High-fidelity of Five Quasimonomorphic Mononucleotide Repeats to High-frequency Microsatellite Instability Distribution in Early-stage Adenocarcinoma of the Colon

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Abstract. *Background: Microsatellite instability (MSI) in colorectal cancer (CRC) is a distinct pathway of carcinogenesis with prognostic implications. MSI is identified through the use of several markers. The aim of this study was to test the fidelity of five markers for high-frequency MSI (MSI-H) across age groups and stages. Patients and Methods: Analysis of the fidelity of five mononucleotide markers to MSI-H and the prevalence of MSI in different parts of the colon was carried out using BAT-26, BAT-25, NR-24, NR-21 and NR-27 in a cohort of predominantly Norwegian patients with stage I-III colon cancer. Results: Of the 121 colon tumors, a total of 33 (27.3%) were MSI-H, only 3 (2.5%) were low-frequency MSI and the rest (n=85, 70.2%) were microsatellite stable. The fidelity for MSI-H (n=33) of each marker was very high, with 100% for BAT-26, 96.9% for BAT-25, 87.5% for NR-24, 97.0% for NR-21, and 97% for NR-27. MSI prevalence was much higher in the proximal compared to the distal colon (43.1% vs. 8.9%; p<0.001). The prevalence of MSI-H decreased with increasing age, from >55% in those <50 years to 21% in those >70 years of age. For early-stage colon cancer (stage I-II, n=83), there was a significant difference in MSI distribution (44% in those <60 years and 22% in those >60 years; p=0.047), and in proximal (52.2%) compared to distal (7%) location (p<0.001). Highest prevalence (75%) of MSI-H was found in the proximal colon of node-negative patients <60 years of age. Conclusion: The five quasimonomorphic mononucleotide markers demonstrated high fidelity for MSI-H, with few cases being low frequency. MSI-H was most prevalent in early-stage, proximal colon cancer and in those <60 years, which may have implications for molecular screening.*

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Colorectal cancer (CRC) is one of the most frequent types of cancer in the Western world and the second most frequent cause of cancer deaths (1). The high incidence, development through precursor lesions (polyps and adenomas) and good survival when treated in the early stages of disease makes the prospect for early diagnosis and intervention attractive (2). Furthermore, the carcinogenesis and stepwise molecular alterations involved are being increasingly understood (3), which makes way for potential biomarkers for early detection with an increasing clinical role (4, 5). A variety of noninvasive molecular approaches to CRC screening are emerging, with potential to improve screening effectiveness and user-friendliness (6). Molecular screening of feces for the early detection of CRC and its precursor lesions has become more attractive (7, 8). New methods, especially next-generation stool-based tests, have been shown to detect both tumors and precancerous lesions with high accuracy. However, the optimal panel of markers in any test is yet to be determined.

Microsatellite instability (MSI) is a well-described genetic pathway in CRC, occurring in about 15-20% of all cases (9), with a predilection for the proximal colon (10). The prognostic role of MSI has been demonstrated in two past meta-analysis (11, 12), yet its predictive role remains somewhat controversial. A number of panels are in use for detecting MSI (13-16), with differences in how sensitive the markers are for detection (17, 18). Based on reports on the use of a panel of five quasimonomorph mononucleotide markers for identification of high-frequency MSI (MSI-H) across several populations (13, 15, 19, 20), the fidelity of these markers were explored in a Norwegian population.

Patients and Methods

Study population. The total population from which the patients in the current study are derived has been thoroughly described previously (21-23). The current study cohort consists of 121 stage I-III colon tumors from a cohort of 186 patients with both colon and rectal cancers for whom DNA was available for MSI analysis (23). Rectal cancer (only 4% had MSI) were excluded from the current study.

