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ORIGINAL ARTICLE

Prospective Study

Longitudinal molecular characterization of endoscopic specimens from colorectal lesions

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Abstract

AIM: To compare molecular profiles of proximal colon, distal colon and rectum in large adenomas, early and late carcinomas. To assess feasibility of testing directed at molecular markers from this study in routine clinical practice.

METHODS: A prospective 3-year study has resulted in the acquisition of samples from 159 large adenomas and 138 carcinomas along with associated clinical parameters including localization, grade and histological type for adenomas and localization and stage for carcinomas. A complex molecular phenotyping has been performed using multiplex ligation-dependent probe amplification technique for the evaluation of CpG-island methylator phenotype (CIMP), PCR fragment analysis for detection of microsatellite instability and denaturing capillary electrophoresis for sensitive detection of somatic mutations in *KRAS*, *BRAF*, *TP53* and *APC* genes.

RESULTS: Molecular types according to previously introduced Jass classification have been evaluated for large adenomas and early and late carcinomas. An increase in CIMP+ type, eventually accompanied with *KRAS* mutations, was notable between large adenomas and early carcinomas. As expected, the longitudinal observations revealed a correlation of the CIMP+/*BRAF*+ type with proximal location.

CONCLUSION: Prospective molecular classification of tissue specimens is feasible in routine endoscopy practice. Increased frequency of some molecular types corresponds to the developmental stages of colorectal tumors. As expected, a clear distinction is notable for tumors located in proximal colon supposedly arising from the serrated (methylation) pathway.

Key words: Colorectal cancer; CpG-island methylator phenotype; DNA; Microsatellite instability; *BRAF*

Core tip: The results indicate that molecular subtyping from endoscopic biopsies is feasible in routine gastroenterology practice to evaluate a patient's prognosis. Subtyping based on Jass classification can be used to evaluate molecular mechanisms of adenoma-carcinoma transition.

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INTRODUCTION

The variability in clinical manifestation of colorectal cancer as well as considerable differences in outcome between some colorectal cancer patients has prompted wide-ranging research into the molecular basis of the disease[1]. The main effort has been directed at mechanisms underlying initiation and progression of colorectal neoplasia from normal colonic mucosa as well as factors defining therapy response and the overall patient's survival[2-5].

There is historic evidence suggesting that more than two-thirds of colorectal cancers begin as colorectal adenomas[6]. The size of adenoma is considered a fundamental risk factor and is directly associated with histological characteristics such as the amount of villosity and dysplasia. Aberrant activation of (proto)oncogenes in key signaling pathways has long been a subject of study in colorectal cancer research. Among others, mutations in two major (proto)oncogenes, *KRAS* and *BRAF*, are frequently found in both carcinomas as well as in adenomas[7]. In 1990, *KRAS* mutations were contributed to the shorter overall survival of colorectal cancer patients[8]. The prognostic value was later restricted only to specific *KRAS* mutation types (Exon 1, codon 12, but not codon 13 mutations)[9]. Later it was discovered that mutations in *KRAS* as well as *NRAS* (both members of a common subgroup, RAS-family) are the major causes of therapy resistance in colorectal tumors treated by monoclonal antiEGFR inhibitors[10,11]. Accordingly, the current NCCA guidelines include recommendations for predictive RAS-testing as a standard of care for colorectal carcinomas[12].

