Higher Frequency of Diploidy in Young-Onset Microsatellite-Stable Colorectal Cancer

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Abstract

Purpose: Colorectal carcinoma (CRC) can be divided into two nonoverlapping groups: those that are chromosomally unstable but microsatellite stable (MSS CIN+) and those that are chromosomally stable but microsatellite unstable (MSI CIN−). However, a third group with neither chromosome nor microsatellite instability (MSS CIN−) makes a substantial contribution to the total CRC burden. The clinicopathologic features of MSS CIN− CRC are not well delineated. We assessed the relationship between age and chromosomal instability (CIN) status as measured by ploidy and allelic imbalance in a series of MSS tumors.

Experimental Design: We studied a prospectively collected series of CRC patients at Mayo Clinic Rochester. A total of 84 samples of MSS CRC in patients ≤50 years old were identified between 1994 and 1997. A consecutive series of 90 MSS CRC in patients ≥65 years old served as a comparison group. CIN status was assessed using two techniques: ploidy analysis by flow cytometry and small chromosome changes as measured by genomewide fractional allelic imbalance.

Results: CRC in the young-onset group was more likely to involve the rectum and to be high stage. MSS tumors in the young-onset group were more often diploid (46%) than those in older patients (26%; $P = 0.006$). This difference was maintained in the subset of MSS CRC that were high stage (42% versus 18%; $P = 0.02$) and in rectal cancers (50% versus 23%; $P = 0.04$).

Conclusion: A greater proportion of young patients with MSS CRC has diploid tumors than patients who develop MSS CRC over age 65.

Colorectal cancer (CRC), the second leading cause of cancer death in the United States, strikes nearly 145,000 individuals each year. The majority of affected patients develop CRC in their mid-60s (1). Age may be understood as an important contributor to CRC initiation and progression in the context of the multistep model of colorectal carcinogenesis (2) because increasing age leads to more time for exposure to damaging environmental factors, random errors in genetic translation, or errors in DNA replication. However, each year 25,000 individuals who are age 50 or younger develop CRC, accounting for up to 17% of the total CRC burden (3–6).

A recent report based on experience from two U.S. cancer registries [National Program of Cancer Registries (NPCR) and the Surveillance, Epidemiology and End Results (SEER)] highlighted the substantial impact that CRC has for young adults and suggested that there are clinicopathologic differences between young and older onset CRC (5). CRC is among the top 10 cancers to affect individuals ages 20 to 49 across all races (5). Young-onset CRC is more likely to present at a later stage and to be more poorly differentiated. Young patients are also more likely to develop rectal cancer compared with the older onset population. This epidemiologic information is consistent with the possibility that young-onset CRC could be biologically distinct from older onset CRC.

CRC can be broadly classified as having either microsatellite instability (MSI) or chromosomal instability (CIN). MSI develops in the setting of defective DNA mismatch repair (MMR) and accounts for 15% to 20% of CRC overall. Base-pair mismatches are likely to occur in short-tandemly repeated sequences called microsatellites (7). Defective DNA MMR is usually related to hypermethylation of promotor sites on *hMLH1* (8), but inherited alteration of the DNA MMR genes (9) also leads to CRC with MSI. MSI tumors do not exhibit large deviations in their chromosomal DNA content, typically are diploid, and do not exhibit the large gains or losses associated with CIN (10). Although there is not a definitive measure for CIN levels, the original classification of CRC into those that showed microsatellite versus chromosomal instability stated that MSI tumors have a diploid DNA content as assessed by flow cytometry. The prevalence of MSI in young-onset CRC is

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reported to be 8% to 17% (4, 11), and the prevalence increases with age, approaching 30% in patients over age 90 (12, 13). An improved prognosis has been reported in both germ line and acquired examples of MSI CRC.

The remaining 80% of CRC have proficient DNA MMR, i.e., these tumors are microsatellite stable (MSS). These MSS tumors are generally assumed to have CIN. CRC tumors with the CIN phenotype often exhibit major abnormalities in the number and structure of many chromosomes, but genomewide instability in the microsatellite repeat regions of genes characteristic of MSI is not seen. CIN has been equated with aneuploidy, a term applied to cells containing an abnormal number of chromosomes (14). CRC aneuploidy is most often characterized by an increased number of chromosomes. Aneuploidy is one of the chromosomal changes that occurs as part of the aging process, may be associated with increasing age, and has been estimated to be present in up to 80% of all CRC (11, 15, 16). The mechanisms underlying these two molecular phenotypes are a crucial component of the framework for molecular genetic subtyping of CRC.

