MUC gene abnormalities in sporadic and hereditary mucinous colon cancers with Microsatellite Instability

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Abstract. Aim of this study was verifying whether mucin producing colon cancers (CRCs) could develop through a molecular pathway involving microsatellite instability (MSI) and MUC gene alterations. Out of 49 CRCs expressing variable amounts of mucin, 22 (44.9%) were MSI-H and 5 (10.2%) were MSI-L. MUC genes were analyzed by Southern blotting and extra bands were evident in the Variable Number Tandem Repetition (VNTR) regions of MUC2 (5 cases) and MUC5AC (2 cases), but not MUC1 and MUC4 genes. Since the somatic VNTR abnormalities were detected in 6 MSI-H and in 1 MSI-L tumors, they seem to be peculiar of mismatch repair defective CRCs. Our finding suggests that alteration and/or loss of structurally normal MUC genes may be an important step in the neoplastic molecular pathway of a subset of CRCs and that mutations involving VNTR repetitive sequences may exist in MSI tumors as a direct and/or indirect consequence of an inefficient MMR system.

Keywords: Colon cancer, MSI, mucin, VNTR, mismatch repair

1. Introduction

Several studies have been carried out to elucidate the molecular pathways involved in the multistep process of the colorectal carcinogenesis and, at present, the pathogenesis of colon cancer (CRC) is one of the best known. In particular, several efforts have been made to understand the molecular bases of microsatellite instability (MSI) and to identify the genes, which are target for mutations in the presence of a defective mismatch repair (MMR) apparatus, and their contribution to tumor progression. It is widely accepted that the entire carcinogenesis process of MMR deficient intestinal cells is sustained by the mutator phenotype. Although there are probably additional mechanisms through which deficient DNA MMR may lead to tumor formation, colon carcinogenesis appears to be driven mainly by loss of postreplicative mismatch correction, with the accumulation of somatic point mutations (expecially small insertions or deletions) in multiple growth regulatory target genes, such as TGFβR-II, E2F4 and BAX [2,14].

CRCs developing in Hereditary Non Polyposis Colorectal Cancer (HNPCC) patients with constitutional mutations in MSH2 or MLH1 genes always display the MSI phenotype. However, MSI, the hallmark of MMR deficiency, may also be due to transcriptional silencing...
of the \textit{MLH1} gene and this phenomenon concerns the majority of the MSI sporadic cases [13,14].

Peculiar histologic features are significantly associated with the MSI colon tumors, both sporadic and hereditary. In particular, CRCs with MSI are more often mucin-producing than tumors with microsatellite stability (MSS) and, conversely, mucinous CRCs display MSI more often than non-mucinous tumors [3,11,15,16,18]. In line with the strong association between HNPCC and MSI, it is not surprising that the mucinous histology, having a high incidence of MSI, has often been reported in HNPCC-related tumors [11].

Mucinous CRCs are characterized by mucin(s) overexpression, but it is not completely clear whether this property may be ascribable to variations in transcription/translation and/or glycosylation of apomucins. Moreover, information on possible gene abnormalities is lacking [1,22]. \textit{MUC} genes are a family of genes containing long stretches of tandem repeats encoding Thr/Ser/Pro-rich domains. Each of these units has a specific length and sequence, and in several \textit{MUC} genes their repetitions vary individually according to a genetic polymorphism [9,20]. These Variable Number Tandem Repeat (VNTR) regions codify for the major portion of the peptide cores that are heavily glycosylated and display antigenic properties. The importance of these molecules to the course of tumor disease is related to their role in cell adhesion and metastasis, their diagnostic and prognostic relevance and their immunotherapeutic potential utility [1,22].

By considering the observed association between MSI and mucinous CRCs, a molecular link between MSI and mucin overproduction may be suspected, but at present, it is not clear which molecular mechanisms are responsible for this phenomenon. Aim of this study was verifying whether mucin-producing CRCs with MSI could develop \textit{MUC} gene alterations.

2. Materials and methods

2.1. Patients and tissues

Fourty-nine CRCs localized in the proximal colon were studied. This series comprises 27 sporadic patients and 22 patients with ascertained or suspected genetic predisposition (clinical diagnosis of HNPCC and/or positive CRC family history and/or early onset and/or multiple HNPCC-related tumors). Fifteen cases of the latter group had been previously screened for \textit{MMR} gene mutations, leading to the identification of 8 mutated tumors (6 \textit{MSH2} and 2 \textit{MLH1}, 7 constitutional and 1 somatic). Data on mucin production were collected by consultation of the pathologic reports, and tumors were classified as ++ (10 cases reported as mucinous histology or with prevalent mucinous components or $\geq 50\%$ mucus), + (28 cases with partial/local mucinous components or $< 50\%$ mucus) and − (11 cases without mucus).

2.2. Molecular analyses

Genomic DNA was extracted from frozen tumors by the DNAeasy tissue kit (Qiagen GmbH, Hilden, Germany) following manufacturer’s instructions. Corresponding blood or normal tissues were also used for DNA extraction and analysis.

