular alterations. As for the single case showing completely different mutations in the adenocarcinoma component from the SSA/P one, these are consistent with a classic colorectal adenoma to carcinoma cascade (i.e. *APC*, *KRAS*, and *TP53*) and suggest that the adenocarcinoma could be arisen from the conventional adenoma component of a mixed polyp. Moreover, the presence of a patchy p16 expression in this CRC could reflect an extreme effort of some cells to prevent uncontrolled proliferation, whereas the diffuse β-catenin nuclear immunoreaction could represent an activation of the WNT pathway [13].

**Table 3**
Primers utilized for qRT-PCR analysis.

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *CACNA1G* | 5'- TTTTTTCGTTTCGCGTTTAGGT -3' | 5'- CTCGAAACGACTTCGCCG -3' |
| *IGF2* | 5'- GAGCGGTTTCGGTGTCGTTA -3' | 5'- CCAACTCGATTTAAACCGACG -3' |
| *NEUROG1* | 5'- CGTGTAGCGTTCGGGTATTTGTA -3' | 5'- CGATAATTACGAACACACTCCGAAT -3' |
| *RUNX3* | 5'- CGTTCGATGGTGGACGTGT -3' | 5' – GACGAACAACGTCTTATTACAACGC – 3' |
| *SOCS1* | 5'- GCGTCGAGTTCGTGGGTATTT -3' | 5' - CCGAAACCATCTTCACGCTAA – 3' |
| *ALU-C4* | 5'- GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA -3' | 5' ATTAACTAAACTAATCTTAAACTCCTAACCTCA – 3' |

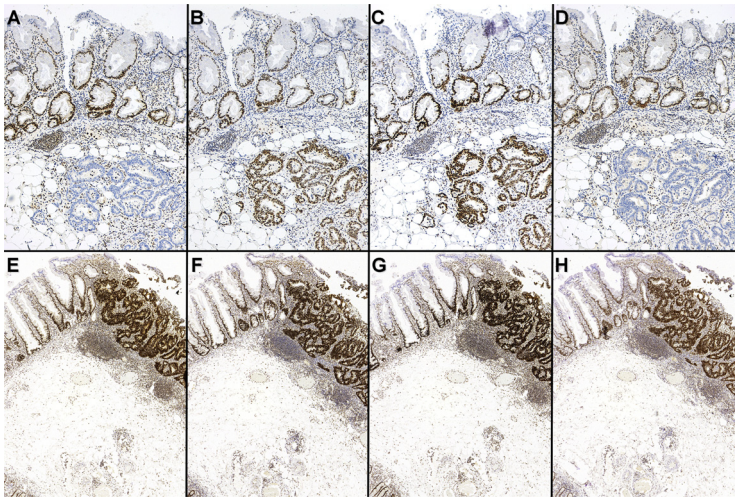


**Fig. 2.** Photomicrographs of mismatch repair deficient (A-D) and proficient (E-H) adenocarcinomas derived from sessile serrated adenomas/polyps. The expression of MLH1, PMS2, MSH2, and MSH6 is conserved in the sessile serrated adenoma/polyp components, while in the adenocarcinoma part of the mismatch repair deficient lesion MLH1 (A) and PMS2 (D) immunostaining is absent. Original magnification 100x (A-D) and 40x (E-H).

Our findings demonstrate that not all the molecular alterations characterizing the sessile serrated pathway (i.e. CIMP, MMRd, and *BRAF* mutation) are required for the development of a SSA/P and for its progression to an invasive adenocarcinoma. Indeed, four of the six lesions were MMRp and another one showed loss of MLH1 and PMS2 expression only in the invasive component. The only alteration constantly detected in the SSA/P was *BRAF* mutation, suggesting its role as a driver for this phenotypic carcinogenetic process. This is in line with the high frequency of *BRAF* mutation in SSA/Ps and SSA/P-derived CRCs reported in the literature [12,14,36–38]. The second step in this serrated adenoma to carcinoma transition is probably the acquisition of CIMP positivity, since this alteration was present in all the CRC showing a sessile serrated pathway profile [34,39,40]. The addition of MMRd or of mutations in other genes determines the further progression of the malignancy [34].

From the prognostic point of view, CRC following the sessile serrated pathway has a more aggressive behavior than conventional CRC and this is depicted by a shorter survival, by an earlier malignant transformation - a conspicuous number of adenocarcinomas develop in polyps smaller than 1 cm - and by a higher occurrence of adverse histological features such as tumor budding, lymphovascular invasion, perineural invasion, and nodal metastasis [30,41–44]. Of note, two out of the six CRCs in this series had a diameter lesser than 1 cm, whereas the mean tumor size was 1.47 cm A possible explanation of the rapid tumor progression of SSA/P-derived CRC is that *BRAF* mutation (present since the inception) is associated with the acquisition of motility and invasiveness by the neoplastic cells through the epithelial to mesenchymal transition process, a phenomenon noticeable at histological level as tumor budding [45–49]. This morphological feature is reported with higher frequency even in early stages in *BRAF* mutated CRCs and is the first step in the metastatic progression via the lymphatic route [45–49].