There is at least one additional molecular subtype of CRC, featuring neither microsatellite nor chromosomal instability (17, 18). Georgiades et al. (17) first described this subgroup of MSS tumors found to have lower levels of loss of heterozygosity(LOH) in combination with a diploid DNA content compared with other MSS tumors that were aneuploid and high levels of LOH. This diploid, low level of LOH group is now termed MSS CIN−, and several additional studies have substantiated its existence (19–24).

Up to 17% of young-onset CRC cases exhibit MSI (4), with many of these young patients having germ line mutations in the DNA MMR system, regardless of family history of CRC. Among the nearly three quarters of young-onset CRC that do not exhibit MSI, most are MSS, and a few are MSI(L) having fewer than 40% of MSI markers having instability. The CIN status of MSS tumors as a function of age at diagnosis has not been systematically evaluated. Chan et al. (20) found that 64% of 22 MSS young-onset CRC were diploid (compared with 13% of MSS older onset), with 7 of those with young-onset MSS diploid CRC having a family history of CRC. Among MSS CRC arising in young Chinese patients, 39% were found to be diploid (25).

Prompted by these findings and the association of aneuploidy with increasing age, we hypothesized that young-onset MSS CRC would have a higher proportion of CIN− tumors than older onset CRC. To test this, we assessed CIN status in both young and older age of onset MSS CRC by two measures used as surrogate markers for CIN: (*a*) flow cytometry to assess ploidy status and (*b*) genomewide allelic imbalance studies to assess global fractional allelic imbalance (FAI).

Materials and Methods

Colorectal cancer sample. Individuals for this study were selected from a prospectively collected series of 514 CRC patients who underwent surgical resection at the Mayo Clinic (Rochester, MN) from December 1995 until April 1997 (12) and from a separate prospective series of CRC patients who underwent surgery from 1987 to 1988 (26). Only patients who did not have FAP by clinical criteria and/or APC testing and who had normal DNA MMR in their CRC were included in this study. Immunohistochemical staining for *hMLH1*, *hMSH2*, and *hMSH6* and MSI testing were done as previously described (12). Microsatellite markers BAT26, D17S250; D5S346; ACTC, BAT40, BAT 25, BAT 34C4, D10S197, MYCL, and D18S55 were used to assess MSI status, and a tumor was called MSS if none of the markers showed MSI and all immunostains showed intact expression of MMR proteins. No MSI(L) or MSI(H) tumors were included in this study.

Eighty-four patients from these two series were identified as MSS, non-FAP young-onset CRC (age ≤50 years at the time of surgery). From the 1995 to 1997 series, the first 90 CRC cancers that fit the criteria of arising at an older age (≥65 years old) and being MSS were identified to serve as the comparison population. These 90 older onset cases were selected without knowledge of clinical features of their tumors, family history, or outcome.

Clinical features, including gender, age at diagnosis of CRC, tumor stage, and site and family history of CRC were collected via a self-administered questionnaire or abstracted from the medical record. Family history was defined as being positive if one or more first-degree relative(s) had CRC, and negative if there were no first-degree relatives with CRC.

Allelic imbalance analysis. Frozen tumor and normal epithelial tissue were used for allelic imbalance studies. Full thickness tumor tissue and normal colonic epithelium were excised from surgical specimens, snap frozen in liquid nitrogen, and stored at −70°C. Tissue sectioning was done in a cryostat at −20°C. Tumor tissue was macrodissected to enrich for tumor density (>70% tumor nuclei). Briefly, a 6-μm-thick frozen tumor section was immunostained with H&E and marked as a guide slide by our pathologist (T.C.S.) to identify regions containing ≥70% tumor nuclei. This slide was then used to identify the region of the corresponding frozen tumor block with only the area earmarked with 70% tumor density being sectioned to be used as the tumor tissue source for DNA. Accompanying normal colonic mucosa, a minimum of 8 cm from the tumor margin was microdissected for epithelial tissue. Sections were placed in extraction buffer, and DNA extraction was done with a proteinase K digestion, phenol/chloroform extraction as previously described (12).

