Hereditary non-polyposis colorectal cancer is predicted to contribute towards colorectal cancer in young South African blacks

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Introduction

Colorectal carcinogenesis involves the stepwise accumulation of mutations and/or epigenetic alterations, leading to the transformation of normal colonic epithelia. This process may develop and progress over a period of 10 to 15 years. Comprehensive studies have examined both morphological and molecular changes associated with the initiation and progression of colorectal cancer. A wealth of knowledge has thus far been accumulated and has led to the detailed classification of four distinct molecular pathways.

The classical pathway, somewhat involved in all of the pathways described, involves the somatic mutational inactivation of the adenomatous polyposis coli (APC) gene in colorectal epithelial cells. This leads to a cascade of events including, amongst others, degradation of β-catenin binding sites and interference with E-cadherin homeostasis during tumour initiation, and ultimately to p53 gene mutations during tumour progression. The familial form of this pathway is the autosomal dominantly inherited predisposition to familial adenomatous polyposis (FAP) that is initiated by germline mutations in the APC gene, and characterised by the presence of adenomatous polyps which develop into colorectal cancer if left untreated.

The next pathway involves the accumulation of mutations due to a mismatch repair (MMR) deficiency, resulting in microsatellite instability (MSI). Tumours with high levels of instability (MSI-H) may develop on a hereditary basis, involving the MMR genes hMSH2, hMLH1, hMSH6, hPMS2 and hPMS1, and thus predispose to hereditary non-polyposis colorectal cancer (HNPCC). The so-called ‘serrated’ pathway involves the silencing of MMR genes through promoter hypermethylation. This pathway is initiated in ‘serrated’ neoplasia through the inhibition of apoptosis, followed by the disruption of DNA repair mechanisms through epigenetic silencing.
Table 1. Primer sequences for the molecular analysis of colorectal cancer, including probes used during real-time polymerase chain reaction (PCR).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sense primer ('5′–3′)</th>
<th>Antisense primer ('5′–3′)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>CAA CTT GAT CCA GGT TCA GG</td>
<td>GAA GAG CCA AGG ACA GGT AC</td>
<td>268 bp</td>
</tr>
<tr>
<td>MINT1 (U)</td>
<td>TTT TGA TGA AGA TGT TTT ATT AGG GTT GT</td>
<td>ACG ACC TCA TCA TAA CTA CCC ACA</td>
<td>263 bp</td>
</tr>
<tr>
<td>MINT1 (M)</td>
<td>AGG TAG AGG TTT TAT TAG GTC CGC</td>
<td>AAA AAC CTG AAC CCC GCG</td>
<td>-90 bp</td>
</tr>
<tr>
<td>MINT2 (U)</td>
<td>GAT TTT GTT AAA GTG TTG AGT TTG TT</td>
<td>CAA AAT AAT AAC AAC AAT TCC ATA CA</td>
<td>-100 bp</td>
</tr>
<tr>
<td>MINT2 (M)</td>
<td>TGG TTA AAG TGT TGA GTT GTC C</td>
<td>AAT AAC GAC GAT TGC TCA GCA</td>
<td>-90 bp</td>
</tr>
<tr>
<td>MINT31 (U)</td>
<td>TAG ATG TGT GGG AAG TGT TTT GTT GT</td>
<td>TAA ATA CCG AAA AAC AAA ACA CCA AC</td>
<td>-90 bp</td>
</tr>
<tr>
<td>MINT31 (M)</td>
<td>TGT TGG GGA AGT GTT TTT CGG C</td>
<td>CGA AAA GGA AAC GCG GCG</td>
<td>-80 bp</td>
</tr>
<tr>
<td>MGMT (U)</td>
<td>TCG AGA TTT CAC TGT AGC TAG CAG ACA ACT GTT CAA ACT GAT GGG ACC CAC TCC</td>
<td>215 bp</td>
<td></td>
</tr>
<tr>
<td>MGMT (M)</td>
<td>TTT GTT TGA TTA TTT TAG GTT TTG TTG GG</td>
<td>ATG CTC TGC CAC CAG TA</td>
<td>215 bp</td>
</tr>
<tr>
<td>KRAS probe</td>
<td>LCr6640- TGG CCT AGG CCA CCA CCT C</td>
<td>GAC TTA TGA ATT AGC TGT ATC GTG AAG GCA C-F1</td>
<td>263 bp</td>
</tr>
<tr>
<td>BRF</td>
<td>CTT ACA TTC ATA ATG GTT GTC C</td>
<td>GAC TTT CTA GTA ACT CAG CAT C</td>
<td>263 bp</td>
</tr>
<tr>
<td>BRF probe</td>
<td>LCr6640- TGG AGA TTT CAC GTG AGG TAG</td>
<td>CAG ACA ACT GTT CAA ACT GAT GGG ACC CAC TCC-F1</td>
<td>263 bp</td>
</tr>
</tbody>
</table>

1U – primers specific for unmethylated DNA, i.e. C modified to T.
2M – primers specific for methylated DNA, i.e. C not modified.

