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SHORT REPORT

Apparent protection from instability of repeat sequences in cancer-related genes in replication error positive gastrointestinal cancers

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Genomic instability at simple repeated sequences has been observed in various types of human cancers and is considered an important mechanism in tumorigenesis. Alterations at microsatellite loci have been reported scattered throughout the genome. Recently, the transforming growth factor-β receptor type II (TGF-β RII) and the insulin-like growth factor II receptor (IGF-IIR) genes were shown to have inactivating mutations within coding microsatellite sequences. The demonstration of mutations in two growth regulatory genes supports the idea that other regulatory genes with repeat sequences may also be targets in tumours with defective mismatch repair. We examined genes involved in tumour suppression, cell adhesion and cell cycle regulation for mutations at small repeat sequences in replication error positive gastrointestinal cancers. Several polymorphisms were found which exhibited instability, but no other instability was present in the regions examined.

Keywords: microsatellite instability; repeats; colorectal cancer; gastric cancer

Microsatellites are short tandemly repeated nucleotide sequences widely distributed throughout the genome. Alterations in the length of these sequences, either by expansion or contraction of the repeat motifs, are characteristic of hereditary non-polyposis colorectal cancer (HNPCC) and a proportion of sporadic carcinomas of the colon, breast, ovary, prostate, stomach, lung, bladder, endometrium and oesophagus (Aaltonen *et al*., 1993; Ionov *et al*., 1993; Patel *et al*., 1994; Thibodeau *et al*., 1993; King *et al*., 1995; Uchida *et al*., 1995; Han *et al*., 1993; Merlo *et al*., 1994; Gonzalez-Zulueta *et al*., 1993; Risinger *et al*., 1993; Meltzer *et al*., 1994).

Microsatellite instability can be observed by a change in allele sizes in tumour DNA compared to constitutional DNA. At least in HNPCC, microsatellite instability is due to mutations in the mismatch repair genes (Fishel *et al*., 1993; Papadopoulos *et al*., 1994). Tumours exhibiting such instability are termed replication error positive (RER+). In such tumours microsatellite instability is extremely widespread and thousands of somatic mutations within anonymous DNA fragments have been detected by arbitrarily primed PCR fingerprinting (Perucho *et al*., 1994). Most microsatellites are within introns or between genes and mutations within these are probably not directly involved in tumorigenesis. Point mutations in genes known to be involved in colorectal tumorigenesis such as APC, p53 and K-ras are no more common in RER+ tumours (Huang *et al*., 1996) and some studies have reported them to be less common (Ionov *et al*., 1993; Young *et al*., 1993; Heinen *et al*., 1995). Thus it seems likely that the mutator phenotype of RER+ cancers leads to cancer development through mutations in genes not yet recognised to play a role in colorectal cancer. Such mutations may be point mutations but it seems likely that repeat sequences would be especially vulnerable to mutation in these tumours.

Recently, mutations within the 10 bp polyA tract in TGF-β RII were found in RER+ colorectal cancer cell lines (Markowitz *et al*., 1995). RII mutations have been subsequently reported to occur in 100 of 111 (90%) RER+ colorectal cancers (Parsons *et al*., 1995), 13 of 16 (81%) RER+ sporadic colorectal cancers and in 3/17 (18%) in RER+ ulcerative colitis-associated neoplasms (Souza *et al*., 1997). The absence of RII cell surface receptors in the RER+ cell lines with the polyA tract mutations is thought to contribute to tumour development by allowing the cells to escape TGF-β's growth-inhibitory effects (Markowitz *et al*., 1995). Parsons *et al*. (1995) found that polyA tracts were otherwise rare in coding regions. Outside coding regions they were frequently mutated in RER+ tumours, with the frequency of alterations proportional to the length of the tract.

More recently, a second growth regulatory gene, IGF-IIR was identified as a non-random target of microsatellite instability in a subset of gastrointestinal cancers (Souza *et al*., 1996). Insertion or deletion mutations were found within two coding microsatellite sequences in 12/92 (13%) RER+ gastrointestinal tumours. Since functional IGF-IIR is required for the extracellular activation of TGF-β (Dennis and Rifkin, 1991), targeting of IGF-IIR by microsatellite instability may represent another step at which disruption of the TGF-β growth control pathway occurs. In addition, E2F-4, a transcription factor, and the mismatch repair genes, hMSH3 and hMSH6, have been shown to exhibit frameshift mutations in repeat sequences in

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RER+ cancers (Yoshitaka *et al*., 1996; Malkhosyan *et al*., 1996).