Table I. *Primers used for microsatellite analysis using five mononucleotide repeats.*

Marker	Gene	Primer sequence	Gene bank number	Average PCR product size (bp)
BAT-26	*hMSH2*	F: 5'-TGA CTA CTT TTG ACT TCA GCC-'3	U41210	120
		R: 5'-AAC CAT TCA ACA TTT TTA ACC C-'3		
BAT-25	*c-KIT*	F: 5'-TCG CCT CCA AGA ATG TAA GT-'3	L04143	124
		R: 5'-TCT GCA TTT TAA CTA TGG CTC-'3		
NR-21	*SLC7A8*	F: 5'-GAG TCG CTG GCA CAG TTC TA-'3	XM_033393	110
		R: 5'-CTG GTC ACT CGC GTT TAG AA-'3		
NR-24	Zinc-finger 2 (*ZNF2*)	F: 5'-GCT GAA TTT TAC CTC CTG AC-'3	X60152	125
		R: 5'-ATT GTG CCA TTG CAT TCC AA-'3		
NR-27	Inhibitor of apoptosis protein (IAP)-1	F: 5'-AAC CAT GCT TGC AAA CCA CT-'3	AF070674	86
		R: 5'-CGA TAA TAC TAG CAA TGA CC-'3		

F denotes forward primer sequence; R denotes reverse primer sequence. The fluorescent markers used were FAM (carboxyfluorescein) for BAT-26, NR-24 and NR-27, and HEX (hexachloro-fluorescein phosphoramidite) for BAT-25 and NR-21.

Gross and microscopic pathologic assessment. All tumors were reviewed by a board-certified pathologist according to WHO classification criteria and staged according to the AJCC/TNM system (24). All tumors underwent gross- and histopathologic investigation according to a structured template, as previously reported (25). No additional pathological features, such as venous or lymphatic invasion, tumor lymphocyte infiltrate, or other histopathological and/or immunohistochemistry markers were investigated at the time of the study.

Location of tumor in the colon. The proximal site in colon was defined as location in the cecum through the left/splenic flexure, and distal colon as descending and sigmoid colon. Tumors located in the rectum were excluded from the current study.

MSI analysis using 5 quasi-monomorphic mononucleotide markers. MSI analysis was performed as previously described (23). Briefly, DNA was isolated using a QIAamp DNA Micro-Kit (QiaGen™, Hilden, Germany) and the manufacturer's protocol for DNA isolation. MSI analysis was performed with five previously described quasimonomorphic markers (BAT-26, BAT-25, NR-21, NR-24, and NR-27) (Table I) (15, 20). PCR amplification was performed under standard conditions using normal DNA for control. The amplified PCR products were run on an automated 16 capillary electrophoresis DNA sequencer (GeneAnalyzer™ 3130XL) and allelic sizes estimated (23). Instability in any marker was visualized as a shift in the product sequence and scored according to recommended methods (18, 26). Instability in ≥2/5 of the markers was regarded as high-frequency microsatellite instability (MSI-H); if positive in only 1/5 markers as lowfrequency (MSI-L); if no positivity (0/5), as microsatellite stable (MSS). The number of positive markers for MSI was assessed, as well as the fidelity for MSI status for each marker.

Age groups. To explore the distribution of MSI frequency across different age groups, the patients were arbitrarily classified as ages <50 years, as age in four 5-year intervals from 50-70 years, and as age ≥70 years, thus creating 6 age groups for investigation.

Ethics. The study was consented to and approved by the Regional Ethics Committee, the Norwegian Social Science Data Service and the Norwegian Data Inspectorate.

Statistical analysis. Data were analyzed with PASW v. 18 for Macintosh (SPSS Inc., Chicago, IL, USA). Categorical variables were compared with the chi-square test. All tests were two-tailed, with statistical significance set at $p<0.05$.

Results

The study included 121 stage I-III colon tumors distributed in the colon as depicted in Figure 1. The prevalence of MSI within each section of the colon (Figure 1) is also demonstrated. MSI prevalence was much higher in the proximal compared to the distal colon (43.1% *vs*. 8.9%; $p<0.001$).

Distribution of MSI and contribution of each marker. Of the 121 colon tumors, a total of 33 (27.3%) were MSI-H, only 3 (2.5%) were MSI-L and the rest (n=85, 70.2%) were MSS. The contribution of each marker is given in Figure 2.

For those with MSI-H (n=33), the percentage of positivity of each marker was very high, with 100% for BAT-26, 96.9% for BAT-25, 87.5% for NR-24, 97.0% for NR-21, and 97% for NR-27.