There is a worrisome trend in South Africa for a disproportionately large number of young black patients to present with CRC. The specific morphological features of the tumours concerned do not seem to indicate the involvement of diet or lifestyle-related factors, and investigations into their pathogenesis may prove to be useful to establish molecular markers for early detection and treatment. This study aimed at investigating CRC in black patients to identify the possible molecular pathways involved, to aid in the establishment of molecular biomarkers. Features such as overall methylation phenotype (CIMP), microsatellite instability (MSI) and BRAF and KRAS gene mutation status were investigated. Results were obtained through methylation-specific polymerase chain reaction (MSP-PCR), microsatellite analysis and real-time PCR.

Materials and methods

Patient selection

Paraffin-embedded tissue samples were collected retrospectively, and included specimens originally diagnosed in the Division of Anatomical Pathology as adenocarcinoma of the colon and/or rectum. Cases were limited to a randomly selected subset of the more recent cases (1999–2003) based on tissue availability, and were stratified according to age at diagnosis (<50 years or >50 years) and ethnicity (black or white), with 44 available kit (CpGenome™ DNA modification kit, Chemicon Int, Temecula, CA) according to the manufacturer’s instructions. Methylation-specific PCR (MSP) was done using primers specific for either the methylated (M) or modified unmethylated (U) DNA described in Table 1. Appropriate positive and negative controls were included in each PCR experiment. The products were visualised on a 3% agarose gel (Saekem LE,Cambrex BioScience,Rockland, ME; MS-4 agarose reaction products,Whitehead Scientific,Cape Town, DE). The integrity of the isolated DNA was routinely evaluated through the amplification of the β-globin gene using the primer pair PC04/GH20 (Table 1).

Methylation-specific PCR

The methylation status of MINT1, MINT2, MINT31, HMLH1 and MGMT was determined by bisulphite treatment of DNA. The methylation status of both non-tumour and tumour tissue of each case was determined separately. These loci were chosen based on published data that showed they offered a means of discriminating the CpG island methylator phenotype (CIMP), and that the MINT loci were unmethylated in normal tissues. Bisulphite modification was performed using a commercially available kit (CpGenome™ DNA modification kit, Chemicon Int, Temecula, CA) according to the manufacturer’s instructions. Methylation-specific PCR (MSP) was done using primers specific for either the methylated (M) or modified unmethylated (U) DNA described in Table 1. Appropriate positive and negative controls were included in each PCR experiment. The products were visualised on a 3% agarose gel (Saekem LE, Cambrex BioScience, Rockland, ME; MS-4 agarose reaction products, Whitehead Scientific, Cape Town, South Africa) (80 V, 80 min) stained with ethidium bromide. The loci were classified as unmethylated if the intensity of the methylated band was visually less than that of the unmethylated band, or as methylated if the intensity of the methylated band was visually more than that of the unmethylated band. CIMP status was determined as CIMP-negative if none of the evaluated loci were methylated, CIMP-low if one locus was methylated, and CIMP-high if two or more loci were methylated.

Microsatellite instability (MSI) testing

The loci recommended by the American Joint Commission on...
Cancer and the International Collaborative Group on HNPCC for microsatellite instability testing include BAT25, BAT26, D5S346 (APC), D17S250 (Mfd15CA) and D2S123. The MSI status of these loci was detected using a multiplex PCR system developed by Roche Diagnostics (HNPCC Microsatellite Instability Test, Catalogue number 2041901). One hundred nanograms of DNA were used to amplify the MSI loci in a single multiplex reaction using the provided primer pairs labelled with different fluorophores (6-FAM, TET or HEX). Analysis of PCR products was performed on the ABI PRISM automated sequencer model 377 (Applied Biosystems, Foster City, CA) according to manufacturer instructions. MSI was defined by the presence of novel peaks, following the PCR amplification of tumour DNA, which was not present in non-tumour DNA. A tumour was classified as high-MSI (MSI-H) if at least 40% (2/5) of the examined loci showed unequivocal instability. Microsatellite-stable (MSS) tumours were those if at least 40% (2/5) of the examined loci showed no microsatellite instability. Tumours with only one marker showing instability were declared low microsatellite instability (MSI-L).