Since 1990, three distinct molecular pathways underlying the malignant transformation of advanced adenomatous polyps into cancerous lesions have been studied[13]. The different pathways are based on independent genomic events leading to the loss of key cellular regulatory mechanisms causing proliferation, invasion and metastasis. The resulting molecular subtypes are denoted by either chromosomal instability (CIN), microsatellite instability (MSI) or CpG-island methylator phenotype (CIMP)[14,15]. The subtypes are typically characterized by disruptions on the DNA level including mutations and allelic losses of major tumor suppressors in CIN[16], mutations of mismatch DNA repair genes in MSI[17] (also referred to as the replication of positive phenotype, RER+) and aberrant methylation of promoter regions of tumor suppressors in CIMP[18]. Over the past decade, clinical associations of these subtypes have been intensively studied. The majority of colorectal carcinomas bear signs of the CIN subtype, most notably somatic mutations of *APC* and *TP53* tumor suppressors and associated losses of alleles at 5q and 17p chromosomal locations [observed as a loss of heterozygosity (LOH)][19]. The CIN type is closely following the fundamental genetic model of colorectal tumorigenesis[20]. While the individual mutations and allelic losses of *APC* and *TP53* tumor suppressors bear no direct prognostic value[21], the "CIN high" phenotype derived from a combination of several markers (mutations and LOH) indicates poor survival compared to the "CIN low" or MSI phenotypes[22].

The CIMP phenotype is on the molecular level notably distinct from the CIN and may also be complemented by MSI[23,24] as a result of *MLH1* promoter methylation[25]. There is sufficient evidence that evaluation of CIMP together with *BRAF* mutation and combined with a presence or absence of MSI gives a strong indication of a patient's survival prognosis. Tumors bearing the CIMP+/*BRAF*+ phenotype exhibit

Table 1 Patient characteristics

Adenomas	94
Gender	
Women	39
Aged	34-98 (median 67.7)
Men	55
Aged	40-89 (median 68.0)
Localization	
Proximal colon	37
Distal colon	42
Rectum	15
Histology	
Tubular	47
Tubulovilous	39
Vilous	4
Serrated	4
Dysplasia	
Low-grade	78
High-grade	16
Carcinomas	127
Gender	
Women	44
Aged	34-98 (median 70.2)
Men	83
Aged	42-90 (median 68.5)
Localization	
Proximal colon	50
Distal colon	38
Rectum	39
Stage	
Early (Ⅰ and Ⅱ)	66
Advanced (Ⅲ and Ⅳ)	61

shorter disease-free survival[26]. Typically arising from serrated lesions and more frequent in the proximal colon (caecum and ascendens) they are the result of a specific molecular process and exhibit a distinct biological behavior[27]. In turn, a concurrent presence of MSI dramatically improves the prognosis of patients with CIMP+/*BRAF*+ tumors[28] as the MSI unstable tumors are less likely to spread to lymph nodes and to develop distant metastases[29]. Aside from the prognostic importance, there is also an ongoing discussion on the importance of CIMP/MSI/*BRAF* phenotyping for prediction of response to chemotherapy treatment[30].

In early 2015, two retrospective studies published a relationship between specific molecular subtypes and the survival of colorectal cancer patients on large patient cohorts[31,32]. Utilizing the knowledge of the above described molecular pathways, the specific molecular types were evaluated based on MSI and CIMP phenotyping in combination with the mutation status of *KRAS* and *BRAF*, as previously suggested by Jass[33]. A significant difference in survival for the different molecular types was indeed confirmed by both studies aimed at patients in stages Ⅲ and Ⅳ, respectively. The five molecular subtypes, now universally referred to as Type Ⅰ-Ⅴ, and a group consisting of the rest, marked as Others, were also characterized by their most likely longitudinal localization and the prevailing gender and age of the patients. Based on the studies mentioned above[31-33], Type 1 is characterized by CIMP+, *BRAF*+, MSI, proximal localization and good prognosis; Type 2 by CIMP+, *BRAF*+, microsatellite stability (MSS) or MSI-Low (MSI-L), proximal localization and poor prognosis; Type 3 by CIMP-, *KRAS*+, MSS or MSI-L, proximal localization and poor prognosis; Type 4 by CIMP-, *KRAS*- and *BRAF*-, MSS or MSI-L, distal localization and median prognosis; Type 5 by CIMP-, *KRAS*- and *BRAF*-, MSI, proximal localization and good prognosis.

While the original Jass characterization gave a unique complex view on the alternative pathways of molecular carcinogenesis, it has, most importantly, now been verified to represent a viable tool in clinical management of the disease. It is, therefore, eminent to adapt appropriate procedures for methodology as well as logistics of testing procedures in current clinical practice. While most studies traditionally rely on molecular testing directed at FFPE sections from resected tissue, endoscopic biopsies as well as endoscopically removed malignant polyps are also more recently being routinely used[34].

