The effect of mismatch repair deficiency on tumourigenesis; microsatellite instability affecting genes containing short repeated sequences

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Abstract. We have investigated microsatellite instability (MSI) in colorectal, gastric, endometrial and ovarian cancer as a result of mismatch repair (MMR) deficiency. We detected frameshift mutations in several genes that carry short repeated sequences and are important in cell fidelity and growth control; hMSH3, hMSH6, BAX, IGFIIR, TGFBIIR, E2F4 and BRCA2. Accumulation of mutations was heterogeneous and mainly restricted to tumours showing MSI at several loci (MSI-H). Both insertions and deletions were evident and occasional intratumour heterogeneity was evident with more than one different additional allele in the tumour. Most MSI-H tumours had acquired mutations in more than one gene and longer repeated sequences were more frequently targets for mutations. The TGFBIIR gene was mutated in 62%, the hMSH3 gene in 43%, the E2F4 gene in 35%, the hMSH6 in 32%, the BAX gene in 32%, the IGFIIR gene in 26%, and the BRCA2 gene in 2% of the MSI-H tumours. Homozygous mutations or mutation of both alleles were evident in all genes except BRCA2, in total 23/105 mutated cases, varying from 7% for BAX to 50% for E2F4. E2F4 mutations were exclusively found in colon tumours and E2F4 polymorphisms was found in 8% of cases. No difference in mutation prevalence was noted between cancer types apart from TGFBIIR mutations, which were frequently found in colon and gastric tumours but not in endometrial tumours, suggesting that endometrial tumours progress by a different route where TGFBIIR mutations are less favourable.

Introduction

Lack of MMR efficiency is observed in a subset of several cancer types, especially colon cancer (1). Colon cancer has been linked to germline mutations in the MMR genes hMSH2, hMLH1, hPMS1, hPMS2 and hMSH6, the human homologues of the *Escherichia coli* MutS and MutL gene families (2,3). Loss of MMR causes cancer indirectly by increasing the mutation rates of genes involved in regulation of cellular growth and/or invasion. Associated with MMR deficiency is the MSI phenotype that is easily detected as shifts in mobility of microsatellite alleles on acrylamide gels where extra bands are present in the tumours. Studies have shown MSI in most tumours from patients with hereditary non-polyposis colorectal cancer (HNPCC) and also to a lesser extent in sporadic colorectal cancer (CRC), as well as other cancer types, some of which are associated with HNPCC (1,4-6). Because of different clinical and pathological features, it has been recommended that tumours should be classified according to the number of markers showing MSI; high frequency MSI (MSI-H) if majority of markers show instability; low frequency MSI (MSI-L) if minority of markers show instability; microsatellite stable (MSS) if all markers are stable (7). There is a unique clinical and pathological phenotype associated with MSI-H but the distinction between MSI-L and MSS is less clear (3).

The human genome has a variety of repetitive elements ranging in size, located in coding and non-coding regions. Genes carrying short tandem repeats in their coding regions are especially prone to mutations in MMR deficient cells, resulting in deletions and insertions that cause frameshifts and premature stop signals in the proteins. This includes the MMR genes hMSH3 and hMSH6 (8) which have tracts of 8 consecutive A and C, respectively, in their coding region. These genes are components of the hMSH2-dependent MMR pathway and contribute to the stabilisation of microsatellite sequences. BAX is a member of the BCL2 gene family that promotes apoptosis. It has a tract of (G)8 that has been found mutated in more than 50% of MSI-H colon tumours (9).

Transforming growth factor- β (TGF β) has an anti-proliferative effect on epithelial cells, mediated by two

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receptors TGF β IR and TGF β IIR (10). The 10 polyadenine cluster region within TGF β IIR has been found mutated in colon and gastric cancer but less frequently in endometrial cancer (11,12). In addition, the gene contains a stretch of 3 GTs which is also a hot spot for mutations (11). The product of the insulin-like growth factor II receptor gene (IGFIIR) is essential for the TGF β growth control pathway (13). Mutations of the (G)₈ repeat within the IGFIIR gene have been associated with gastric cancer progression (14).