Paired normal DNA and tumor DNA were analyzed for allelic imbalance using 97 dinucleotide repeat markers from the ABI Prism Linkage Mapping set (Perkin-Elmer Corp., Foster City, CA). Forty-five of the young-onset tumors and all the older onset CRC were evaluated with an initial marker set of 97 dinucleotide repeat markers. An additional 41 young-onset CRC cases were evaluated using another 97 markers, with 58 of these being common to the initial marker set. To develop a marker panel that could be used on paraffin-embedded samples for future studies, 39 markers from the initial marker set were replaced with markers that amplified PCR products <200 bp and that approximated the chromosomal location of the 39 markers they replaced.

Calculation of FAI scores. To calculate the allelic imbalance ratio, the ratio of two alleles in a tumor was divided by the ratio of the two alleles from the normal epithelium. Allelic imbalance was called positive if the calculated tumor-to-normal ratio was ≥2. For statistical purposes, if this value was between 0 and 1, the inverse was taken so that all values would be equal to or greater than 1. The global arm FAI score was used as the measure of the global FAI score in this study and was based on the number of chromosomal arms affected, i.e., number of imbalanced arms/number of informative arms.

Flow cytometry. Flow cytometry was done as previously described (27) on 50-μm-thick sections of paraffin-embedded tumor samples, and results were obtained for all reported cases. Tumors were deparaffinized to make a single-cell suspension of nuclei and then stained with propidium iodide (28). From each tumor, a minimum of 10,000 cells were evaluated by flow cytometry. DNA histograms were than analyzed with Modfit software version 5.4(Verity Software, Topsham, ME). Tumors were assessed as diploid, tetraploid, or aneuploid as previously described (28). For the purposes of analysis, the designation of a tumor as aneuploid was inclusive of tumors classified as either aneuploid and tetraploid tumors.

Statistical analysis. Data are summarized as proportions with confidence intervals for categorical data and as medians (interquartile range) for continuous data. FAI was dichotomized using the median FAI score in the older onset sample. Rank sum tests were used to assess associations between continuous variables and dichotomous variables. χ^2 or Fisher's exact tests were used as appropriate to assess association of two categorical variables. The Breslow-Day (29) homogeneity of odds ratios test, which does not distinguish response and predictor variables, was used to assess the association between dichotomous variables controlling for another dichotomous variable. Due to sample variation and exploratory analysis, *P* values are considered suggestive of significance, and multiple comparisons are not incorporated in the results presented. All analyses were done using SAS (Cary, NC) and S-plus (Mathsoft, WA) software packages.

Results

Patient characteristics. Descriptions of the two patient samples in this study are shown in Table 1. Statistically significant differences were observed between the two groups. The majority of young-onset tumors presented as later stage tumors, with 69% of the young-onset CRC being diagnosed with regional or distant metastasis, compared with 49% of the older onset cases presenting as late-stage tumors ($P = 0.01$). The majority of young-onset tumors were rectal, whereas the majority of the older onset tumors were proximal (young onset: 24% proximal, 21% distal, and 55% rectal; and older onset: 48% proximal, 26% distal, 26% rectal; $P = 0.0003$). Young-onset CRC tended to be poorly differentiated, although this difference was of borderline statistical significance ($P = 0.05$). The family history of CRC and gender did not differ between the two samples.

Table 1. Clinical and molecular characteristics of 84 young- and 90 older-onset CRC study patients

Characteristic	CRC onset ≤50 years, *n* (%)	CRC onset ≥65 years, *n* (%)	*P*
Gender			
Male	52 (62)	53 (59)	0.68
Female	32 (38)	37 (41)	
Tumor site			
Proximal colon	20 (24)	43 (48)	0.0003
Distal colon	18 (21)	23 (26)	
Rectum	46 (55)	23 (26)	
Tumor stage			
Early stage ($T_{0\text{-}3}N_0M_0$)	26 (31)	46 (51)	0.01
Late stage ($T_{0\text{-}4}N_{1\text{-}2}M_{0\text{-}1}$)	57 (69)	44 (49)	
Tumor grade			
Well differentiated	30 (36)	46 (51)	0.05
Poorly differentiated	53 (64)	44 (49)	
Family history of CRC*			
Positive family history	32 (39)	23 (26)	0.08
Negative family history	50 (61)	64 (74)	
Ploidy			
Diploid	39 (46)	21 (26)†	0.006
Aneuploid	45 (54)	61 (74)	
FAI‡			
Low FAI	49 (58)	46 (51)§	0.36
High FAI	35 (42)	44 (49)	

*Positive family history of ≥1 first-degree relative with CRC.
†*n* = 82 older onset with ploidy.
‡FAI is measured as a dichotomous variable using the median of the older onset global FAI score.
§*n* = 90 with FAI.

