ORIGINAL ARTICLE

Screening for microsatellite instability target genes in colorectal cancers

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Background: Defects in the DNA repair system lead to genetic instability because replication errors are not corrected. This type of genetic instability is a key event in the malignant progression of HNPCC and a subset of sporadic colon cancers and mutation rates are particularly high at short repetitive sequences. Somatic deletions of coding mononucleotide repeats have been detected, for example, in the *TGFβRII* and *BAX* genes, and recently many novel target genes for microsatellite instability (MSI) have been proposed. Novel target genes are likely to be discovered in the future. More data should be created on background mutation rates in MSI tumours to evaluate mutation rates observed in the candidate target genes.

Methods: Mutation rates in 14 neutral intronic repeats were evaluated in MSI tumours. Bioinformatic searches combined with keywords related to cancer and tumour suppressor or CRC related gene homology were used to find new candidate MSI target genes. By comparison of mutation frequencies observed in intronic mononucleotide repeats versus exonic coding repeats of potential MSI target genes, the significance of the exonic mutations was estimated.

Results: As expected, the length of an intronic mononucleotide repeat correlated positively with the number of slippages for both G/C and A/T repeats (p=0.0020 and p=0.0012, respectively). *BRCA1*, *CtBP1*, and *Rb1* associated *CtIP* and other candidates were found in a bioinformatic search combined with keywords related to cancer. Sequencing showed a significantly increased mutation rate in the exonic A9 repeat of *CtIP* (25/109=22.9%) as compared with similar intronic repeats ($p \leqslant 0.001$).

Conclusions: We propose a new candidate MSI target gene *CtIP* to be evaluated in further studies.

Germline mutations in at least five DNA mismatch repair genes (*MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6*) play a role in susceptibility to hereditary non-polyposis colorectal cancer (HNPCC).[1–6] Defects in the DNA repair system lead to genetic instability and a mutator phenotype. This type of genetic instability is a key event in the malignant progression of HNPCC and a subset of sporadic colorectal cancers, and mutation rates are particularly high at short repetitive sequences. The phenomenon is called microsatellite instability (MSI).[7–10]

Deletions and insertions in coding region mononucleotide tracts lead to frameshift type mutations and to truncated protein products. Mutations in MSI target genes should give a growth advantage to cells carrying these defects, thus favouring their selection and subsequent formation of tumours. Approximately 90% of the DNA mismatch repair deficient colorectal carcinomas (CRCs) display mutations in the *TGFβRII* gene.[11] Somatic deletions of coding mononucleotide repeats have been detected, for example, in the *IGFIIR* and *BAX* genes.[12 13] Also mononucleotide repeats in DNA mismatch repair genes *MSH6* and *MSH3* have been found to be mutated.[14] In previous works, *TCF-4*, *CHK1*, *RIZ*, *MBD4*, *BLM*, *Caspase-5*, *PTEN*, *FAS*, *APAF-1*, *BCL-10, RAD50*, and *WISP-3* have all been proposed to be MSI target genes.[15–25] In addition, Mori *et al*[26] and Park *et al*[27] have recently proposed several novel candidates. Explorations of the genome for additional targets of MSI would increase understanding of the mutator pathway in human tumorigenesis.

Many studies have been carried out to identify new genes with somatic alterations in MSI tumours. According to Boland *et al*,[28] MSI target genes should fulfil five criteria: a high mutational frequency, biallelic inactivation, a role in a growth suppressor pathway, alterations in MSI negative tumours also, and in vitro or in vivo functional evidence. These criteria have been discussed and the idea of haploinsufficiency has also recently been presented.[29 30] High mutational frequency and functional evidence have been accepted without ambiguity as criteria for novel MSI target genes.