E2F4 is a member of a family of transcriptional factors regulating a number of genes involved in cell cycle control and DNA synthesis (15). The gene contains a repeated trinucleotide sequence (CAG) encoding a stretch of 13 consecutive serines (16). There have been indications that E2F4 has oncogenic effect (16). Somatic mutations in the E2F4 gene have been found in CRC and gastrointestinal tumours (17,18). Germline mutations in the BRCA2 gene predispose to breast cancer but mutations are rare at the somatic level (19). The gene contains in its coding region several short repeated sequences that are putative targets for mutations associated with MMR deficiency.

The aim of our study was to compare the frequency and effect of MMR deficiency with respect to multiple gene mutations in cancer types reported to appear in HNPCC, such as: colorectal, gastric, ovarian and endometrial cancer. We assessed the MSI status in the tumours and examined somatic frameshift mutations in 7 genes (hMSH3, hMSH6, BAX, IGFIIR, TGF β IIR, E2F4 and BRCA2). These genes all carry small repeated sequences in their coding regions and are mutational targets in tumours of the microsatellite mutator phenotype.

Materials and methods

Samples. Included in the study were 191 tumour and corresponding normal samples, comprising of 92 colorectal, 49 gastric, 33 ovarian and 17 endometrial tumours. The mean age at diagnosis was 61.5 years for colorectal cancer patients (partly age selected in order to increase the number of MSI-H cases), 71.9 years for gastric cancer patients, 58.2 years for ovarian cancer patients and 64.7 years for endometrial cancer patients. Tissue was obtained fresh on the day of surgery or from paraffin-embedded material. DNA for PCR was prepared using the proteinase K treatment (20).

Microsatellite instability. For MSI screening we used 9 polymorphic markers distributed over the genome, obtained from Research Genetics: D2S123, D2S382, BAT26, D3S1210, D3S1217, D6S292, D7S480, D9S157 and D13S153. A tumour was considered MSI-H if two or more markers exhibited MSI, MSI-L if one marker exhibited MSI and if all markers were stable the tumour was considered MSS (7).

Mutation screening. Mutational screening was performed on 9 short repeated sequences located in the coding regions of 7 genes. The following repeats were amplified; hMSH3 codons 381-383 (A)₈; hMSH6 codons 1116-1118 (C)₈; BAX codons 38-41 (G)₈; IGFIIR codons 1314-1316 (G)₈; TGF β IIR codons 125-128 (A)₁₀ and codons 532-534 (GT)₃;

Figure 1. The frequency of MSI-H, MSI-L and MSS in colorectal, gastric, ovarian and endometrial tumours. n, total number of tumours.

E2F4 codons 307-319 (CAG)₁₃; BRCA2 codons 602-604 (A)₈ and codons 3030-3032 (A)₈. The markers have been described previously (8,9,12,14,17,21) except for the BRCA2 markers (codons 602-604; 5'GCAGGTTTAATATCCACTT TGAA'3 and 5'AGTTCTGATTTTGGTCTTTTCG'3; codons 3030-3032; 5'CATCTTGCAACTTCAAAATCTAA'3 and 5'CATCTGAAACCGGTAGTTGTTG'3), manufactured by TAG Copenhagen A/S.

The amplified PCR products were subjected to electrophoresis on a denaturing 6% acrylamide gel, transferred to a N⁺ hybond membrane (Amersham, UK) and detected using the enhanced chemiluminescence (ECL) technique (Amersham, UK). This analysis enables identification of both deletions and insertions as small as 1 nt.

From the intensity of the bands we could estimate that some tumours had greater intensity of mutant than normal bands and thus could be scored as two allele mutants. Occasionally it was difficult to determine the heterozygous or homozygous nature of the mutations, presumably due to the existence of normal cells in the tumour samples.

Fisher's exact two tailed test was used to evaluate mutations in different cancer types and also to assess the relationship between mutations and MSI. For statistical calculations MSI-L and MSS were combined against MSI-H. A χ^2 test was used to compare the frequency of polymorphisms between patients and a control group.