In three tumors, MSI-H was based on only 4 markers as one marker was missing due to failed sample or lack of reading (one each for BAT-26, BAT-25 and NR-24, respectively). In all these cases the other four markers (4/4) were positive for MSI.

Role of age groups. The relative distribution across age groups is given in Figure 3. Notably, although of low frequency, MSI-L occurred only in those >60 years of age.

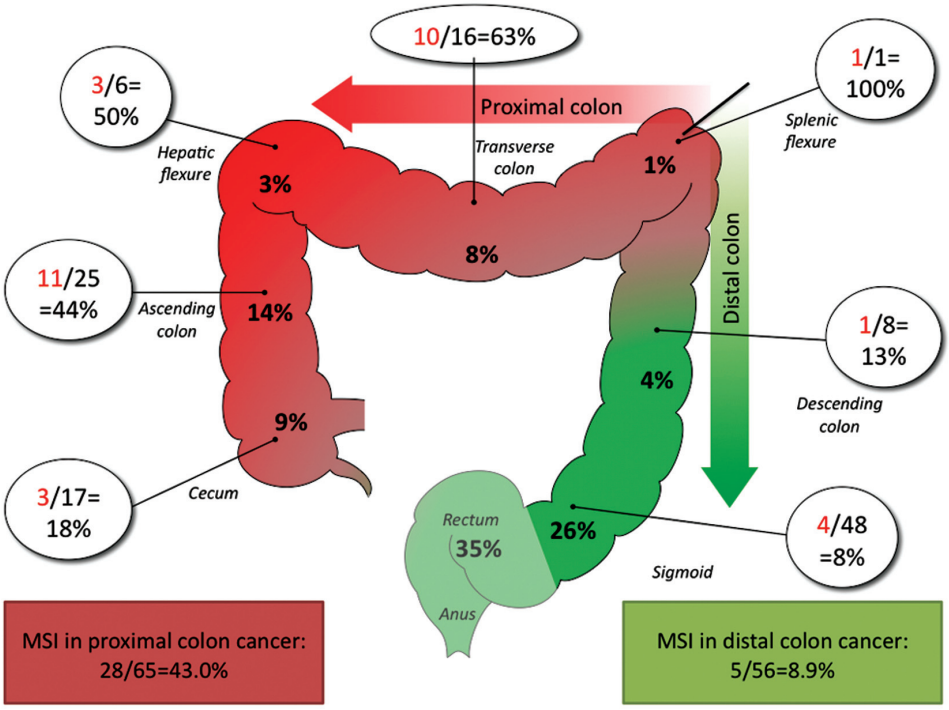

Figure 1. *Prevalence and distribution of MSI-H across the colon.*

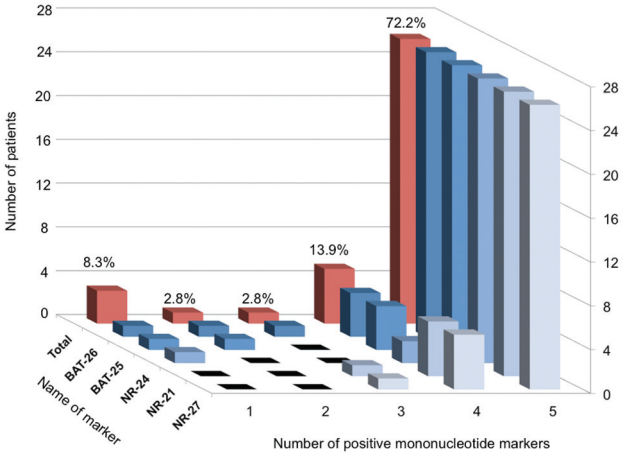

Figure 2. *Contribution of each mononucleotide marker to the prevalence of MSI frequency. One marker positive denotes MSI-L (low frequency), while positivity in 2-5 markers denotes MSI-H (high frequency). Numbers are shown for those patients with a positive marker only.*