With the identification of several targets of defective DNA mismatch repair, the aim of our study was to identify further key mutation targets in other cancer-related genes. We examined DNA from RER+ sporadic colorectal cancers and RER+ gastric adenocarcinomas for insertion or deletion mutations at mono-, di-, tri- and tetra-nucleotide repeat sequences in genes involved in tumour suppression, cell adhesion and cell cycle regulation. Since triplet repeat expansions in 5′ UTR and 3′ UTR of disease-related genes have been well documented as a cause of disease (Kruyer *et al*., 1994; Krahe *et al*., 1995; Sutherland and Richards, 1995), we did not exclude the possibility that inactivating mutations may occur outside the coding regions of our genes of interest. We examined coding and non-coding regions of p53, von Hippel-Lindau tumour suppressor (VHL) gene, N-cadherin, β-catenin, spot-1, bcl-2, c-met proto-oncogene, waf-1, estrogen receptor, retinoic acid receptor alpha (RARα) and transforming growth factor-β receptor type I (TGF-β RI) for alterations at repeat motifs using PCR amplication and denaturing polyacrylamide electrophoresis.

The p53 tumour suppressor gene is one of the most commonly mutated genes in human cancer (Levine *et al*., 1994). Deletion and insertion mutations at simple repeated sequences account for 10% of all p53 mutations (Jego *et al*., 1993). We examined two mononucleotide repeats in p53, one in exon 11, the other in the 3′ UTR and a pentanucleotide repeat within intron 1, for sequence length variations in RER+ tumours. The VHL disease tumour suppressor gene is frequently mutated in clear renal cell carcinomas (Latif *et al*., 1993) and less frequently in lung cancer (Sekido *et al*., 1994). We investigated the possibility of VHL gene inactivation occurring as a result of genomic instability at polyA repeat sequences in the promoter region. Cadherins and catenins are important regulators of cell adhesion of different cell types in developing and adult tissues. α-catenin binds to and is regulated by the tumour suppressor gene, APC (Polakis, 1995). Mononucleotide and tri-nucleotide repeats in the coding and non-coding regions of N-cadherin and β-catenin were analysed for genetic alterations. The nuclear protein spot-1 interacts specifically with p53 via its His-Thr domain (Elkind *et al*., 1995). This His-Thr domain encoded by p(CA)n, is the first reported p(CA)n repeat to encode protein. We examined the dinucleotide repeat for sequence length variations. The oncoprotein bcl-2 acts specifically to suppress apoptotic cell death (Hockenbery *et al*., 1990). We investigated the possibility of bcl-2 deregulation occurring in colorectal cancers as a result of unstable repeat motifs in the 3′ UTR of the gene. Given that a 5′ rearrangement in the tyrosine kinase growth factor, c-met, was found to have transforming potential in an osteosarcoma cell line (Cooper *et al*., 1984) and that c-met is overexpressed in 50% of colorectal cancers, we sought to establish whether the c-met gene could be up-regulated in RER+ tumours by genetic alterations within a polyA tract in its coding region. The waf-1 encoded protein, p21, is a target for p53 and negatively regulates the cell cycle in response to DNA damage (El-Diery *et al*., 1993). Retinoids have been shown to inhibit the proliferation of head and neck cancers (Lotan, 1993), induce remission in promyelocytic leukaemia (Huang *et al*., 1988) and inhibit the growth of estrogen receptor-positive breast cancer cell lines (Fontana *et al*., 1990). Retinoids exert their effect by binding to retinoic acid receptors (RARs) and retinoid X receptors. RAR-α is one of three distinct receptor subtypes in the RAR family that can control transcriptional events by interacting with specific RA-responsive elements in the regulatory regions of target genes (Leid *et al*., 1992). A poly GT sequence within the 5′ flanking region of the hRAR-α was examined for instability. The estrogen receptor (ER) has growth-regulatory properties in multiple cell types (Ma *et al*., 1993; Zajchowski *et al*., 1993; Xu and Thomas, 1994) and ER inactivation due to methylation changes results in deregulated growth in aging colorectal mucosa (Issa *et al*., 1994). We examined several mono- and tri-nucleotide repeat sequences within the N-terminal transcriptional activation domain for insertion and deletion mutations. TGF-β is a potent inhibitor of normal epithelial and lymphoid cell growth. The TGF-β RI encodes a membrane-bound serine threonine kinase receptor that binds activated TGF-β and forms a heterodimeric complex with TGF-β RII (Alexandrow and Moses, 1995). With the identification of inactivating mutations in a polyA tract in TGF-β RII, we examined a small adenine mononucleotide repeat in TGF-β RI for similar sequence length variations.