Longitudinal clinicopathological heterogeneity of colorectal cancer has been reported as early as 2002[35]. Biological diversity stemming from embryonic origins may be responsible for different mechanisms of tumorigenesis in proximal and distal colon and rectum resulting in different manifestation, response to therapy and the overall prognosis[36]. In this work, we present data from molecular phenotyping and mutation analysis of tissue samples acquired during colonoscopy. We present molecular profiling of colorectal carcinomas as well as of their precursor lesions, large adenomatous polyps. We evaluate molecular profiles at proximal, distal and rectal tumor localizations and assess overall feasibility and clinical utility of such molecular classification in routine endoscopy practice.

MATERIALS AND METHODS

Study population

The prospective study design was reviewed and certified by the Scientific and Ethics boards of the Military University Hospital. All patients admitted into the study have signed an informed consent. Patients were treated at the endoscopy unit and consecutive samples were collected during a 2-year prospective study. Tissue samples were obtained either as endoscopic biopsies or by endoscopic polypectomy (EPE) or endoscopic mucosal resection (EMR). The inclusion criteria was based solely on primary morphology evaluations by the endoscopist. The large adenomas (AA) were assigned as being any size greater than 1 cm[6]. Stage Ⅰ and Ⅱ carcinomas were jointly assigned as early carcinomas (EC) and Stage Ⅲ and Ⅳ were assigned as late

Table 2 Overview of the molecular testing results *n* (%)

Marker	Localization	Advanced adenoma[1]	Early carcinoma[2]	Late carcinoma[3]
MSI	Proximal	0 (0)	5 (20)	7 (24.14)
	Distal	0 (0)	0 (0)	0 (0)
	Rectum	0 (0)	0 (0)	0 (0)
CIMP	Proximal	13 (30.95)	15 (65.22)	16 (59.25)
	Distal	7 (16.28)	10 (45.45)	6 (35.29)
	Rectum	2 (13.33)	11 (50.22)	8 (47.06)
BRAF	Proximal	7 (10.94)	4 (17.39)	7 (24.14)
	Distal	1 (1.49)	0 (0.00)	0 (0.00)
	Rectum	4 (17.39)	1 (4.20)	2 (11.11)
KRAS	Proximal	25 (35.71)	11 (44.00)	15 (51.72)
	Distal	28 (43.08)	11 (44.00)	4 (23.53)
	Rectum	12 (50.00)	10 (41.67)	7 (38.88)
APC	Proximal	24 (35.82)	5 (20.83)	5 (17.24)
	Distal	22 (34.38)	8 (34.78)	6 (37.5)
	Rectum	9 (37.50)	7 (29.17)	8 (44.44)
TP53	Proximal	5 (7.46)	8 (33.33)	8 (27.59)
	Distal	2 (3.13)	8 (34.78)	8 (50.00)
	Rectum	1 (4.17)	11 (45.83)	10 (55.55)

[1]> 1 cm; [2]Stage Ⅰ or Ⅱ; [3]Stage Ⅲ or Ⅳ.

carcinomas (LC). The description of patients from this study is listed in Table 1.

Tumor characteristics

In order to follow a prospective strategy of all evaluations, we have decided to use adenomatous polyp size beyond 10 mm as the only inclusion criteria that allows immediate decision about molecular testing during the endoscopy procedure. DNA from fresh biopsies or FFPE sections was extracted following a standard histopathology evaluation to ensure adequacy (viability, quantity, tumor cell fraction) for the testing. On FFPE sections, tumor-positive areas were clearly marked by a pathologist prior to microdissection. DNA was extracted from fresh and FFPE specimens using a standard spin-column procedure using a commercial kit (JETquick Tissue DNA spin, GENOMED G.m.b.H, Loehne, DE).