Sequencing. Tumours with aberrantly migrating bands were subjected to sequencing using Thermo Sequenase radio-labelled terminator cycle sequencing (Amersham, UK). PCR products were prepared by enzymatic clean-up by mixing with exonuclease I (10.0 U/ μ l) and shrimp alkaline phosphatase (2.0 U/ μ l), incubated at 37°C for 15 min and the exonuclease inactivated by heating to 80°C for 15 min. The reaction mixture was composed of 1 μ l template, 2 μ l of 10X reaction buffer provided by the manufacturer, 1 μ l primer (0.5 μ M), 2 μ l Thermo Sequenase polymerase (4 U/ μ l) and a mixture of dNTPs and [α -³²P]ddNTP, in a total volume of 20 μ l. This was subjected to 30 cycles comprising of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final extension at 72°C for 5 min. The products was electrophoresed on a 6% denaturing acrylamide gel and exposed to X-ray film.

Figure 2. Alterations of microsatellite sequences. N, normal DNA; T, tumour DNA. Case numbers shown above each matching normal-tumour pair. In each case novel alleles are evident in the tumour tissue. A, mutations in the hMSH6 gene, tumour 531 has no mutation, tumour 582 has two additional alleles with 1 bp insertion and 1 bp deletion and loss of the wild-type copy, tumour 645 contains heterozygous 1 pb insertion. B, mutations in the BAX gene, tumour 462 has no mutation, tumour 481 contains two additional alleles with 1 and 2 bp deletions, tumours 98 and 99 have 1 bp heterozygous deletions. C, mutations in the IGFIIR gene, tumour 582 has no mutation, tumour 645 has 1 bp heterozygous deletion and tumour 481 has 1 bp heterozygous insertion.

Results

Fig. 1 shows the results of the MSI screening. A total of 191 tumours were screened and 47 (25%) were MSI-H. Out of 92 colorectal samples 35 (38%) were classified as MSI-H, also 8/49 (16%) gastric, 1/33 (3%) ovarian and 3/17 (18%) endometrial samples. All samples were screened for mutations in the repeats of the 7 genes; hMSH3, hMSH6, BAX, IGFIIR, TGFBIIR, E2F4 and BRCA2.

Frameshift mutations were mainly restricted to tumours showing MSI at several loci. Out of 47 MSI-H tumours 42 (89%) had mutations in at least one gene but also 2/144 (1.4%) of the MSI-L/MSS tumours ($p < 0.001$, Fisher's exact test). One MSI-L colon tumour sample had a 1 bp deletion in the BAX gene and one MSS ovarian cancer sample had a 1 bp insertion in the hMSH6 gene.

Figure 3. Cycle sequencing of the hMSH3 gene. N, normal DNA; T, tumour DNA. Case numbers shown above each sample. The normal sample no. 234 contains an eight-adenine microsatellite, tumour 234 contains a 1 bp heterozygous deletion and tumour 582 has a homozygous 1 bp deletion.

Table I shows the results from the mutation screening of tumours showing MSI-H and mutations in at least one gene. Four MSI-H colorectal tumours and one ovarian tumour did not show mutations in any of the 7 genes. Both small insertions and small deletions were found in MSI-H tumours (Figs. 2 and 3). The longer repeated sequences were preferentially a target for mutations as represented by the frequent mutations (62%) in the 10 polyadenine cluster region in the TGFBIIR gene in the MSI-H tumours. The frequency of detected gene mutations in MSI-H tumours was: hMSH3, 43%, hMSH6, 32%, BAX, 32%, E2F4, 35%, IGFIIR, 26%, and BRCA2, 2%. No mutations were found in the GT repeat of the TGFBIIR gene. One mutation was found in the BRCA2 gene, in an MSI-H endometrial sample (no. 609) that had a 1 bp insertion in the (A)8 stretch at codons 3030-3032.