Microsatellite markers, MYCL, AT3, F13B and D2S123 were selected to screen a prospective series of 184 sporadic colorectal cancers for microsatellite instability. Patients meeting the criteria for HNPCC were excluded from this study (Vasen *et al*., 1991). DNAs from gastric adenocarcinomas were screened for RERs at microsatellite loci D2S123, D2S147, D2S119, D10S197 and D11S904 (Rhyu *et al*., 1994). For the purposes of this study, tumours with alterations at one or more loci were selected to analyse each nucleotide repeat motif. These tumours were then divided into two groups, those with alterations at two or more loci which were designated

Table 1 Assessment of repeat sequence alterations in RER+ gastrointestinal cancers

Gene[a]	*Region*	*Type of repeat*	*Status of repeat motif in RER+ cancers*
p53	intron 1	(AAAAT)6 – 10	conserved
p53	exon 11	(A)5,(A)6	conserved
p53	3′ UTR	(T)18	polymorphic
p53 RD*	coding	(GTTT)8	conserved
VHL	promoter	(A)15,(A)8	polymorphic
N-cadherin	5′ UTR	(TCC)4,(GCC)8(TCC)2	conserved
spot-1	coding	(CA)39	conserved
β-catenin	exon 2	(GGT)3(TAA)2	conserved
β-catenin	3′ UTR	(T)19,(T)27	polymorphic
bcl-2	3′ UTR	(CA)5,(CA)7,(A)9,(A)10	conserved
c-met	coding	(A)16	conserved
waf-1(a)	5′ UTR	(T)9,(T)10	polymorphic
waf-1(b)	5′ UTR	(T)10,(A)8	conserved
estrogen receptor	exon 1	(GCC)3,(G)6,(C)6, (CCG)3	conserved
RAR alpha	5′ UTR	(GT)5	conserved
TGF-β RI	coding	(A)6	conserved

[a]GenBank accession nos. X54156, M22898, X67536, U19763 X54315, X87838, M14745, J02958, U24179, M12674, X56057, L17075
*responsive domain

RER+ (Aaltonen *et al*., 1993; Liu *et al*., 1995b), and those with alterations at one locus only. Primers were designed to amplify the various repeat elements outlined in Table 1. After amplification, PCR samples were analysed on 5% denaturing polyacrylamide gels. The gels were dried and exposed to Kodak XAR film. Primer sequences and PCR conditions are available upon request.

Microsatellite instability was found in 53/184 (29%) of sporadic colorectal cancers. Of these, 19 (10%) were RER+ with alterations at two or more loci. The loci most frequently showing instability were MYCL (35/184 or 19%), AT3 (21/184 or 11%) (Figure 1a). Of these 53 tumours, 40 were chosen for further analysis on the basis of available DNA (19 tumours with two or more RERs and 21 tumours with only one locus