Measures of genomic instability. Two measures of genomic instability were done: (*a*) flow cytometry, which detects overall increases or decreases in DNA content between normal and tumor cells, by assessing larger, gross chromosomal gains or loss, duplications, and amplifications present in the tumor DNA relative to the adjacent normal DNA; and (*b*) a 97-marker genomewide scan of allelic imbalance (gains and losses) between normal and tumor tissue DNA. Because the majority of other studies that have addressed the phenomenon of CIN status have used flow cytometry as one measure of CIN (17, 18, 20) and studies that have used allelic imbalance as an adjuvant method have used different markers (17), the allelic imbalance data are reported mainly in reference to the flow cytometry data.

Significant differences in ploidy status were observed between the two groups. Forty-six percent of young-onset CRC were diploid compared with only 26% of the older onset group ($P = 0.006$; Fisher's exact test; Table 1). Among late-stage tumors, young-onset CRC were significantly more likely to be diploid than older onset tumors ($P = 0.02$; Table 2).

Among older onset CRC tumors, the proportion of aneuploid tumors was similar across tumor sites (68% of proximal, 82% of distal, and 77% of rectal; Table 3). Seventy-two percent of the young-onset distal colon cancers were aneuploid, mirroring the trend seen in the older onset CRC in all sites of the colon and rectum. However, in young-onset proximal and rectal tumors, the proportion of aneuploid tumors was 45% and 50%, respectively. Although these proportions are significantly different only when comparing young- and old-onset rectal tumors ($P = 0.10$, 0.71, and 0.04 for proximal, distal, and rectal tumors, respectively), the data also suggest a difference in trend between old- and young-onset tumors at the proximal site.

A significant association was seen between family history and young-onset diploid CRC, with 83% of the diploid young-onset cases having a family history of CRC in at least one first-degree relative, whereas only 17% of the diploid older onset cases shared a similar family history ($P = 0.03$). Gender was not significantly related to tumor ploidy status or age of CRC onset.

Recognizing that global FAI scores represent a continuum of values, we characterized a tumor as having high or low FAI by using the median score of the older onset group (0.158) as the lower limit of the high-FAI group. Within the older onset population, 44 were in the high-FAI group, and 46 were in the low-FAI group. A total of 35 young-onset cases were classified as having high FAI based on the median of the older onset group, with 49 young-onset cases having low FAI. The distribution of global FAI score did not differ between the young- and older onset groups when evaluated as a continuous variable using the rank sum test ($P = 0.51$) or when assessed as a dichotomous variable using the Fisher's exact test ($P = 0.36$) using the median global FAI score.

When assessing global FAI as a dichotomous variable, 46% of the subjects with low FAI scores had diploid tumors, whereas only 24% of subjects with high FAI scores had diploid tumors ($P = 0.004$), although global FAI score and ploidy status of a tumor did not have a one-to-one correlation, i.e., in some cases,

Table 2. Interaction of CRC stage and age on chromosomal stability

	Early-stage CRC			Late-stage CRC		
	Diploid *n* (%)	Aneuploid *n* (%)	*P*	Diploid *n* (%)	Aneuploid *n* (%)	*P*
Age of onset						
≤50 years old	14 (54)	12 (46)	0.07	24 (42)	33 (58)	0.02
≥65 years old	14 (32)	30 (68)		7 (18)	31 (82)	
FAI status for all ages						
Low FAI	19 (47)	21 (53)	0.14	22 (44)	28 (56)	0.01
High FAI	9 (30)	21 (70)		9 (20)	36 (80)	
Young onset						
Low FAI	11 (69)	5 (31)	0.11	19 (59)	13 (41)	0.003
High FAI	3 (30)	7 (70)		5 (20)	20 (80)	
Older onset						
Low FAI	8 (33)	16 (67)	0.81	3 (17)	15 (83)	1
High FAI	6 (30)	14 (70)		4 (20)	16 (80)	

a diploid tumor had a high FAI score, or an aneuploid tumor had a low FAI score.

In the context of the age of cancer onset, the correlation of FAI and ploidy status was particularly clear, and the consistency between these two measures of CIN is illustrated to be most concordant in the young-onset population ($P = 0.02$, Breslow-Day test for homogeneity of odds ratio), with the majority of young-onset diploid tumors compared with fewer than half of the older onset diploid CRC having low FAI scores (Fig. 1). The correlation between the FAI score, i.e., the majority of low-FAI tumors being diploid and of high-FAI tumors being aneuploid, was most consistently maintained among the young-onset CRC and particularly according to the tumor stage (Table 2) and by site (Table 3), with the only exception occurring in the distal colon.