Microsatellite instability testing

Microsatellite instability was evaluated using MSI Analysis System, Version 1.2 (Promega corporation, Madison, WI, United States). The multiplex PCR kit produces fluorescently labelled amplicons of five nearly monomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two additional polymorphic markers (Penta C and Penta D) for specimen identification[37]. PCR amplicons were resolved on a 16-capillary sequencer (ABI PRISM 3100, Applied Biosystems, Foster City, CA, United States) according to the manufacturers protocol. The data was evaluated by GeneMarker software (Softgenetics, State College, PA). Only samples exhibiting unstable alleles at 2 or more markers were assigned as MSI, otherwise the assignment was MSI-L (1 marker instable) or MSS (no unstable markers detected).

CpG island methylator phenotype testing

The CIMP phenotype evaluation was based on multiplex ligation-dependent probe amplification technique (MLPA) utilizing a non-bisulfite conversion approach. A commercial MLPA kit was used (SALSA MLPA ME042 CIMP, MRC Holland, NL) and the MLPA data was evaluated by GeneMarker software using an appropriate MLPA CIMP panel (available for download from the Softgenetics website). The investigated genes were as suggested by Ogino[38]. A CIMP-high phenotype was assigned to a sample showing any of the MLPA probes methylated for at least 6 out of 8 evaluated genes (*RUNX3*, *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *SOCS1* and *CDKN2A*)[39].

KRAS, BRAF, APC and TP53 mutation testing

Somatic mutation testing in *KRAS, BRAF, APC* and *TP53* genes was performed by denaturing capillary electrophoresis (DCE) using a previously described protocol[40-43]. The technique is based on a principle of differential denaturation of wildtype and mutant alleles, similar to the high-resolution melting technique[44]. In brief, the target sequences harboring the mutation sites were amplified using GC-clamping at one of the primers and a fluorescence label at the other primer. The PCR amplification program was concluded by a heteroduplex formation step in which the product mixture was heated for 8 min at 95 ℃, then kept at 65 ℃ for 30 min and finally cooled at 0.1 ℃/s down to 15 ℃. Each amplicon was then subjected to capillary electrophoresis separation at optimized separating temperature leading to the resolution of homo- and hetero- duplex forms in case of a mutation presence. In order to speed up the screening process, amplicons with similar separating temperatures were analyzed in different capillaries during the same run. The target amplicons included exons 2, 3 and 4 of *KRAS* gene, the V600E mutation (exon 15) of *BRAF* gene[41], codon span 1250-1550 (mutation cluster region) of *APC* gene[42,45] and exons 5 to 8 of *TP53* gene[43]. According to the Catalog of somatic mutations in cancer (COSMIC) this testing panel should detect more than 88% of somatic mutations in the studied genes[46].

RESULTS

Over the 2-year duration of the project, a total of 6080 colonoscopies were performed yielding 297 tissue specimens. The set included 159 large AA, 74 EC and 64 LC (see Methods for details of the AA/EC/LC assignment).

The success rates for DNA extractions were 96.3% (104/108) for fresh tissue and 93.7% (177/189) for FFPE sections. The amounts of extracted DNA were typically between 500-1000 μL volumes of 5-10 ng/μL. A complete set of results consisting of MSI, CIMP, *BRAF*, *KRAS*, *APC* and *TP53* data was obtained for 246 out of 281 extracted DNA samples (87.6%). The

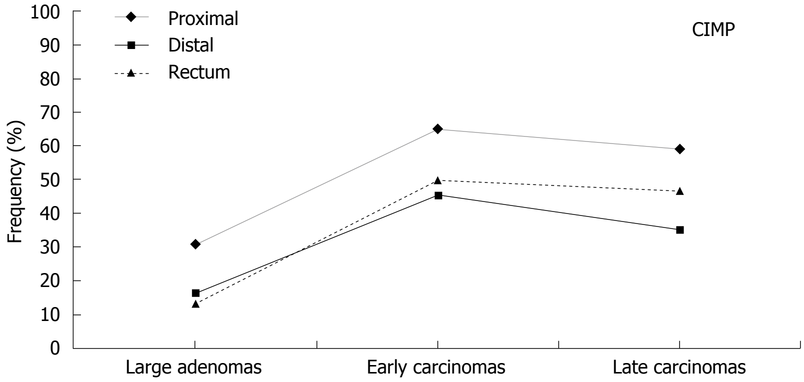

Figure 1 Longitudinal frequency of CpG-island methylator phenotype phenotype in different tumor types. CIMP: CpG-island methylator phenotype.