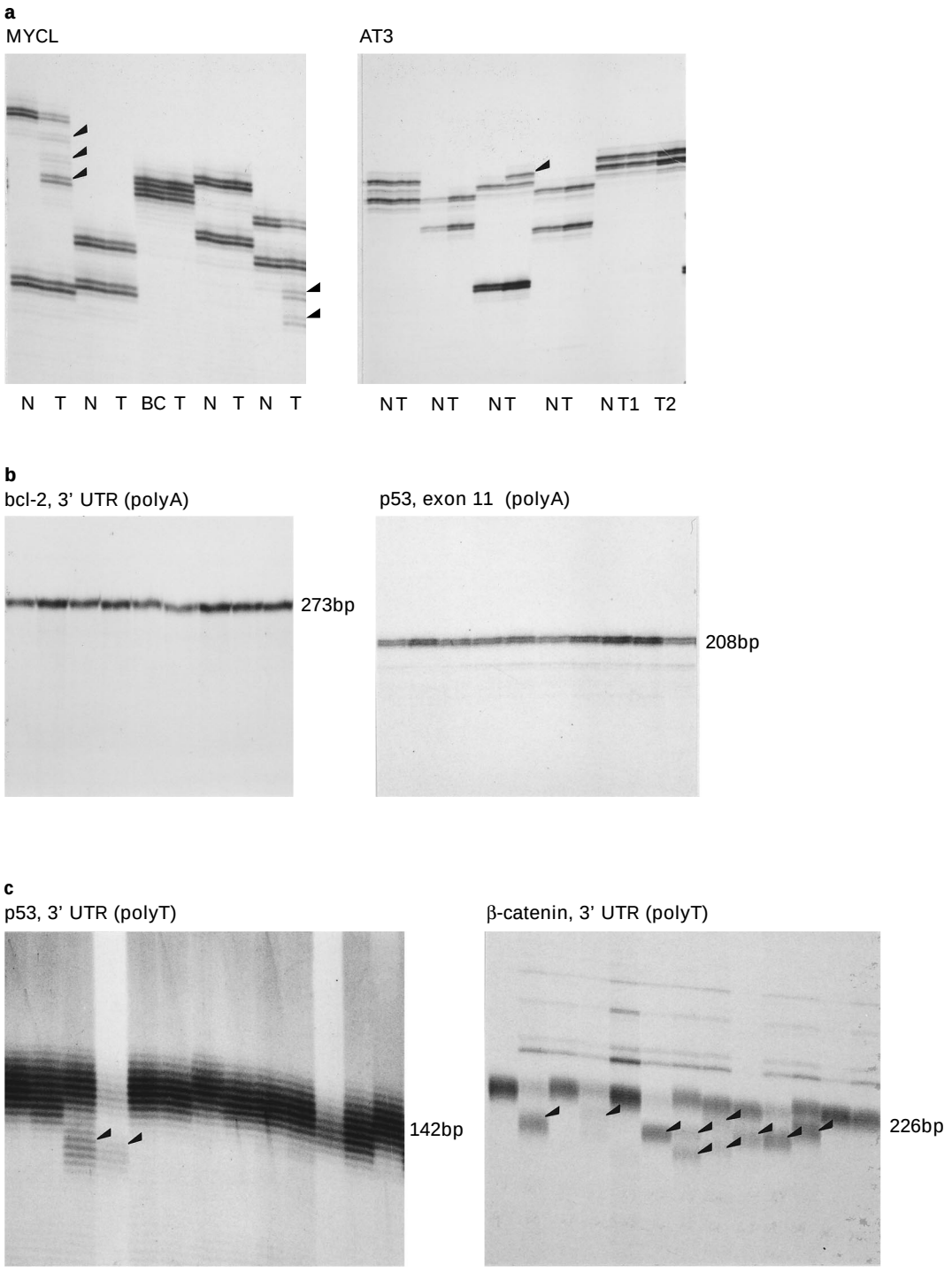

Figure 1 Analysis of microsatellite instability in sporadic colorectal cancer. N, normal colonic mucosa DNA; BC, normal peripheral blood cell DNA; T, tumour DNA. Novel bands due to alterations in repeat sequence length are indicated by the arrowheads. (**a**) Amplification of microsatellite markers MYCL and AT3 in paired normal (N or BC) and tumour (T) DNA from individuals with colorectal cancer. Novel bands are tumour specific. Amplification of mononucleotide repeat motifs in bcl-2 and p53 (**b**), and p53 and *β*-catenin (**c**), in a representative number of RER+ tumours. No sequence length variation is seen in any of the RER+ tumours in **b** in contrast to **c**, where variation in allelic length is frequently observed. Sequence variants were also detected in anonymous blood donor DNA indicating that these repeat motifs are polymorphic

showing instability). As reported previously, 16/52 (31%) gastric adenocarcinomas demonstrated microsatellite instability (Rhyu *et al*., 1994). Similarly, nine of these 16 were selected for the study on the basis of DNA availability. Analysis of repeat motifs in the 40 colorectal and nine gastric tumours revealed variations in the length of amplified products in four genes; p53, VHL, β-catenin and waf-1(a) (Table 1, Figure 1c). To determine whether the sequence variants were polymorphic, we tested DNA samples from 40 anonymous blood donors. All four repeat structures were found to exhibit length variation in the normal DNA controls. The polymorphism detected at the polyT tracts in the 3′ UTR of β-catenin was unstable in 15/19 (79%) of RER+ colorectal tumours with two or more errors compared with 17/19 (89%) and 15/19 (79%) at the MYCL and AT3 screening loci respectively further supporting the classification of these tumours as RER+. Interestingly, no such instability was seen in those tumours with alterations at only one locus. The sensitivity of these markers does not compare favourably with the BAT markers described by Parsons *et al*. (1995). However, the tumours examined in that study consisted mostly of HNPCC derived lesions, and may behave differently to sporadic lesions in which it has been difficult to attribute the RER+ phenotype to mutations in genes responsible for HNPCC (Liu *et al*., 1995a). There was no evidence of alterations within the non-coding regions of p53 (intron 1), N-cadherin, bcl-2, waf-1 (b) and RARα nor within the coding regions of p53, spot-1, β-catenin, c-met, estrogen receptor or TGF-β1 RI (Table 1, Figure 1b).