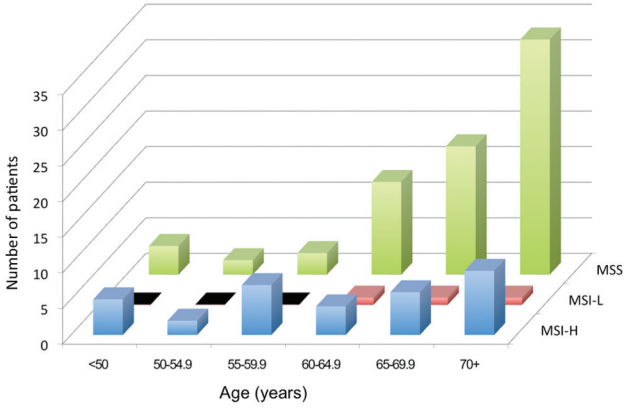

Figure 3. *Distribution of MSI frequency according to age group.*

The percentage of MSI-H decreased with increasing age, from >55% in those <50 years to 21% in those >70 years of age.

Tumor location. For the proximal colon tumors only (n=65), the MSI-H was 28 (43%), MSI-L 2 (3%) and the rest were MSS (n=35, 54%). In the proximal location of the colon, the MSI-H frequency was higher (12/22; 55%) for those <60 years and those ≥60 years of age (16/43; 37%) but not significantly so (p=0.182), nor was the difference in age significant for distal colon cancer (p=0.352).

Stage distribution. For early-stage colon cancer (stage I-II, n=83), there was a significant difference in MSI distribution according to age, with 44% MSI in those <60 years and 22% in those >60 years (p=0.047), and in proximal (21/40=52.2%)

compared to distal (3/43=7%) location ($p<0.001$), but not for gender distribution ($p=0.579$).

Excluding the node-positive cases (pN+, stage III) in the proximal colon and including stage I-II only (n=40), the frequency of MSI-H was higher in those <60 years (9/12; 75%) compared to those ≥60 years of age (12/28; 43%) but this was not statistically significant ($p=0.062$).

Discussion

The very high fidelity of all markers (in the range from 97-100%) except for NR-24 (at 88%) is in line with other studies, and reflects the usefulness of these markers in testing for MSI in a clinical setting. This study confirms the results obtained from previous populations evaluating a set of five quasimonomorphic mononucleotide markers for the identification of MSI-H colon tumors (13, 15, 19). The low prevalence of MSI-L is a result of high concordance between the markers, with 73% being positive for all five markers, and in the group of MSI-H identified by 4/5 positive markers, three patients lacked the fifth marker for evaluation (marker did not score or project on evaluation) so a missing value for any marker did not contribute to false-positive MSI-L, nor to false negative MSI-H in this study. Limitations of the study include the lack of comparison to other panels, such as the Bethesda panel that includes dinucleotide markers (16). However, other studies have compared the two panels with favorable results in regards to the panel containing only mononucleotide repeats. Rectal tumors were also excluded from the cohort. This is based on the fact that rectal tumors rarely have MSI (only 4% in our past series) (23) and that pre-operative radiotherapy may confound the results. Furthermore, focus on colon cancer, and particularly on tumors of the proximal colon, was made on the grounds that this location is more difficult to reach for early detection, and findings of marker distribution (such as MSI) may in the future be explored as useful for molecular screening/testing (27). Results in this study point to the high prevalence of MSI in the proximal colon and in those <60 years, which supports previous findings (28). As we have reported the clinical results in regard to the MSI findings in the current study population previously (10, 23, 29), the aim of the current investigation was to further investigate the fidelity of the markers in the panel for the prevalence of MSI-H according to location, age group and stage in a Norwegian population. Testing for MSI is as not as yet routinely performed in Norway unless there is a clinical suspicion on the development of disease on a background of hereditary cancer predisposition. However, with the increasing focus of the importance of MSI in sporadic colorectal cancer (in particular of the colon) (11, 12), it is likely only a question of time before these methods will be introduced on a more widespread basis. Lastly, as molecular screening is becoming of age, it will be important to include markers with high prevalence in those groups that are target screening populations. The high prevalence of MSI in proximal, early-stage colon cancer, and particularly those <60 years of age, is attractive for such screening. Further investigation should explore if these, or other alternative markers projecting this pathway of carcinogenesis, could be exploited as a molecular screening tool.

Acknowledgements

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