By comparison of intronic mononucleotide repeats with exonic repeats of potential target genes, the significance of selection can be estimated, though a caveat to this approach is the varying mutation frequency in intronic mononucleotide repeats.[31] Mutations in the intronic repeats are most likely not selected for because they typically do not have an effect on tumour development. Comparing the results of exonic repeats to these intronic controls and published data on mutation rates in other mononucleotide repeats of identical length should assist in showing the possible selection for mutated exonic mononucleotide repeats.

In this work we present our results on background mutation level in MSI tumours. We screened 14 intronic $(A/T)_{6-9}$ and $(G/C)_{6-9}$ mononucleotide repeats from MSI positive CRCs to gain more understanding on the background mutation rates of various repeats. In light of the results of this effort, two previously proposed MSI target genes with relatively low reported mutation frequencies, *IGFIIR* (G8) and *BLM* (A9), were also scrutinised. In addition, bioinformatic searches were used to identify novel candidate MSI target genes. As a result of the search combined with keywords related to cancer, and subsequent laboratory analyses, a new candidate MSI target gene *CtIP* is proposed.

MATERIALS AND METHODS

Intronic mononucleotide repeats and known MSI target genes

First, monomorphic intronic mononucleotide repeats were screened from 93 MSI positive colorectal cancers derived from

Abbreviations: CRC, colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability

Table 1 Intronic mononucleotide repeats were screened from 93 samples of tumour DNA of MSI positive colorectal cancer patients. 0–5.7% of samples were mutated in mononucleotide repeats and the length of an intronic mononucleotide repeat correlated positively with the number of slippages for both G/C and A/T repeats (p=0.0020 and p=0.0012, respectively)

Intronic repeat	Gene (GenBank accession No)	Mutations
G/C 6	*LPAP* (*CD45-binding protein*) (X97267)	0/93=0%
G/C 6	*Fast skeletal troponin T* (*TNNT3*) (AF026276)	0/93=0%
G/C 7	*Human blood coagulation factor XII gene* (M17466)	2/93=2.2%
G/C 7	*Ciliary neurotrophic factor alpha receptor* (HSCNFAR05)	0/92=0%
G/C 8	*CMP-N-acetylneuramini acid hydroxylase* (AB009668)	2/81=2.5%
G/C 9	*Regulatory factor 2 of sodium/hydrogen exchanger isoform A3* (AB016243)	4/84=4.8%
A/T 6	*Neurofibromatosis 1* (*NF1*) (AF004526)	0/93=0%
A/T 6	*DNA topoisomerase II beta* (*TOP2B*) (AF087143)	0/93=0%
A/T 7	*LPAP* (*CD45-binding prot*) (X97267)	0/93=0%
A/T 7	*Leptin receptor* (*OBR*) (U62501)	0/93=0%
A/T 8	*DNA topoisomerase II beta* (*TOP2B*) (AF087143)	5/93=5.4%
A/T 8	*Chemokine receptor* (*CXCR-4*) (AF005058)	0/93=0%
A/T 9	*EWS* (AB016208)	5/88=5.7%
A/T 9	*Osteonidogen* (AB009814)	4/93=4.3%

a population based series of 1044 colorectal cancers,[32 33] to determine the background mutation rate of 14 $(A/T)_{6-9}$ and $(G/C)_{6-9}$ tracts in MSI tumours. Studied intronic mononucleotide repeats are shown in table 1. Second, previously recognised MSI target genes *IGFIIR* and *BLM* were scrutinised, because the reported mutation frequencies are relatively low (9.3%-22% and 16%-18%, respectively).[12 19 20] The MSI status of the tumours had been determined by using the BAT26 mononucleotide marker. *MSH2* mutations had been excluded by genomic sequencing, and *MLH1* mutations had been found from 17 cases.[32 33]