Discussion

We compared 84 young-onset MSS CRC to 90 older onset CRC using flow cytometry to assess ploidy and determined that the proportion of diploid to aneuploid tumors in young patients differs from older ones. Young-onset MSS CRC were more often diploid than older onset CRC; nearly half of our young-onset MSS CRC were CIN− by ploidy, whereas older onset CRC were significantly more likely to be aneuploid and exhibit larger scale chromosomal abnormalities.

That we found a significant number of MSS CRC that do not exhibit aneuploidy is consistent with the concept that MSS diploid CRC is an entity distinct from MSS aneuploid CRC. Hawkins et al. reported that MSS diploid CRC without respect to age seemed to be associated with metastases (35% of diploid MSS CRC, 8% of aneuploid MSS CRC, and 0% MSI(H) CRC; $P = 0.03$), but not to the T or N stage (18). From their results, Hawkins et al. (18) drew the conclusion that the metastatic tendency of MSS diploid CRC may represent a relatively static genetic state in which the tumor does not evolve to a more aggressive tendency for invasiveness, but that the invasiveness is an inherent, putative feature of the carcinoma. A similar association of MSS CIN−, here referred to as low CIN, with higher stage tumors had been reported in the index article proposing MSS CIN− CRC by Georgiades et al. (17) and offered as evidence that MSS CIN− CRC is not a precursor step to a CIN+ tumor. In our young-onset cases, nearly half of the young-onset CRC cases were diploid regardless of stage, and this supports the concept espoused by Georgiades and Hawkins that MSS CIN− tumors are not an intermediary step to a CIN+ tumor, but rather a carcinogenic pathway distinct from that of MSS CIN+ CRC.

Our study corroborates the finding by Chan et al., that an increased proportion of distal and rectal MSS tumors are diploid in younger onset CRC patients compared with those

Table 3. Interaction of CRC site and age on chromosomal stability

	Proximal colon			Distal colon			Rectum		
	Diploid *n* (%)	Aneuploid *n* (%)	*P*	Diploid *n* (%)	Aneuploid *n* (%)	*P*	Diploid *n* (%)	Aneuploid *n* (%)	*P*
Age of onset									
≤50 years old	11 (55)	9 (45)	0.1	5 (28)	13 (72)	0.71	23 (50)	23 (50)	0.04
≥65 years old	12 (32)	25 (68)		4 (18)	18 (82)		5 (23)	17 (77)	
FAI status for all ages									
Low FAI	19 (49)	20 (51)	0.08	5 (23)	17 (77)	1	18 (60)	12 (40)	0.005
High FAI	4 (22)	14 (78)		4 (23)	14 (77)		10 (26)	28 (74)	
Young onset									
Low FAI	10 (67)	5 (33)	0.13	4 (36)	7 (64)	0.6	17 (74)	6 (26)	0.001
High FAI	1 (20)	4 (80)		1 (14)	6 (86)		6 (26)	17 (74)	
Older onset									
Low FAI	9 (37)	15 (63)	0.48	1 (9)	10 (91)	0.59	1 (14)	6 (86)	1
High FAI	3 (23)	10 (77)		3 (27)	8 (73)		4 (27)	11 (73)	

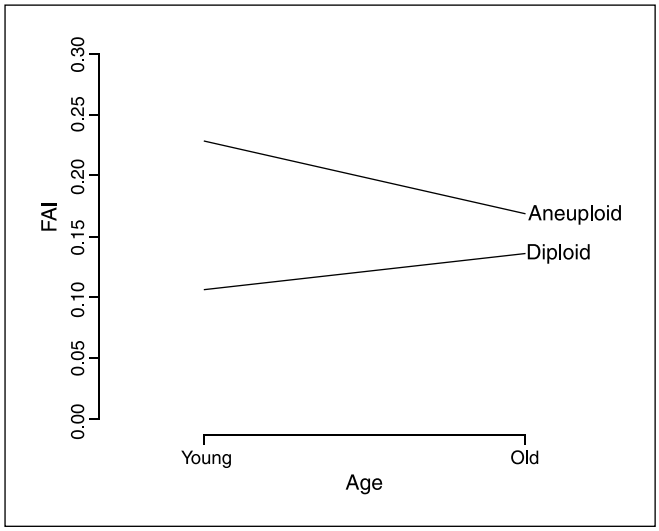

Fig. 1. Significant correlation between FAI score and ploidy status in young-onset CRC cases. Among the young-onset cases, FAI assessed as a dichotomous variable and ploidy scores are more concordant ($P = 0.02$; Breslow-Day test for homogeneity of odds ratio) with most young-onset diploid tumors compared with only half of the older onset diploid cases having low FAI scores.