The discovery of microsatellite instability at random loci distributed throughout the genome in HNPCC and a subset of sporadic colorectal cancers has led to a plethora of reports describing microsatellite alterations in a wide variety of tumour types. These findings suggest that instability at microsatellite repeats is a structural and not a functional feature of the repeat motifs. It is also feasible that genetic alterations, as a consequence of defective mismatch repair, could affect other sequences within the genome that are functional (Field *et al*., 1995). Recently, the growth-regulatory genes, TGF-β RII and IGF-IIR, were identified as non-random targets for the RER mutator mechanism in cancers of the colon, stomach and UC-associated neoplasms (Markowitz *et al*., 1995; Meyeroff *et al*., 1995; Souza *et al*., 1996, 1997). The demonstration of microsatellite instability affecting sequences within a functional gene suggests that alterations may be occurring in other specific target genes, however it is apparent from Genbank searches during the current study and also by others (Parsons *et al*., 1995) that similar 10 bp runs in coding sequences are exceedingly rare.

In the present study, we found microsatellite instability in 29% of primary colorectal tumours. The highest level of alterations were found in the tetra- and trinucleotide repeats of MYCL and AT3 loci respectively. Our observation that the majority of alterations occurred more frequently at higher order repeats is in agreement with previous studies (Wooster *et al*., 1994; Huddart *et al*., 1995). Having identified a panel of tumours with microsatellite instability, we screened repeat structures in genes involved in tumour suppression, cell adhesion and cell-cycle regulation for mutations which may give rise to functional alterations. We could find no evidence for the mutator mechanism affecting the function of these genes. Polymorphisms were detected in the 3′ UTR of p53, β-catenin, the VHL promoter and in the 5′ UTR of waf-1(a) at mononucleotide runs. These polymorphisms were found to be as susceptible to instability in RER+ cancers as microsatellite sequences. Interestingly, the repeat motifs within the non-coding regions of p53 (intron 1), N-cadherin, bcl-2, waf-1(b) and RARα were highly conserved. The motifs present in N-cadherin (compound trinucleotide repeat), waf-1(b) (mononucleotide runs) and RARα (dinucleotide repeat) are located in the 5′ UTR of the genes and may represent part of a regulatory sequence. The mononucleotide runs present in the 3′ end of bcl-2 may be involved in stability of the mRNA. Excepting mutations in the TGF-β RII and IGF-IIR genes, the present study suggests that repeat sequences in coding regions or non-coding regions likely to be important to gene function are rare in RER+ cancers. Some of the sequences we examined may have been protected due to their short length. Previous work has reported that the instability of mononucleotide sequences decreases with the number of repeats present in a sequence, perhaps accounting for the lack of instability in the short mononucleotide runs in bcl-2 and waf-1, though mononucleotide runs of 5 bp are susceptible to deletions in p53 (Greenblatt *et al*., 1996). The number of repeats in the trinucleotide N-cadherin sequence is also small and although it is a higher order repeat may be subject to the same mechanisms which conserve small mononucleotide repeats. However, other sequences we examined such as the long CA repeat in the spot-1 gene and the polyA tract in the c-met gene, were not mutated in RER+ cells. It may be that mutations in these repeat sequences adversely affect cell viability and are therefore selected against. Alternatively, these regions may undergo faster rates of repair (Tornaletti and Pfeifer, 1994; Gao *et al*., 1994), or have methylation patterns that are either protective or allow repair to take place (Modrich, 1991).

Our results indicate that despite microsatellite instability occurring at random microsatellite loci in more than one quarter of gastric and sporadic colorectal cancers, we found no evidence for the accumulation of mutated repeat sequences in those genes examined in this study. Although this study does not exhaust the search for key targets of defective mismatch repair, it does exclude a significant number of candidate repeat regions from mutations due to the RER+-associated mutator mechanism.

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