Mutations in at least one gene were found in 31/35 (89%) of MSI-H colon tumours. hMSH3 was mutated in 15/34 (44%), hMSH6 in 10/35 (29%), BAX and IGFIIR in 11/35 (31%) and TGFBIIR(A)10 in 22/35 (63%) of colon tumours. E2F4 was only mutated in colon tumours (41%). Eight gastric

Table I. MSI-H samples with gene mutations.

Sample no.	Tissue type	MSI	hMSH3 ^a (A)8	hMSH6 ^a (C)8	BAX ^a (G)8	IGFIIR ^a (G)8	TGFBIIR ^a (A)10	E2F4 ^a (CAG)7-14		Total
			T	T	T	T	T	N	T	
9	Colon	3/9	8	8	8	8	10, 11	13	13	1/6
11	Colon	4/9	8	9 ^b	8	8, 9	11 ^b	13	13, 14	4/6
12	Colon	8/9	8	8	8	8, 9	9 ^b	13	13	2/6
25	Colon	6/6	7 ^b	7, 8, 9 ^c	7, 8	8	9 ^b	13	10, 13	5/6
26	Colon	2/3	8	8	7, 8	7, 8	9, 10	13	11 ^b	4/6
28	Colon	6/6	8	8	8	8	10, 11	13	13	1/6
31	Colon	8/9	8	8	8, 9, 10 ^c	8	9, 10	13	12, 13	3/6
38	Colon	3/7	8	8	8	8	10	13	13, 14	1/6
39	Colon	2/9	8	8, 9, 10 ^c	7, 8	7, 8	10	13	13	3/6
47	Colon	3/7	8	8	8	8	10	7, 13 ^d	7, 13	1/6
49	Colon	7/9	7, 8	8	8	8	9, 10	13	11, 12, 13 ^c	3/6
50	Colon	4/9	8	8	8	8	9, 10	13	13	1/6
55	Colon	9/9	NI	8	7, 8	7, 8	9, 10	13	13	3/5
69	Colon	4/9	8	8	8	8	10	13, 14 ^d	13, 14	1/6
92	Colon	4/6	7, 8	8	7, 8	8	10	13	13	2/6
94	Colon	7/8	7, 8	7 ^b	8	8, 9	8, 9, 10 ^c	11, 13 ^d	11, 13	4/5
95	Colon	4/4	7, 8	8, 9	7, 8	8	9, 10, 11 ^c	13	11, 12, 13 ^c	5/6
98	Colon	7/7	7, 8	8, 9, 10 ^c	7, 8	8, 9	8, 9, 10 ^c	13	13, 14	6/6
99	Colon	7/7	7, 8	7, 8	7, 8	7, 8	9, 10	13	10 ^b	6/6
139	Colon	6/9	6, 8	8	8	8	9, 10	13	12 ^b	3/6
234	Colon	5/7	7, 8	8	8	8	8, 10	13	13	2/6
394	Colon	5/8	8	7, 8	8	8	10	13	13	1/6
481	Colon	6/8	8	8	6, 7, 8 ^c	8, 9	8, 10	13	12 ^b	4/6
582	Colon	7/8	7 ^b	7, 9 ^{b,c}	8	8	9, 10	13	12 ^b	4/6
645	Colon	6/6	6, 7, 8 ^c	8, 9	8	7, 8	9, 10	13	12 ^b	5/6
705	Colon	3/8	8	8	8	8	9 ^b	13	13	1/6
778	Colon	6/7	7 ^b	8	7, 8	8	10	13	14 ^b	3/6
779	Colon	7/8	7, 8	8	8	7, 8	8, 10	13	13	3/6
718	Colon	2/8	8	8	8	8	10	13, 14 ^d	13, 14	1/6
826	Colon	3/4	7, 8	8	8	8	10	13	13	1/6
891	Colon	9/9	8	8	8	8	9 ^b	13	13	1/6
283	Gastric	7/9	8	8	7, 8	8	8, 9, 10 ^c	13	13	2/6
314	Gastric	5/8	8	8, 9	8	8	10	13	13	1/6
451	Gastric	4/4	7 ^b	8, 9	9 ^b	9 ^b	11 ^b	13	13	4/6
474	Gastric	8/9	7, 8	8	8	8, 9	10, 11	13	13	3/6
556	Gastric	6/9	7, 8	7, 8	8	8	9, 10	13	13	3/6
750	Gastric	7/8	8	8	7, 8	8	9, 10	13	13	2/6

Table I. Continued.