with older onset disease (20). We found that a substantial proportion of the young-onset rectal, but not distal colon tumors were diploid compared with the majority of older onset rectal cancers being aneuploid. Proximal colon and rectal cancer in our young group consistently were more likely to be CIN− than CIN+ by ploidy and FAI status both individually and in combination. However, the majority of the young distal colon samples were CIN+ by ploidy and FAI, mimicking the distribution seen in all older onset CRC regardless of site. Whether this finding reflects an age-dependent predisposition for young-onset MSS CRC that is CIN− (by both ploidy and FAI) to develop in certain regions of the colorectum will require confirmation in a larger sample. However, the fact that young-onset CRC is most likely to arise in the rectum according to the SEER and NPCR U.S. cancer registries (5) does support the possibility that there may be biological, perhaps at the level of carcinogenesis, differences that lead to these age-related site and stage disparities.

In a population-based study of patients with young-onset CRC (age < 45 years), known to be negative for germ line mutations of *hMLH1* and *hMSH2*, Jenkins et al. (30) described a strong familial association in young-onset MSS CRC. Data presented by Chan et al. (20) also support that there may be a significant hereditary component within the specific subgroup of young-onset MSS CIN− CRC, with 50% of those with MSS CIN− CRC having a family history of CRC. In our study population, we found that cases of diploid tumors in the young were significantly more likely to have a family history of CRC in a first-degree relative compared with the older onset group. Recently, it was shown that MSS CRC has a significant, although lower risk for CRC than those patients with Lynch syndrome, the autosomal dominantly inherited CRC syndrome caused by germ line mutations in the DNA MMR genes (31). Traditionally, the Amsterdam criteria (AC) define a kindred as at risk for defective DNA MMR CRC if three persons are affected with CRC and (*a*) at least one person develops CRC at <50 years of age; (*b*) one person with CRC is the first-degree relative of the other two, and (*c*) at least two generations are affected with CRC. Lindor et al. found that among kindreds meeting AC with MSS CRC, the standardized incidence ratio of CRC was 2.3 for first-degree relatives not in the original triad that met AC. This condition of AC-positive MSS CRC was termed familial colorectal cancer type X (31). It may be that the MSS diploid young-onset population in particular will provide the context in which to explain this syndrome or to discover another genetic susceptibility to CRC.

The distinction between MSS CIN− versus MSS CIN+ tumors is difficult to define. Although one definition of CIN indicates that CIN is a rate that can only be determined by measuring the copy-number heterogeneity in colonies from single cells (32) or through cytogenetic meta-analyses of multiple karyotypes (33), practically, CIN as measured by flow cytometry does seem to have important implications about tumor behavior. Recently, CRC diploidy was found to be significant for prognostication, being associated with improved survival, independent of the MSI status of a tumor (10). We used flow cytometry as a gross measure of the phenomenon of the presence or absence of CIN, but limitations of flow cytometry may include contamination of tumor DNA with benign cells that could lead to a diploid result when aneuploid tumor cells are diluted by excess benign diploid cells. Hypomorphic mutations in *hMLH1* polymorphisms have been reported to be associated with risk for MSS CRC (34) and would not be detectable with either flow cytometry or our FAI panel. The implications of the increased diploid DNA content detected in our young-onset group require additional investigation in a larger population to determine the impact that ploidy status might have on tumor behavior, including aggressiveness and response to treatment. Neither AI, LOH, nor flow cytometry would be able to detect balanced chromosomal rearrangements.