incomplete molecular profiles were largely due to failed CIMP examination in FFPE mainly as a results of low amounts or low quality of DNA. Results for individual markers obtained for each tumor subtype at proximal, distal and rectal localizations are listed in Table 2.

CIMP, BRAF and MSI

The distribution of CIMP+ phenotypes for the three evaluated tumor types along the proximal and distal colon and rectum is shown in Figure 1. In all three types the CIMP+ frequency in proximal colon is 15% higher than in distal colon or rectum. In all three sections there is a 2-3 fold jump in frequency between large adenomas and early carcinomas while only a relatively small change (< 10%) between early and late carcinomas.

The *BRAF* mutations were found in 12 of 154 large adenomas (7.8%), 5 of 74 (6.8%) early carcinomas and in 9 of 64 (14.1%) advanced carcinomas. A CIMP+/*BRAF*+ combination was mostly found in proximal colon with frequency gradually increasing with the tumor progression from 5.3% (2/38) in large adenomas to 13% (3/23) and 26% (7/27) in early and late carcinomas, respectively.

In agreement with previous reports MSI has only been found in early and late cancers, but not in adenomatous tissue[47]. In carcinomas, MSI was detected only in the proximal localization at 16.0% in early cancers (4/25) and 24.1% in late cancers (7/29). MSI was accompanied by CIMP+ phenotype in 81.2% (9/11) and 88.9% (8/9) of CIMP+ carcinoma had *MLH1* promoter methylation.

APC, KRAS and TP53

Mutations in *APC*, *KRAS* and *TP53* were observed in all tumor groups across proximal and distal colon as well as in the rectum. Similarly to a recently published study[25], we have found a higher frequency of *APC* and *KRAS* mutations in CIMP+ carcinomas with a presence of *MLH1* methylation when compared to CIMP+ without *MLH1* methylation. The difference was 20%; 2/10 *vs* 33.3%; 9/27 for *APC* (P = 0.74) and 21.4%; 3/14 *vs* 59.3%; 16/27 for *KRAS* (P = 0.031). Regardless of the tumor localization, *TP53* mutation rates showed a significant increase from large adenomas (5.1%; 8/155) to early and late carcinomas (36.5%; 27/74 for early and 41.3%; 26/63 for late, $P < 0.001$, $\chi^2 = 49.928$). Also in an agreement with previous findings[48] *TP53* mutations were detected more frequently in the group of CIMP- carcinomas compared to the CIMP+ carcinomas (39.6%; 38/96 *vs* 27.1%; 13/48), but the result was not statistically significant.

DISCUSSION

Principal contributions of various pre-analytical factors to the success of molecular genetic testing from FFPE sections have long been studied[49]. Among others, the principal importance of the quality of the formalin solution (buffered to neutral pH) and the duration of fixation has been recognized[50]. The negative effects of fixation are intensified for small volume samples, typically acquired by endoscopic biopsies. At the same time, upon extraction, the small biopsy specimens often yield low amounts of DNA limiting the extent of the molecular testing. For complex molecular profiling, such as the subtyping performed in this study, a prioritization of the individual tests, as already practiced in molecular testing of other cancer types[51], is clearly a necessity for future routine use.

Most cases of inconclusive results in this study were, indeed, due to the low DNA quality or amount. A dedicated mutation technology typically based on single-plex PCR usually requires only minute amounts of DNA. The MSI detection approach utilizing a multiplex PCR followed by capillary electrophoresis is also low to medium in the demand of DNA. On the other hand, CIMP evaluation by MLPA requires by far the highest amounts of input DNA. With a very limited availability of other reliable CIMP-detection techniques, this is clearly the limiting factor.