These data suggest the opportunity to develop specific strategies for the prevention and the treatment of this subtype of CRC. For what concerns the CRC screening setting, the adequate follow up schedule for patients with SSA/P has not yet been established. For example, the European Society of Gastrointestinal Endoscopy only recommends to report the presence of cytological dysplasia in SSA/P, without providing any advice on the surveillance to be implemented [50]. Due to its premalignant nature, SSA/P should no longer be managed as a hyperplastic polyp, as it still happens in some countries, but probably it should be controlled at least as a conventional adenoma [51]. According with this point of view, the British Society of Gastroenterology took a cautious position by advising to perform a colonoscopy at 3 years in patients with SSA/P without cytological dysplasia equal to or greater than 1 cm or with SSA/P with cytological dysplasia (regardless of size) [52]. This is the same time interval set for conventional adenoma follow up. And it might be not adequate for SSA/P with cytological dysplasia. Indeed, it might require a closer follow up because of the reported higher rate of malignant transformation compared with conventional adenoma and the over-representation of CRC with serrated pathway among interval cancers [27,53,54]. Anyway, the frequency of CRC arising in SSA/P seems to be less than 1%, thus the cost-benefit ratio between the frequency of endoscopic follow up and the reduction of CRC burden in the population should be considered to guarantee the sustainability of the screening program [55,56]. Another pending issue is the definition of the risk of CRC development in patients with multiple synchronous or metachronous SSA/P [24]. All these points need to

**Table 4**
Immunohistochemical and molecular characterization of the sessile serrated adenoma/polyp and adenocarcinoma components of the six lesions.

| Case | CIMP | | MMR | | Mutations | | p16 | | β-catenin | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *SSA/P* | *CRC* | *SSA/P* | *CRC* | *SSA/P* | *CRC* | *SSA/P* | *CRC* | *SSA/P* | *CRC* |
| **#1** | Pos | Pos | MMRd | MMRd | $BRAF^{V600E}$ | $BRAF^{V600E}$ | Neg | Neg | Neg | Neg |
| **#2** | Pos | Pos | MMRp | MMRd | $BRAF^{V600E}$ | $BRAF^{V600E}$ | Neg | Neg | Neg | Neg |
| **#3** | Pos | Pos | MMRp | MMRp | $BRAF^{V600E}$ | $BRAF^{V600E}$ $TP53^{R248Q/L}$ | Neg | Neg | Neg | Neg |
| **#4** | Pos | Pos | MMRp | MMRp | $BRAF^{V600E}$ | $BRAF^{V600E}$ | Neg | Neg | Neg | Neg |
| **#5** | Pos | Pos | MMRp | MMRp | $BRAF^{V600E}$ | $BRAF^{V600E}$ $APC^{R1450*}$ | Neg | Neg | Neg | Neg |
| **#6** | Neg | Neg | MMRp | MMRp | $BRAF^{V600E}$ | $APC^{E1353*}$ $TP53^{R248W}$ | Neg | Pos (patchy) | Neg | Pos |

CIMP = CpG island methylator phenotype; CRC = colorectal cancer; MMRd = mismatch repair-deficiency; MMRp = mismatch repair-proficiency; SSA/P = sessile serrated adenoma/polyp.

be addressed by specifically designed studies.

As for the therapy, targeting *BRAF*-mutated CRC with single inhibitor proved to be ineffective [57]. A possible explanation is that BRAF in CRC is involved in several signalling pathways thus conferring resistance to the treatment [58]. Probably, different anti-BRAF molecules or combinatorial approaches with other agents blocking other pathways will have better results [59,60].

The relatively small sample size of the present study may appear a limitation. However, it should be considered the overall rarity of this type of lesions, the strict histological criteria applied for their selection (combination of SSA/P and submucosal invasive CRC with exclusion of all cases with a component of conventional adenoma, SSA/P with cytological dysplasia, or traditional serrated adenoma), and that they were all collected from a single Institution among the endoscopically removed cases of the CRC screening program to assure the maximum homogeneity (all patients were asymptomatic, with FOBT positivity and without serrated polyposis syndrome and history of previous CRC). The major points of strength of this study are: i) the microdissection of the two components of each lesion was performed by a pathologist; ii) the investigator who performed the molecular analyses was unaware of the clinicopathological features of the samples; iii) all the molecular aspects of the serrated neoplasia pathway (i.e. CIMP, DNA mismatch repair system, and *BRAF* gene) were analyzed with appropriate techniques; iv) a wide sensitive mutational analysis covering more than one hundred hot-spot regions in the eleven most commonly mutated genes in CRC was performed.

In conclusion, our findings further support the hypothesis that SSA/P, even without cytological dysplasia, is a premalignant lesion. Thus, it should be definitively accounted only as sessile serrated adenoma. Appropriate follow up schemes should be established for this type of lesion in the CRC screening programs.

## Authors' contributions

All the authors of this research paper participated directly in the execution of the study as well as in the analysis of the results.

## Potential competing interests0

The authors have no competing interests to declare.

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