We had expected that, in many cases, the FAI and ploidy data would be similar. Because a low global FAI score reflects that most of the genomewide markers assessed in tumor DNA did not show allelic imbalance, it might indicate that few, if any, large-scale genomic gains and losses had occurred, and thus, a diploid DNA content would be expected. Ploidy and FAI correlated well in our young-onset cases. However, we found disagreement between ploidy and FAI scores, specifically in the older onset cases in which the majority of older onset cases were aneuploid and often independent of the FAI score. This discrepancy could result because allelic imbalance can detect unbalanced structural chromosomal changes, whereas flow cytometry cannot. Possibly, the discordant findings of aneuploidy and a low FAI score result because flow cytometry can identify a small clonal population of aneuploid cells in a predominantly diploid tumor, but that the FAI score is skewed by the greater share of diploid tumor cells. A high FAI score in a diploid tumor may also represent tumor heterogeneity, balanced gains and losses, or possibly micro-chromosomal changes detected by FAI, but not large enough to alter ploidy status. As in our study, a split made on the basis of DNA ploidy analysis may not be correlative with low-level chromosomal change detected by comparative genomic hybridization (CGH) or LOH. Jones et al. have reported disagreement in some tumors between the DNA content measured by flow cytometry and another measure of CIN, CGH microarrays (23). Some MSS tumors with a diploid DNA content were found to have a

large number of chromosomal gains and losses which the authors speculate may represent balanced gains and losses or low-level aneuploidy not detectable by flow cytometry (23).

Further refinement of the appropriate methodology to clarify MSS CIN+ or CIN− tumors via CGH analysis, methylation analysis, LOH studies, fluorescent *in situ* hybridization studies, and/or flow cytometry is necessary to more clearly define MSS CIN− and CIN+ CRC. Rowan et al. (35) propose that CIN− tumors as determined by ploidy analysis can be subdivided into CIN stable and CIN low on the basis of the total number of chromosomal changes detected by CGH.

Possibly, the combination of ploidy status and another more sensitive measure of chromosomal stability is required to classify a MSS tumor as CIN− versus CIN+.

Clinically, our young-onset population mirrored that of the general U.S. population reported by Fairley et al., specifically with regard to the findings that young-onset CRC tended to present at higher tumor stage, tended to be located in the rectum and were more frequently poorly differentiated than older onset CRC cases. However, our sample sizes in subgroup analyses are quite small, limiting statistical power. Because CRC frequency differs in young African American and Asian/Pacific Islander compared with a U.S. Caucasian population, we acknowledge that our study results likely apply only to Caucasians, and that different factors may contribute to CRC risk in African Americans and Asian/Pacific Islanders.

Our results corroborate that not all MSS tumors are aneuploid (17, 18). The clinical features of tumor site, stage, and possibly family history in MSS tumors may be associated with CIN status depending on the age of onset of CRC and may help to explain the age-related differences in these clinical parameters. Further studies assessing larger groups for the interplay of age, stage, and site based on these parameters of MSS CIN− or MSS CIN+ tumors are needed. At this point, there is substantial evidence to support subcategorizing MSS cancers that could be used to refine tumor phenotyping on the basis of genetic stability, i.e., MSS CIN− versus MSS CIN+ CRC. MSS tumor phenotyping may be useful for clarifying etiologic mechanisms of MSS and possibly defining hereditary CRC among the young-onset MSS diploid CRC population.