PCR reactions of intronic mononucleotide repeats as well as *IGFIIR* were carried out in 10 µl of reaction volume containing 50 ng genomic DNA, 10 × PCR buffer (Applied Biosystems (AB), Branchburg, NJ), 1 µmol/l of each primer, dCTP at 20 µmol/l, 200 µmol/l each of dATP, dGTP, dTTP (Amersham Pharmacia Biotech, Piscataway, NJ), 0.7 µCi [α-^{32}P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England), and 0.5 units of Ampli*Taq*GOLD polymerase (AB). The $MgCl_2$ concentrations were 1.25-5 mmol/l. The following PCR cycles were used for amplification: 95°C for 10 minutes, 37 cycles of 95°C for 45 seconds denaturation, 59°C or 61°C for 30 or 45 seconds annealing, and 72°C for one minute extension. Final extension was 72°C for 10 minutes. Primer sequences and PCR conditions are available upon request. Ten µl of PCR product was mixed with 7 µl loading buffer, and the 53-126 bp PCR products were run on 6.7% polyacrylamide gels; 0.6 × TBE was used as a running buffer in the gel run at a constant power of 80 watts. All aberrant bands were sequenced, and in all aberrant cases the corresponding normal DNA was analysed to confirm the somatic nature of the changes. The enzyme used in the PCR for sequencing was Pfu Turbo polymerase (Stratagene, La Jolla, CA), and the PCR products were about 200-300 bp in length. ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing ready reaction kit (AB) were used for direct sequencing. Cycle sequencing products were electrophoresed on 6% Long Ranger gels (FMC BioProducts) and analysed on an Applied Biosystems model 373A, 377 or 3100 automated DNA sequencer (AB).

For sequencing *BLM* A9 mononucleotide repeats, the following touch down PCR cycles were used for amplification: 95°C for one minute, four cycles of 95°C for 45 seconds denaturation, annealing temperature 65-61°C for 45 seconds, and 72° for 45 seconds extension, 30 cycles of 95°C for 45 seconds denaturation, annealing temperature 61°C for 45 seconds, and 72°C for 45 seconds extension. Final extension was 72°C for 10 minutes. Primer sequences are available upon request.

Novel candidate MSI target genes

Thirdly, we applied bioinformatic techniques to identify novel candidate MSI target genes. Human RNA sequences submitted after 1997 were extracted from GenBank (release 116) primate division. Entries with the word "partial" in the description were discarded in order to decrease the sequence redundancy. The annotated coding sequences (CDS lines in the feature table) were searched for mononucleotide tracts of 8 bp or longer. Medline identifiers from the sequence records containing those repeats were used to obtain Medline records. Title, abstract, and MeSH terms were searched for 83 manually selected keywords related to cancer. This approach resulted in the identification of 251 sequences. All the steps above with the exception of compiling the list of keywords were done automatically with a script written in PERL. Other searches were based on sequence homology to 34 known tumour suppressor or CRC related genes. Data are available on request. The list of 251 and 841 hits, respectively, was further scrutinised by eye to select a smaller subset of candidates. Eight possible new MSI target genes were studied by PCR and sequencing (table 2).

Screening for novel MSI target genes was performed with a set of 30 MSI positive CRCs by direct sequencing of the repeat region. PCR reactions were carried out in a 50 µl reaction volume containing 150 ng genomic DNA, 10 × PCR buffer (AB), 500 µmol/l of each dNTP (Finnzymes, Espoo, Finland), 1 µmol/l of each primer, 1.25-5 mmol/l of $MgCl_2$, and 5 units of AmpliTaqGOLD polymerase (AB). The following PCR cycles were used for amplification: 95°C for 10 minutes, 37 cycles of 95°C for 45 seconds denaturation, annealing temperature for 45 seconds, and 72°C for 45 seconds extension. Final extension was 72°C for 10 minutes. The sequencing was performed as described earlier and the corresponding normal tissue DNA of the tumour cases with mutations was analysed. For *CtIP*, an additional 79 samples were sequenced (total 109 samples). Pfu Turbo proofreading polymerase (Stratagene) was used to confirm the nature of the mutations. Primer sequences, length of PCR fragments, annealing temperatures, and $MgCl_2$ concentrations are listed in table 2.