Assignment of Jass molecular subtypes

According to the original work of Jass[33] and the recent publications by Phipps *et al*[32] and Sinicrope

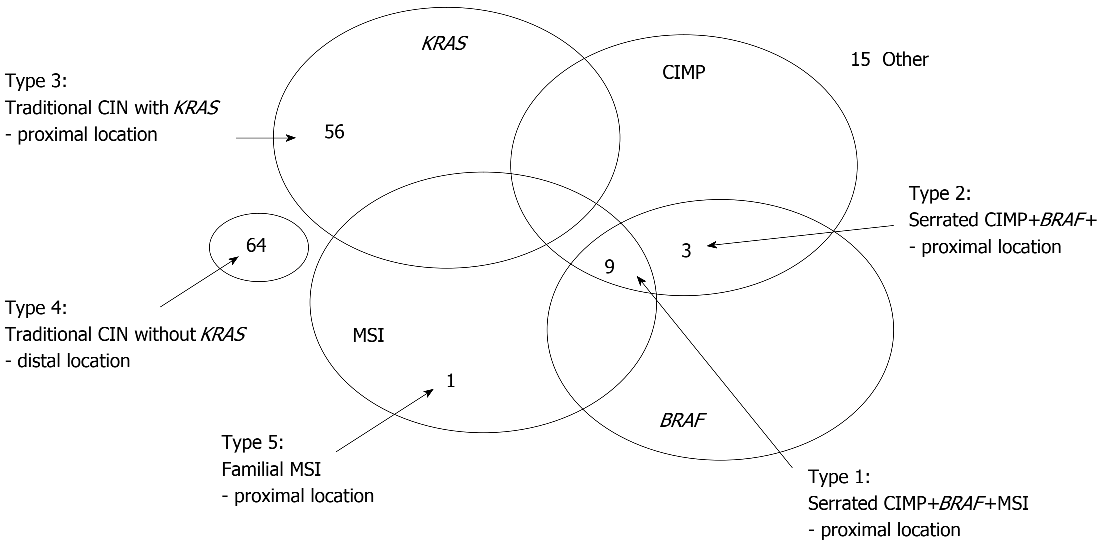

Figure 2 Molecular classification of colorectal carcinomas (all stages) using classification according to Jass and others[30-32]. MSI: Microsatellite instability; CIN: Chromosomal instability; CIMP: CpG-island methylator phenotype.

et al[31], we have applied their principles to our data to assign the molecular subtypes. The classification is based on a combined evaluation of CIMP/MSI/*BRAF*/*KRAS* testing. The resulting spectrum of molecular subtypes for carcinomas in our study is presented in Figure 2. Even with the smaller size of our prospective group, the relative distribution among the 6 different groups (Types 1-5 and Others) corresponds to the data presented in those large retrospective cohorts. The Type 4 and Type 3, both characteristic of the CIN pathway, were the most frequent at 43.2% and 37.8%, respectively, followed by Types 1 and 2, resulting from the CIMP-serrated pathway, at 6.0% and 2.0%, respectively.

The probability of developing future advanced adenomas or cancers increases with the size of adenoma and can range from 1.5% to 7.7% for sizes below 5 mm, 3% to 15.9% for sizes between 5 and 20 mm and 7% to 19.3% for adenomas over 20 mm in size[6]. We have evaluated the Jass-types separately for the groups of large adenomas, early carcinomas and late carcinomas to visualize the degree of molecular irregularities along the tumor progression route. The evaluation workflows for all groups are shown in Figure 3.