Sample no.	Tissue type	MSI	hMSH3 ^a (A)8	hMSH6 ^a (C)8	BAX ^a (G)8	IGFIIR ^a (G)8	TGFβIIR ^a (A)10	E2F4 ^a (CAG)7-14		Total
			T	T	T	T	T	N	T	
808	Gastric	3/3	8	8	8	8	9, 10, 11 ^c	13	13	1/6
904	Gastric	3/5	7, 8	8	7, 8, 9 ^c	8	9, 10	13	13	3/6
326	Endom.	9/10	7 ^b	8	8	8	10	13	13	1/6
607	Endom.	9/10	8	8, 9	8	8	10	13	13	1/6
877	Endom.	6/9	8	8, 9	8	8	10	13, 14 ^d	13, 14	2/6
			5/19	3/15	1/15	1/13	6/29	7/14		23/105
Double hit (%)			(26)	(20)	(7)	(8)	(21)	(50)		(22)

MSI, number of markers exhibiting microsatellite instability. N, non-tumour tissue; T, tumour tissue; NI, non-informative. ^aNumbers beside columns represent the number of repeats detected within the given gene. ^bTumour showing loss of wild-type allele, presumably representing mutation at both alleles or mutation of one allele and loss of the other. ^cTumour showing heterogeneity with respect to allele size. ^dPolymorphism.

tumours were identified as MSI-H (16%) and all of them had a mutation in at least one of the tested genes. The gene repeat most frequently mutated in these gastric tumours was TGFβIIR(A)10 in 7/8 (88%) but hMSH3 mutations were found in 4/8 (50%) and BAX mutations in 4/8 (50%) of the tumours. hMSH6 was mutated in 3/8 (38%) and IGFIIR in 1/8 (13%) of gastric tumours. Three out of 17 (18%) endometrial tumours showed MSI-H, hMSH3 and hMSH6 were mutated in 1/3 (33%) and 2/3 (67%) of these tumours, respectively, but mutations in other gene repeats were not found. Only 1 of the 33 (3%) ovarian samples showed MSI but none of the investigated genes were mutated in this sample.

In tumours showing MSI, the mutation spectrum was similar in the different tumour types except for TGFβIIR(A)10 where mutations were significantly less frequent in endometrial cancer compared to colon (p-value 0.02, Fisher's exact two-tailed test) and gastric (p-value 0.007, Fisher's exact two-tailed test) tumours. Also, mutations in the E2F4 gene were restricted to colon cancer.

Mutations in the E2F4 gene were evident in 14 colon MSI-H tumour samples but the repeat length was found to be polymorphic in all tissue types, both MSI-H and MSI-L/MSS samples. The polymorphism was found in 8% of all samples, 7/92 colorectal, 4/49 gastric, 1/33 ovarian and 3/17 endometrial samples. In these cases the non-tumour samples showed the most common band containing the 13 CAG repeat and an additional band (7, 11 or 14 CAGs) at equal intensity. There was no significant difference in the frequency of polymorphisms found in these patients and a control group, 15/191 and 10/169 respectively (p-value 0.48, χ^2 test).

In some instances intratumoural heterogeneity was evident with respect to mutations, represented by more than one additional allele detected in the tumour (Table I). Mutations at both alleles or mutations at one allele and deletion of the wild-type allele was detected in all genes except BRCA2, in total 23/105 (22%) mutated cases; E2F4, 7/14 (50%); hMSH3, 5/19 (26%); TGFβIIR, 6/29 (21%); hMSH6, 3/15 (20%); IGFIIR, 1/13 (8%) and BAX, 1/15 (7%). One gastric tumour (no. 451) had homozygous mutations in 4 genes; hMSH3, BAX, IGFIIR and TGFβIIR. One tumour (no. 582) showed two additional alleles for hMSH6 and loss of the wild-type copy (Fig. 2).