**Real-time PCR analysis for KRAS and BRAF gene mutation status**

Detection of mutations in codon 12 and 13 of exon 1 of the KRAS gene (GGT to GAT transition), and in exon 15 of the BRAF gene (T1796A or V600E mutation) was performed using real-time PCR and melting curve analysis. Positive controls included DNA from a papillary thyroid carcinoma for the BRAF V600E mutation, while an oesophageal carcinoma served as included DNA from a papillary thyroid carcinoma for the BRAF gene (T1796A or V600E mutation) was performed using real-time PCR and melting curve analysis. Positive controls included DNA from a papillary thyroid carcinoma for the BRAF gene (T1796A or V600E mutation) was performed using real-time PCR and melting curve analysis. Positive controls included DNA from a papillary thyroid carcinoma for the BRAF gene (T1796A or V600E mutation) was performed using this technique.

**Statistical analysis**

Statistical comparisons between the ethnic groups were completed using the two-sided Fisher's exact test. Patients were analysed based on ethnicity (Black vs White) and age (<50 years vs >50 years). All statistical analyses were completed using Stata Intercooled 7.0 (Stata, College Station, TX, USA). The differences were considered statistically significant when \( P < 0.05 \).

**Results**

Table 2 summarises the results obtained and data available on the sample population.

**Methylation studies**

The overall methylation status was determined by examining five distinct loci. These included the methylated in tumour (MINT) markers 1, 2 and 31, as well as the promoter regions of the hMLH1 and MGMT genes. Although not statistically significant, a high level of the CpG island methylator phenotype (CIMP-H) was more common in tumours located on the right in the young black patients [6/13 (\( P = 0.46 \)) compared to 2/11 (\( P = 0.18 \)) for left-sided tumours]. The other groups were too small to predict a trend. The majority of young blacks presented with low methylation status (CIMP-L) [16/25; \( P = 0.64 \)] (Fig. 1). Methylation of the MGMT promoter region was often observed in young blacks (19/36; \( P = 0.41 \)). Methylation of the hMLH1 promoter region was found in only 28% of young blacks (7/25), however, The positive predictive values of loss of protein expression of the MGMT and hMLH1 were 0.79 and 0.84, respectively (Fig. 1).

**Microsatellite instability**

The distinction between the mutator and methylator pathways is characterised by the microsatellite instability (MSI) status of key markers. MSI was analysed at the five recommended loci and included Bat25, Bat26, DSS346 (APC), D17S250 (Mfd15CA) and D2S123. Overall, in agreement with features of the mutator pathway, MSI-H was more often observed in young patients (7/25 black and 2/3 white) in comparison with patients older than 50 years of age at diagnosis (1/7 blacks and 1/8 whites). There
A previous comprehensive study on the morphological features associated with CRC in these patients had revealed that young black patients appeared to present with morphological features associated with HNPCC in a significant proportion of cases.\(^8\) These included proximally located (right-sided), high-grade tumours with a mucinous appearance, as reported for HNPCC tumours.\(^7\) The molecular features associated with this disease include mutations within the mismatch repair genes, mainly \textit{hMLH1} and \textit{hMSH2} that subsequently result in high levels of microsatellite instability (MSI-H). Diagnosis of HNPCC mainly relies on the Amsterdam and Bethesda criteria of which several revised editions are available.\(^14\) As a purely retrospective study, a lack of available family history complicated the diagnosis of HNPCC based on the above-mentioned criteria. This is often the case in academic, provincial and rural hospitals in developing countries such as South Africa where language barriers, inadequate understanding of the causes of death, or a lack of diagnosis are often encountered as limiting factors. We therefore postulated that through the use of molecular biology the diagnosis could be made directly in association with the morphological features identified.