A notable change in the distribution patterns of the molecular types can be observed between large adenomas and early carcinomas. The main difference appears to be a result of an increase in CIMP+/*BRAF*- phenotypes from large adenomas (13.8%, 13/94) to early carcinomas (50.0%, 31/62). When explored further, an additional increase in a *KRAS* positive subgroup can be noticed. Accordingly, the rate of CIMP+/*BRAF*-/*KRAS*+ increases from 10.6% (10/94) in large adenomas to 30.6% (19/62) in early carcinomas. At the same time, this increase is complemented by the decrease of CIMP-/*BRAF*-/*KRAS*+ from 49.0% (38/94) in large adenomas to 16.1% (10/62) in early carcinomas, but also partially by the decrease in CIMP-/*BRAF*-/*KRAS*- from 39.3% (37/94) in large adenomas to 30.6% (19/66) in early carcinomas. In other words, methylation, partially accompanied by *KRAS* mutation, takes place during malignant transformation of at least some colorectal tumors during the transition from large adenomas to early carcinomas.

In addition to the Jass types an interesting molecular subgroup has recently been identified including carcinomas with CIMP+ phenotype with unmethylated *MLH1* harboring *KRAS* mutations[25]. We have identified high frequency of *KRAS* mutations in the CIMP+/unmethylated *MLH1*- group within early carcinomas (10/17; 58.8%) as well as late carcinomas (6/10; 60%). According to the previous reports such cancers arise mainly from *KRAS*-mutated traditional serrated adenomas and exhibit poor prognosis. This is in contrast to the CIMP- carcinomas.

Characterization of molecular types according to location

Combined with the information on mutator pathways a full longitudinal image of the colorectal cancer landscape can be elucidated[52]. Data from our study have confirmed the predominant manifestation of the CIMP-associated Type 1 and Type 3 in the proximal colon. At the same time, tumors bearing the CIN characteristics are evenly distributed throughout the colon and rectum. It is clear that further research will lead to more molecular tests to be performed routinely in the diagnosis and therapy of colorectal neoplasia. The molecular subtyping of adenomas and carcinomas using the Jass classification may lead to the discovery of molecular markers specific for the malignant conversion of colonic tissue from precursor lesions to malignant tumors. Such markers would be viable tools to complement endoscopic screening and the diagnosis of colorectal cancer patients.