MSI was evaluated using the standard NCI panel [4] by fluorescent-PCR analyses on an ABI 310 automatic sequencer (Applera, Foster City, CA, USA).

The VNTR regions of the \textit{MUC1}, \textit{MUC2}, \textit{MUC4}, and \textit{MUC5AC} genes, characterized by repetitions of 24–69 bp highly polymorphic in length, were studied by Southern blotting. For VNTR sizing, normal and tumor DNAs were digested using either \textit{Hinfl} or \textit{PvuII} restriction enzymes, electrophoresed through 0.8–1% agarose gels, blotted and hybridized to $^{32}$P-labelled probes by standard methods. \textit{TaqI} digestion was also used on a limited number of cases. The \textit{MUC} probes were obtained by PCR and plasmid cloning (PGEM-T vector Systems, Promega, Madison, WI, USA) and contained a minimum of 3 gene-specific Tandem Repeat units (details will be given on request).

3. Results

3.1. MSI

Twenty-two tumors (44.9\%) had high-level MSI (MSI-H), 5 (10.2\%) had low-level MSI (MSI-L), and the remaining 22 (44.9\%) were microsatellite stable (MSS). The high prevalence of MSI (55.1\%) was clearly ascribable to the peculiarity of the series analyzed, which was a collection of 49 CRCs comprising exclusively proximal tumors, about three fourths mucin producing, and nearly one half from ascertained or presumed hereditary cases.

Twenty-three out of the 27 MSI-H/MSI-L CRCs had variable amount of mucus (++) or (+). Conversely, among the 38 mucin producing CRCs, there was a slight excess of MSI (23 MSI-H/MSI-L, 60.5\%) in compari-
C. Pastrello et al. / MUC gene abnormalities in sporadic and hereditary mucinous colon cancers with Microsatellite Instability

Fig. 1. Southern blot analysis of the MUC2 VNTR. DNA was digested by HinfI (5 panels on the left) or TaqI (right panel). Ctrl, cases not displaying abnormalities; N, normal DNA; T, tumor DNA. Abnormal fragments in tumor DNAs are indicated by arrows and partial loss of heterozygosity is indicated by an asterisk. In the TaqI panel, the 2.3 kb band represents a constant fragment, whereas the alternative 2.1 and 1.7 kb bands derive from a sequence RFLP within the VNTR region.

The 11 CRCs without mucus (4 MSI-H/MSI-L, 36.4%). Fourteen of the patients with MSI tumors had an HNPCC-related personal and/or family history, and 7 of them had a constitutional MSH2 or MLH1 gene mutation. With the exception of a somatic MSH2 mutation in a (supposedly) hereditary tumor, MLH1 promoter hypermetylation was probably the major contributor to the instability of the remaining 6 hereditary and 13 sporadic MSI cases.

3.2. MUC genes

Southern blot analyses were carried out to study the VNTR region of the MUC1, MUC2, MUC4 and MUC5AC genes. HinfI digestion could be applied to all 49 cases for the analysis of MUC1, MUC2 and MUC5AC genes, whereas PvuII restriction was limited to the study of MUC4 and MUC5AC genes in the 40 tumors for which sufficient amount of DNA was available. Moreover, TaqI was used to integrate the MUC2 analysis of 4 tumors.

Extra bands were evident at the DNA tumor level in MUC2 (5 cases) and MUC5AC (2 cases) (Figs 1, 2 and Table 1), but not MUC1 and MUC4 genes. All these tumors had a mucinous component (3 ++ and 4 +). Interestingly, 6 out of the 7 somatic MUC gene mutations were detected in MSI-H tumors and only one was displayed by a MSI-L tumor (Table 1). Therefore, abnormalities of the VNTR sequences of MUC2 and MUC5AC genes, consisting of both increased and decreased allele sizes, seem to be peculiar of the CRCs with mucin and MSI (7/27 MSI-H/MSI-L and 0/22
Mors has also been suggested by Biemer-Hüttemann et al., who reported selective increased expression of MUC2 and MUC5AC as a model for the “secretory mucins”, which are normally produced and secreted by goblet cells and, together with other gel-forming mucins, are the major constituents of mucus. We also chose MUC1 and MUC4 as models for the “membrane-bound mucins”, which are transmembrane glycoproteins with anti-adhesive properties, exerting a role in tumor progression and metastasis.

MSS; $p = 0.0123$. Fisher exact test). Since only 2 cases were from patients with an MMR constitutional defect (Table 1), the MUC mutations appear independent on the mechanism leading to MMR inactivation.

### 4. Conclusion

In this study, the MUC analysis was restricted to 4 genes coding mucins that are variably produced in gastrointestinal normal epithelia and whose expression appears deregulated in colon tumors [1,3,22]. We chose MUC2 and MUC5AC as a model for the “secretory mucins”, which are normally produced and secreted by goblet cells and, together with other gel-forming mucins, are the major constituents of mucus. We also chose MUC1 and MUC4 as models for the “membrane-bound mucins”, which are transmembrane glycoproteins with anti-adhesive properties, exerting a role in tumor progression and metastasis.