Statistical analysis

The independence and significance of correlation of mutation frequency with repeat length were estimated by logistic regression analysis. The occurrence/non-occurrence of mutation was considered as the dependent variable in each repeat length group. χ^2 test statistic was used for other analyses. $p<0.05$ was considered statistically significant.

Table 2 Screening for eight candidate MSI target genes was performed in MSI positive CRCs. Primer sequences, length of PCR fragments, annealing temperatures, and $MgCl_2$ concentrations are listed. Genes marked with • are from the search with keywords related to cancer, and genes marked with * from the tumour suppressor homology search

Gene (repeat) (GenBank accession No)	Function	Primer sequence	Length of PCR fragment (bp)	Annealing °C	$MgCl_2$ (mmol/ l)	Mutations
BCL10 (T7) (AF097732)•	Apoptotic signalling gene	F: agc cac gaa caa cct ctc c R: tgt aga tct ggt ggc aaa gg	240	59	1.25	0/29=0%
PTP-BAS (A8) (D21209)•	Protein tyrosine phosphatase	F: tgg gat ttc tgt cca tca aag R: caa cga cga tat gtg gca gt	250	61	2.5	0/30=0%
CtIP (A9) (U72066)•	CtBP (phosphoprotein) binding protein	F: ttg tcc cct tct ctt tta cag c R: ggg gct cca aat gtt tat ca	274	61	2.5	25/109=22.9%
BAI1 (C7) (NM_001702)*	Brain specific angiogenesis inhibitor	F: tct gcg tgt cct cct cct ac R: agg gca atg ttg cag aac tt	346	59	2.5	0/30=0%
P73 (C6) (AF077616)*	p53 related protein	F: gct gat gag gac cac tac cg R: ctt cac acc ggc acc aag	484	59	2.5	0/30=0%
GIOT-2 (T7) (AB021642)*	Transcription repressor	F: cgc tgg aaa gaa acc cta tg R: aaa cag gga agg ctt tag aac a	221	57	1.25	0/29=0%
PAIP (T6) (AF013758)*	Polyadenylate binding protein interacting protein	F: taa gcc cca ggt ggt tgt ag R: caa cca ttc agg gtc tct gc	228	61	2.5	0/30=0%
RIP3 (A6) (NM_006871)*	Receptor interacting serine/threonine kinase	F: tat ccc aga gtc agg cca ag R: ctg aag atg tgc ctg ctg tc	221	59	2.5	0/30=0%

RESULTS AND DISCUSSION

Intronic mononucleotide repeats

The results are summarised in table 1. As expected, the length of an intronic mononucleotide repeat correlated positively to the number of slippages for both G/C and A/T repeats ($p=0.0020$ and $p=0.0012$, respectively). Compatible with previous findings,[31] long G/C repeats ($n \geq 8$) were found to be more polymorphic than the respective A/T repeats. In general, the mutation frequencies were similar to the ones observed by Suzuki *et al*.[34] To facilitate interpretation, we focused on repeats which were monomorphic in the germline. A/T 10 and G/C 10 repeats were typically polymorphic in the germline and were not included in this study. Multiple G8 and G9 repeats were tested to detect monomorphic ones, and this selection is a potential cause of bias towards inclusion of relatively stable repeats. However, Suzuki *et al*[34] did not find any difference between the frequency of somatic mutations in polymorphic and monomorphic loci.

Known MSI target genes

Mutations in the G8 tract in the proposed MSI target gene *IGFIIR* were analysed in an MSI positive CRC panel of 92 cases. Mutations (+1bp/−1bp) of the coding G8 repeat were found in nine patients (9.8%). This finding is similar to other studies[12] and the observed mutation rate was higher than in the intronic G8 repeat (2.5%), but the difference did not reach significance. However, when taking into consideration the combined *IGFIIR* results from this and earlier studies (28/216=13%),[12 20 35] *IGFIIR* mutation rate and intronic mutation rate were significantly different ($p \leq 0.01$).