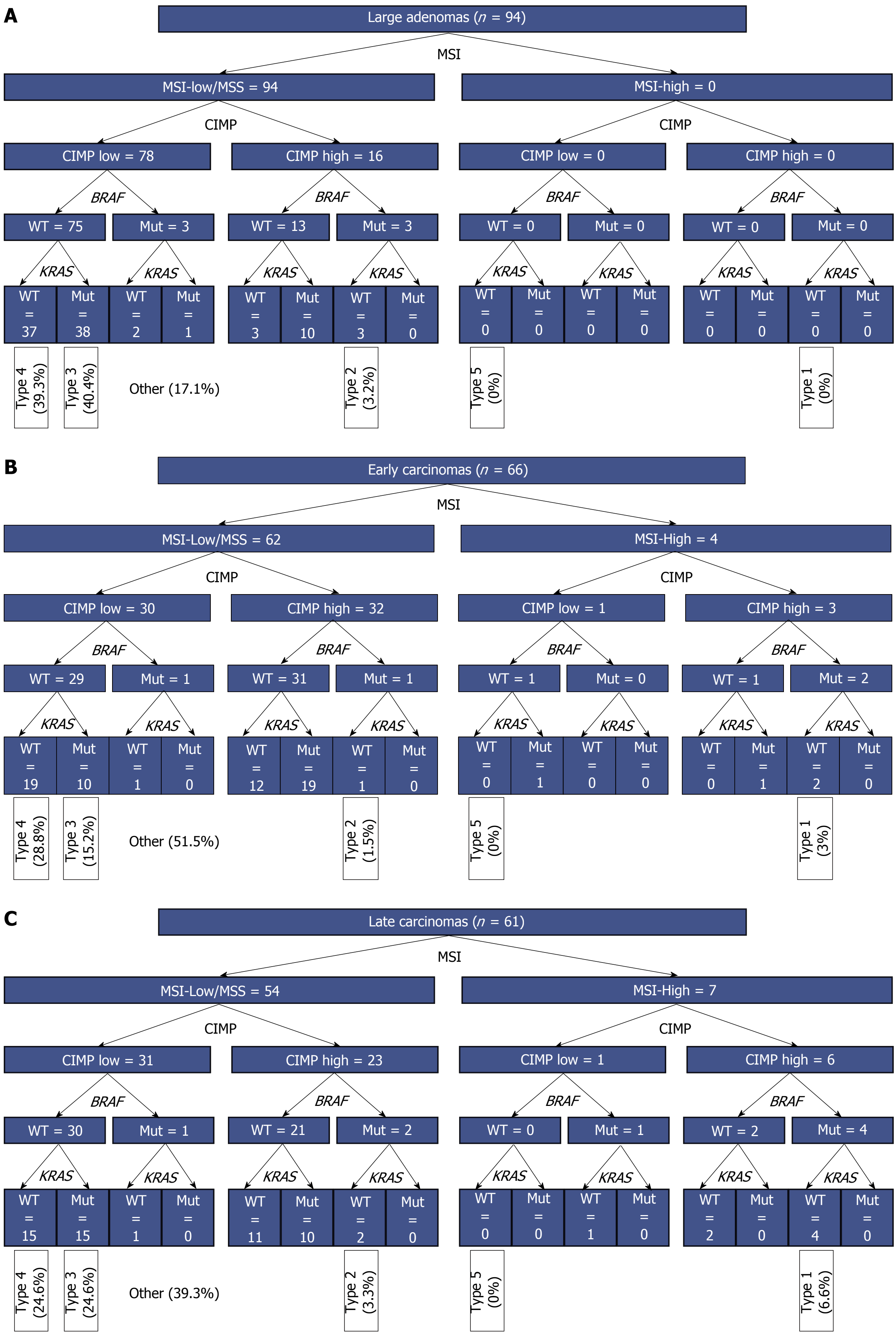

Figure 3 Evaluation workflows for assignment of Jass types in large adenomas (A), early carcinomas (B) and late carcinomas (C).

COMMENTS

Background

Recent advances in molecular profiling have resulted in definition of molecular types of colorectal cancer based on genetic and epigenetic aberrations. Resulting from separate developmental pathways the different types are associated with distinct prognostic features, which can be utilized in clinical practice.

Research frontiers

In a prospective study, endoscopic specimens from colorectal carcinomas as well as pre-malignant lesions were subjected to molecular profiling directed at evaluation of microsatellite instability (MSI) and CpG-island methylator phenotype (CIMP) status in combination with somatic mutations of *KRAS*, *BRAF*, *TP53* and *APC* genes.

Innovations and breakthroughs

The distribution of molecular types was evaluated for precursor lesions (large adenomas) and for early and late carcinomas with respect to their localization in proximal colon, distal colon and rectum.

Applications

The study demonstrates feasibility of molecular profiling in routine gastroenterology practice. The study results further suggest distinct molecular changes occurring during the malignant transition from large adenoma to early carcinoma, in particular DNA methylation affecting *KRAS*-mutated tumors.

Terminology

Somatic aberrations: Changes in DNA composition (base sequence or methylation) occurring within cells as a result of external factors and not the inheritance. CIMP: A molecular subtype characterized by methylation at certain positions within the DNA sequence. MSI: A molecular subtype characterized by unequal numbers of repetitions of short DNA sequences obtained for different cells within a tissue. The MSI occurs due to somatic aberrations in genes securing a proper function of the DNA repair system. Promoter methylation of *MLH1* gene is a frequent cause of MSI.

Peer-review

The authors studied molecular profiles of proximal and distal colon and rectum in colorectal adenomas and carcinomas that were obtained by routine endoscopic biopsy. They analyzed CIMP, MSI and mutations of *KRAS* and *BRAF*, and then classified into molecular subtypes in colorectal tumors. Most importantly, longitudinal molecular characterization was clearly shown in colorectal tumors based on CIMP/MSI/*BRAF*/*KRAS* classification. This approach to the molecular classification of colorectal cancer should accelerate understanding of causation, have an impact on clinical management, and facilitate the development of new ways to prevent and treat colorectal cancer.

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