Under our experimental conditions, 25.9% of MSI-H/MSI-L CRCs displayed DNA anomalies restricted to the genes coding for the secretory gel forming mucins. It is noteworthy that a major involvement of the secretory mucins in the pathogenesis of the unstable tumors has also been suggested by Biemer-Hüttemann et al. [3], who reported selective increased expression of the MUC2 and MUC5AC mucins, but not MUC1 and MUC4, in sporadic CRCs with MSI-H. This immunohistochemical study was realized by means of monoclonal antibodies directed toward the VNTR region (MUC2) or the COOH-terminus (MUC5AC) of the corresponding apomucins. However, it is not clear if this high immunoreactivity derives from an increased mRNA/protein synthesis or an increased exposure of the core protein epitopes due to changes in glycosylation or structural modifications of the apomucin.

In at least 4 of our MUC-mutated tumors (C210T, C213T, C480T and CRC10T) the relative amount of the anomalous fragments was considerable, suggesting that one of the two MUC alleles can be abnormal in at least 50% of tumor cells (100% in CRC10T). This observation opens the question on the possible existence of a positive selective pressure in favor of the unstable tumor cells with a MUC-gene anomaly. Notably, nullizygous mice with targeted inactivation of the MUC2 gene displayed aberrant intestinal crypt morphology, altered cell maturation and migration and increased tumor formation with spontaneous progression to invasive cancer [24]. Thus, normal MUC2 gene could really be involved in the suppression of colon carcinogenesis.

The exact nature of the VNTR abnormal fragments is presently unknown, but their discovery restricted to MSI tumors implies that the effect of MMR gene inactivation in some CRCs may go beyond the classical mutator phenotype, characterized by alteration in the size of microsatellite DNA sequences. In some cases, they could simply derive from point mutations generating new RFLPs. This could be the case of MUC5AC gene, which displayed abnormalities with only one or the other of the two enzymes used in the analysis (HinfI or PvuII), cutting inside a complex sequence with tandem repeats alternating to Cys-rich domains. Alternatively, the aberrant fragments could be due to a real change of the VNTR size and this could have occurred for some MUC2 mutations. The MUC2 gene exhibits a VNTR size polymorphism that is easily detectable by the enzyme HinfI, whereas changes of the VNTR length are expected to affect the TaqI digestion-pattern mainly at the level of the smaller fragments (≤50 kb) containing multiple repeats of the tandem repeat units [23]. Unfortunately, qualitative and/or quantitative variations of this pattern were hardly appreciable on our experimental conditions, and this did not allow us to definitely con-
firm the VNTR size changes with two different digestion approaches. However, TaqI enzyme also revealed a sequence RFLP located within the 3' portion of VNTR region[23] and the novel 1.7 kb TaqI-fragment in tumor C210T and the apparent loss of heterozygosity in tumor C213T (Fig. 1) could also be related to the MUC2 VNTR mutations/rearrangements detected by HinfI.

Defective processes of homologous recombination might explain our findings in some MSI tumors. In fact, apart from postreplicative mismatch correction, MMR proteins coordinate a complex network of physical and functional interactions that can be relevant to carcinogenesis[2], including post-replicative DNA-damage signaling and apoptosis[12,26], base excision repair[5] and prevention of recombination between nonidentical sequences (homeologous recombination)[21]. In agreement with these theories, in yeast MMR machinery imposes a barrier to recombination between sequences of low divergence[6]. Accordingly, loss of MMR in mammals results in an increased frequency of recombination between diverged sequences and in longer gene conversion tracts[7,8]. Homologous recombination is an important DNA-repair pathway leading to correction of double-strand-breaks (DSBs) produced by exogenous damaging agents and by replication errors, and it has been recently suggested that deletion or insertion mutations associated with DSBs represent a novel mechanism by which MSH2-deficiency could promote cancer-associated mutations[25]. Thus, our findings might be consistent with a direct role of the MMR system in the control of reparative recombination involving VNTR sequences.

Alternatively, MMR deficiency itself could not be directly responsible for the observed effect, but loss of correct homologous recombination or other DSB repair processes could be the result of events downstream the loss of MMR, such as mutations of RAD50, BLM, MRE11 and XRCC2 target genes[10,17,19]. According to this hypothesis, 5 MUC-mutated tumors had also RAD50 (2 cases) and/or MRE11 (4 cases) somatic mutations[10].

In conclusion, this study indicates a possible link between MSI and MUC gene abnormalities and suggests that alteration and/or loss of structurally normal MUC genes may be an important step in the neoplastic molecular pathway of a subset of colon tumors. Although frameshift mutations in several growth regulatory target genes represent the most significant consequence of the mutator phenotype, mutations involving VNTR repetitive sequences may also exist in MSI tumors as a direct and/or indirect consequence of an inefficient MMR system.

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References


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