IGFIIR is a cell surface receptor which allows a serine proteinase granzyme B to enter cells and induce the apoptosis of target cells. The mutated G8 repeat causes a premature stop codon and cytotoxic T cell mediated apoptosis of target cells is prevented.[36] If apoptosis is prevented, cell growth is likely to be promoted.

Bloom's syndrome is a rare autosomal recessive disorder. The cells of BLM patients show genomic instability, and the patients have a high incidence of different cancers. BLM has DNA stimulated ATPase and ATP dependent DNA helicase activities. With RPA it is suggested to function in unwinding DNA during replication, recombination, or repair.[37] The Blooms syndrome gene's (*BLM*) coding A9 repeat has been reported to be mutated in MSI positive colon cancer samples (10/63=15.9%, 7/39=18%).[20 24] In this study, *BLM* was found to be mutated in 18 out of 93 (19.4%) samples. When comparing this mutation rate of *BLM* to intronic A9 repeats studied here, *BLM* appeared as a true target gene ($p \leq 0.001$). This finding is supported by the notion that Bloom's syndrome patients are predisposed not only to lymphomas and leukaemias, but also to colorectal tumours.[38]

Novel candidate MSI target genes

A total of 251 hits to genes with coding repeats were found by the MSI target gene search combined with keywords related to cancer, and 841 hits by search selecting for homology to 34 tumour suppressor or CRC related genes. Based on these two lists, publications were searched for interesting novel MSI targets. Three genes (*BCL10, PTP-BAS, CtIP*) and five genes (*BAI1, P73, GIOT-2, PAIP, RIP3*), respectively, were screened for mutations in mononucleotide repeats (table 2). Sequencing showed a significantly increased mutation rate in the A9 repeat in the middle of the *CtIP* gene (25/109=22.9%). The frequency of *CtIP* A9 as compared with intronic A9 mutations was significantly different ($p \leq 0.001$). Second hits at *CtIP* shorter mononucleotide repeats (5-6 bp) were searched for but not found (data not shown).

The screened MSI positive CRC samples did not contain uniformly mutated MSI target genes or intronic repeats; indeed different samples contained individual mutations. Mutations in different samples seemed to occur randomly. Correlation between the clinicopathological characteristics and MSI target gene mutations[18] as well as determining the spectrum of MSI target genes in a given cancer type could provide important insights into the malignant process. The tumour samples with *CtIP* mutation did not differ significantly from other MSI CRC samples. The degree of differentiation (grade I 16%, II 52%, III 28%, IV 4%) was similar to other MSI cancers (8%, 72%, 20%, 0%, respectively) and poorer as compared with MSS cancers in our whole population based

Table 3 Previously proposed MSI target genes which contain exonic A9/T9 repeats (proportion of mutated MSI samples 10% or more)

MSI target genes (exonic A9/T9 repeat)	Mutations/samples	Reference	This study
TCF4	19/49=39%	Duval *et al*[15]	
RIZ	8/24=33%	Chadwick *et al*[17]	
RAD50	13/39=33%	Kim *et al*[24]	
WISP-3	11/36=31%	Thorstensen *et al*[25]	
NADH-ubiquinone oxidoreductase B14.5B subunit	12/43=28%	Mori *et al*[26]	
KIAA0977	10/42=24%	Mori *et al*[26]	
CtIP			25/109=22.9%
GRK4/GPRK2L	19/91=21%	Park *et al*[27]	
BLM	10/63=16%	Calin *et al*[20]	
	7/39=18%	Kim *et al*[24]	18/93=19%
CBF2	14/91=16%	Park *et al*[27]	
KIAA0905	6/43=14%	Mori *et al*[26]	
Exportin T	6/43=14%	Mori *et al*[26]	
RECQL1	11/93=12%	Park *et al*[27]	
MCT	4/36=11%	Mori *et al*[26]	
ERCC5	8/80=10%	Park *et al*[27]	
CHK1	1/10=10%	Bertoni *et al*[16]	