HNPCC is an autosomal dominant cancer-susceptibility syndrome that accounts for approximately 1% to 6% of all CRC cases diagnosed.\(^8\) It is recognised at an early age (~45 years), in multiple individuals within a family and arises from adenomas with an advanced transformation rate, which is due to mutation and the subsequent inactivation of DNA mismatch repair genes, especially \textit{hMLH1} and \textit{hMSH2}.\(^15\) Loss of immunohistochemical nuclear expression of these gene products often indicate the germline-inactivated gene, and it was previously reported that young blacks frequently showed loss of expression of the \textit{hMLH1} (23%; 29/128) \((P = 0.121)\) and \textit{hMSH2} (12%; 16/129) \((P = 0.013)\) proteins.\(^8\) In the 25 young black CRC patients of this study, nine (36%) showed such a loss of expression (Table 2). A hallmark feature used in the molecular diagnosis of HNPCC is the high level of microsatellite instability.\(^16\) Twenty-nine per cent of the young black patients in the current study presented with MSI-H in comparison with only 14% of older black patients (Fig. 1). It is of interest that two of the three young whites presented with the same molecular phenotype but did not show the morphological features associated with an HNPCC diagnosis. This is partially explained through the serrated neoplasia pathway that involves high levels of MSI and frequently methylated DNA regions.

The concept of serrated neoplasia is well established and

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**Fig. 1.** Examples of methylation-specific PCR (MSP) at the \textit{hMLH1} promoter region CpG island in two unaffected (N) and colorectal cancer (T) samples. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated DNA; the presence of product in those lanes marked M indicates the presence of methylated DNA. In both cases de novo methylation was observed in the tumour tissue.

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**Fig. 2.** Microsatellite instability in South African patients with CRC. Microsatellite status was examined at five loci using a multiplex PCR system. Tumours with more than two unstable loci were termed microsatellite instability high (MSI-H).

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**Table 3.** \textit{BRAF}(V600E) and \textit{KRAS}(codon 12 and 13 exon 1 G → A) gene mutations in South African patients with CRC.

<table>
<thead>
<tr>
<th></th>
<th>Young African</th>
<th>Young Caucasian</th>
<th>Old African</th>
<th>Old Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant \textit{BRAF}</td>
<td>4% (1/25)</td>
<td>0% (0/3)</td>
<td>0% (0/8)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>Mutant \textit{KRAS}</td>
<td>32% (8/25)</td>
<td>0% (0/3)</td>
<td>25% (2/8)</td>
<td>25% (2/8)</td>
</tr>
</tbody>
</table>
Involves the methylation and subsequent silencing of CpG islands often found in gene promoter sequences. Genes silenced in this way include amongst others the hMLH1 and DNA repair gene O-6-methylguanine DNA methyltransferase (MGMT), that is associated with high or low levels of MSI respectively. Only one of the 25 young black patients showed methylation within the hMLH1 gene in conjunction with the MSI-H phenotype. 

Kras gene mutations in codon 12 and 13 of exon 1 were frequently observed in young blacks (Table 3). This was different from the young white patients presented with the BRAF–V600E mutation (Table 3). Deng et al. recently showed that tumours from HNPPC patients do not harbor the BRAF–V600E mutation. This finding was subsequently confirmed by Domingo et al., who suggested BRAF screening as a strategy for simplifying HNPPC genetic testing since the presence of the BRAF mutation would suggest an alternative explanation. During the same time, Oliveira et al. showed that KRAS is mutated in 40% (63/158) of HNPPC families that contain germline mutations in one of the mismatch repair genes. BRAF and KRAS gene mutation status can conveniently be used to distinguish between hereditary CRC in the form of HNPPC and sporadic MSI-H CRC. The main distinguishing factor between these two possibilities is the presence of promoter methylation within the hMLH1 gene found in sporadic CRC. Several groups have shown a relatively low frequency of KRAS mutations in sporadic CRC in a background setting of hMLH1 promoter methylation when compared to HNPPC. The methylation status in young black patients reported here was predominantly low, with infrequent methylation of the hMLH1 gene.

The diagnosis of HNPPC is predominantly based on fulfillment of the revised forms of the Amsterdam and Bethesda criteria, pertaining primarily to a family history of colorectal cancer. As stated earlier, it is often problematic to obtain family history from affected individuals in developing countries due to factors such as language barriers, fears of isolation and being prejudiced against and overall patient reluctance to follow-up after initial diagnosis. The need therefore exists for diagnostic criteria that rely on morphological and molecular features for accurate diagnosis. Studies into the morphological features of colorectal cancer in young black patients have revealed features in keeping with the sensitive Bethesda criteria for a diagnosis of HNPPC. Several patients showed high levels of microsatellite instability, providing support for the hypothesis that these patients may present with a hereditary form of CRC, most likely HNPPC. 

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