References

1. Matanoski G, Tao XG, Almon L, et al. Demographics and tumor characteristics of colorectal cancers in the United States, 1998–2001. Cancer 2001; 107:1112–20.
2. Vogelstein B, Fearon ER, Kern SE. Allelotype of colorectal carcinomas. Science 1989;14:207–11.
3. Moore PA, Dilawari RA, Fidler WJ. Adenocarcinoma of the colon and rectum in patients less than 40 years of age. Am Surg 1994;50:10–5.
4. Gryfe R, Kim H, Hsieh ETK, et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. N Engl J Med 2000;342:69–77.
5. Fairley TL, Cardinez CJ, Martin J, et al. Colorectal cancer in US adults younger than 50 years of age, 1998–2001. Cancer 2006;107:1153–61.
6. O' Connell JB, Maggard MA, Liu JH, Etzioni DA, Livingston EH, Ko CY. Rates of colon and rectal cancer are increasing in young adults. Am Surg 2003;9: 866–72.
7. Peltomaki P. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. Hum Mol Genet 2001;10:735–40.
8. Thibodeau SN, French AJ, Cunningham JM, et al. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. Cancer Res 1998;58:1713–8.
9. Peltomaki P, Lothe RA, Aaltonen LA, et al. Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome. Cancer Res 1993;53:5853–5.
10. Sinicrope FA, Rego RL, Halling KC, et al. Prognostic impact of microsatellite instability and DNA ploidy in human colon carcinoma patients. Gastroenterology 2006;131:729–37.
11. Kim WH, Lee HW, Park SH, Kim YI, Chi JG. Microsatellite instability in young patients with colorectal cancer. Pathol Int 1998;48:586–94.
12. Cunningham JM, Kim CY, Tester DJ, et al. The frequency and mechanism of defective DNA mismatch repair in unselected colorectal carcinomas. Am J Hum Genet 2001;69:780–90.
13. Kakar S, Burgart LJ, Thibodeau SN, et al. Frequency of loss of hMLH1 expression in colorectal carcinoma increases with advancing age. Cancer 2003;97: 1421–7.
14. Duesberg P, Rausch C, Rasnick D, Hehlmann R. Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A 1998;95:13692–7.
15. Baker DJ, Jeganathan KB, Cameron JD. BubR1 insufficiency causes early onset of aging-associated phenotypes and infertility in mice. Nat Genet 2004; 36:744–9.
16. Geigl JB, Langer S, Barwisch S, Pfleghaar K, Lederer G, Speicher MR. Analysis of gene expression patterns and chromosomal changes associated with aging. Cancer Res 2004;64:8550–7.
17. Georgiades IB, Curtis LJ, Morris RM, Bird CC, Wyllie AH. Heterogeneity studies identify a subset of sporadic colorectal cancers without evidence of chromosomal or microsatellite instability. Oncogene 1999;18: 7933–40.
18. Hawkins NJ, Tomlinson I, Meagher A, Ward RL. Microsatellite-stable diploid carcinoma: a biologically distinct and aggressive subset of sporadic colorectal cancer. Br J Cancer 2001;84:232–6.
19. Choi S-W, Lee KJ, Bae Y-A, et al. Genetic classification of colorectal cancer based on chromosomal loss and microsatellite instability predicts survival. Clin Cancer Res 2002;8:2311–22.
20. Chan TL, Curtis LC, Leung SY, et al. Early-onset colorectal cancer with stable microsatellite DNA and near-diploid chromosomes. Oncogene 2001;20: 4871–6.
21. Goel A, Arnold CN, Neidzwiecki D, et al. Characterization of sporadic colon cancer by patterns of genomic instability. Cancer Res 2001;63:1608–14.
22. Risques RA, Moreno V, Ribas M, Marcuello E, Capella G, Peinado MA. Genetic pathways and genome-wide determinants of clinical outcome in colorectal cancer. Cancer Res 2003;63:7206–14.
23. Jones AM, Douglas EJ, Halford SER, et al. Array-CGH analysis of microsatellite-stable, near-diploid bowel cancers and comparisons with other types of colorectal carcinoma. Oncogene 2005;24:118–29.
24. Chang S-C, Lin J-K, Yang SH, Wang H-S, Li AF-Y, Chi C-W. Relationship between genetic alterations and prognosis in sporadic colorectal cancer. Int J Cancer 2005;118:1721–7.
25. Xie D, Leung S, Zeng W, et al. The expression of DNA mismatch repair genes and detection of DNA ploidy in young patients with colorectal carcinoma. Zhonghua Bing Li Xue Za Zhi 2000;29:412–5.
26. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. Science 1993;260:816–9.
27. Newton HB, Rosenlum MK, Malkin MG. Turcot's syndrome. Flow cytometric analysis. Cancer 1991;68: 1633–9.
28. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 1983; 31:1333–5.
29. Agresti A. Categorical data analysis. New York: John Wiley & Sons; 1990.
30. Jenkins MA, Baglietto L, Dite GS, et al. After MSH2 and hMLH1—what next? Analysis of three-generational, population-based, early-onset colorectal cancer families. Int J Cancer 2001;10:166–71.
31. Lindor NM, Rabe K, Petersen GM, et al. Lower cancer incidence in Amsterdam-1 criteria families without mismatch repair deficiency: famililal colorectal cancer type X. JAMA 2005;293:1979–85.
32. Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. Nature 1997;386:623–7.
33. Gisselsson D, Jonson T, Petersén Ä, et al. Telomere dysfunction triggers extensive DNA fragmentation and evolution of complex chromosome abnormalities in human malignant tumors. Proc Natl Acad Sci U S A 2001;98:12683–8.
34. Lipkin SM, Rozek LS, Rennert G, et al. The MLH1 D132H variant is associated with susceptibility to sporadic colorectal cancer. Nat Genet 2004;36: 694–9.
35. Rowan A, Halford S, Gaasenbeek M, et al. Refining molecular analysis in the pathways of colorectal carcinogenesis. Clin Gastroenterol Hepatol 2005;3: 1115–23.