data set of 1044 CRCs (22%, 69%, 9%, 0%, respectively).[32 33] The proportion of mucinous cancers was 20%, 21%, and 8% in *CtIP* mutation positive CRC, other MSI positive CRC, and MSS lesions, respectively. The preference for mucinous and poorly differentiated histopathology in MSI lesions has been extensively documented previously by Kim *et al*[39] and others.[8–10] The patients with and without somatic *CtIP* mutation had similar age distributions at the time of carcinoma diagnosis, and comparable site distribution with other MSI tumours; 19 *CtIP* mutated tumours out of 25 (76%) were proximal, versus 68 out of 86 (79%) in other MSI cases. Differing sex distribution was not observed (14 out of 49 in males versus 11 out of 60 in females).

CtIP was identified as a candidate from the search combined with keywords related to cancer. In the MSI positive CRC tumour samples, the 1 bp deletion rate of 22.9% in the A9 repeat suggests that *CtIP* could be a new MSI target gene. Many of the suggested MSI target genes contain a mutable mononucleotide A9 repeat. As compared with other similar previously proposed MSI target genes, *CtIP* appears to be a reasonable candidate (table 3).

CtIP modulates transcriptional regulation, DNA repair, and cell cycle checkpoint control. CtIP has been found to associate with BRCA1, CtBP1, and Rb1.[40] ATM protein mutated in ataxia telangiectasia phosphorylates CtIP and so modulates BRCA1 mediated regulation of the DNA damage response *GADD45* gene.[41] If CtIP is truncated, the association with the three tumour suppression activity proteins is likely to change. *CtIP* has not been found to be mutated in an earlier MSI target gene study. Ikenoue *et al*[42] studied 13 primary colorectal tumours with high frequency of MSI, but no mutations were found in the mononucleotide repeat.[42] If the *CtIP* mutation results from this study and that of Ikenoue *et al*[42] are combined (25/122=20.5%), the frequency of *CtIP* A9 and intronic A9 mutations remains significantly different ($p \leq 0.001$).

Mismatch repair defective tumours display a mutator phenotype, in contrast to mismatch repair proficient colorectal tumours. Mismatch repair proficient tumours may also contain MSI target gene mutations, but because of the different biological backgrounds of the respective lesion types such absence of defects does not exclude the possibility of a real target gene.[29] Thus the role of *CtIP* in microsatellite stable lesions was not evaluated in this work. In this study biallelic inactivation of *CtIP* (or any other gene) in MSI positive CRCs was not found, but the association with tumour suppression activity proteins may decrease by the reduced amount of CtIP. This might lead to a defect in the cellular response to DNA damage, and to tumorigenesis. By a similar mechanism heterozygous mismatch repair gene mutations appear to reduce the amount of gene products involved in the repair process.[30] This alone may decrease replication fidelity. Also haploinsufficiency of the *NSD1* gene induces overgrowth in Sotos syndrome, implying that NSD1 acts as a corepressor of genes that promote growth.[43] Miyoshi *et al*[44] have proposed that the initiation of polyposis in Peutz-Jeghers syndrome mouse model is not the result of biallelic loss of the Peutz-Jeghers syndrome gene *Lkb1*. Gastrointestinal hamartomas appear to develop in heterozygous mice because of *Lkb1* haploinsufficiency.

It should be mentioned that in our study biallelic inactivation of *CtIP* might have been difficult to detect. We used primary tumours which contained some contaminating normal tissue. Microdissection, which would make detection of biallelic changes more feasible, was not applied. *CtIP* mutated MSI tumour cell lines would be useful in evaluating possible biallelic inactivation of this gene.

As compared with the mutation frequency cut off level for real MSI target genes proposed by Duval *et al*[45] (12%), the observed *CtIP* mutation frequency (22.9%) is of significance. Further work, such as functional analyses, is required to elucidate the importance of *CtIP* in the development of colorectal and other cancers.

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