Discussion

MSI is thought to be caused by lack of mismatch repair efficiency and is associated with HNPCC. We analysed MSI and gene mutations in HNPCC tumours i.e. colorectal, gastric, ovarian and endometrial tumours. In our study colorectal tumours showed a high frequency of MSI-H which can partly be explained by a low mean age at diagnosis in the selected patient group. Our data show that tumour progression is different in MSI-H than in MSI-L/MSS tumours, and that mutations in short repeated sequences within several genes are specifically associated with cancer of the microsatellite mutator phenotype. Also longer mononucleotide repeats appear to be more susceptible to mutations, suggesting that these mutations are the result of instability rather than selection. We find that most of the MSI-H samples had acquired mutations in more than one gene tested. In MSI-H tumours, the polyadenine cluster region (A)10 in the TGFβIIR gene was most frequently

mutated. This may reflect the importance of TGF β IIIR as a tumour suppressor gene, or it may be because it has the longest repeat. In a large proportion of tumours we observed homozygous mutations or mutations of both alleles of the given gene. This was evident in all genes analysed, except BRCA2, and a particularly high proportion of tumours carrying a mutation in the E2F4 gene showed double hit mechanism. Mutations of both alleles fit well with the general idea that inactivation of a tumour suppressor gene takes place prior to tumour progression. The detection of a homozygous mutation in the tumour material analysed here could possibly be due to the same structural alteration of both copies of the gene, or to mutation at a short repeated sequence at one allele and loss of the other allele. The case where two alleles are detected within the same short repeated sequence distinct to wild-type, probably represents a different mutation at each allele although the possibility of two distinct carcinoma subpopulations existing within the tumour, with different mutations, cannot be excluded.

All 7 genes analysed are important for maintaining genome integrity, in the regulation of cellular growth or for tumour cell invasion in cancer. Mutations in the MMR genes hMSH3 and hMSH6 occur in 55% of the MSI-H tumours. These mutations could facilitate further acceleration of mutational rate and speed up tumour progression. BAX mutations may block the capability to trigger apoptosis upon receiving a death signal. We find one gastric tumour homozygous for BAX mutations and possibly totally lacking a functional protein but heterozygous BAX mutations may also contribute to tumour progression due to reduced BAX-BLC2 ratio (9).

TGF β is connected to the IGFIIIR pathway and inhibits the growth of multiple epithelial cells. When growth control is lost, cells are more likely to grow uncontrolled and to become tumorigenic. We found that 62% of the MSI-H tumours have acquired mutations in either or both TGF β IIIR and IGFIIIR that might affect their growth control pathways. Mutations in TGF β IIIR are frequent in colon and gastric tumours but are not detected in endometrial tumours, supposing a different route for endometrial tumours to progress compared to colon and gastric tumours.

The serine repeats in the E2F4 gene are positioned in the least conserved segment of the gene, which is likely to function as a transactivation domain (15). Mutations were found exclusively in colon tumours and polymorphism was detected in all tissue types. These polymorphisms have all been described previously (22). The number of serine repeats does not seem to have a major effect on the protein function even though occasionally only 7 CAGs are left in the gene. Somatic mutations in the BRCA2 gene seem to be of minor importance in the progression of these tumour types since mutation was found only in one endometrial tumour.

Six of the MSI-H colorectal samples have a known family history of colorectal and gastric cancer. All of them have at least one gene mutated. Two of them (no. 98 and 99) have accumulated mutations in 6 genes and have also a very strong MSI phenotype (7/7). These samples are from sisters with a family history of colon cancer.

In this study we screened for mutations in 7 genes and found that the accumulation of mutations was heterogeneous, which

could reflect the underlying basis of the MMR deficiency, for example if different MMR genes were mutated. There are short tandem repeats in the coding region of additional genes that could play a critical role in selecting pathways for tumour progression. The MSI-H tumours found with no mutations may progress through different pathways with mutations in other genes. Further studies will help in identifying additional targets of MSI and to further clarify the role of MMR in cancer progression.

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