

p53 mutations and DNA ploidy in colorectal adenocarcinomas

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Abstract. The p53 tumour suppressor gene has an important role in the the maintenance of genome stability and its mutational inactivation may be at the origin of aneuploidy in cancer cells. The aim of this study was to determine whether p53 mutations were associated to DNA aneuploidy, as assessed by flow cytometry, in colorectal adenocarcinomas. Analysis of p53 mutations spectrum of the sorted nuclei was done by Denaturing Gradient Gel Electrophoresis (DGGE) and DNA sequencing. Overall, we studied 20 adenocarcinomas, the corresponding control mucosa, and 7 lymph node metastases.

Five tumours (25%) were DNA diploid, while 15 tumours (75%) were composed of DNA aneuploid and diploid subpopulations. DNA diploid control mucosa and adenocarcinomas showed no p53 mutations, while 60% of the tumours with DNA aneuploidy had p53 mutations. Therefore, p53 mutations occurred significantly more often in DNA aneuploid than in DNA diploid tumours ($p < 0.04$, Fisher's exact test). Incidences of DNA aneuploidy and p53 mutations in lymph node metastases were 60 and 86%, respectively. In all tumours showing a p53 mutation, the wild-type allele was not or only barely visible in DNA aneuploid cells suggesting that, in such cells, aneuploidy is accompanied by complete p53 functional inactivation.

The present observations suggest that p53 mutations may have a role in the origin of aneuploidy at late stages of colorectal carcinogenesis.

1. Introduction

Human colorectal cancer, during its well defined progressive stages [33,41], is characterized by a series of inherited and/or acquired gene defects [5,6,11,21,22,30,35,40,42,43]. Among these, p53 mutations appear to represent an important event for late tumour progression [21,42]. A role of p53 in the control of genome stability has been proposed [29] and is supported by several pieces of evidence. Fibroblasts from Li Fraumeni patients, heterozygous for p53 mutation, showed aneuploidy at early passages of subcultivation [3]. Furthermore, studies on Barrett's esophagus showed that loss or inactivation of p53 is associated

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with tetraploidy and aneuploidy [4,34]. Finally, the results obtained by Cross et al. [17] indicate that the p53 protein is a component of the spindle checkpoint in mouse cells in culture and might be associated to aneuploidy. It is therefore conceivable that aneuploidy and other manifestations of genomic instability often found in neoplasms, might be triggered by loss of the p53 function.

Two recent studies [12,16] suggested that immunocytochemical positivity for p53 protein in colorectal adenocarcinomas, usually associated with p53 gene alterations [13], preceded and apparently facilitated the generation of DNA tetraploid subclones. In another study, Costa et al. [15] found, instead, that p53 mutations or loss of heterozygosity (LOH) were not generally associated with DNA aneuploidy. Moreover, Meling et al. [31] found that LOH in the vicinity of the p53 gene was associated with DNA aneuploidy only in those colorectal carcinomas in which the LOH extended to both arms of chromosome 17. In early adenomas, aneuploidy was observed in about 30% of cases [24], whereas p53 mutations were rare [42,43]. There is, therefore, no consensus on the association of p53 mutations and aneuploidy in colorectal cancer. It is known, in fact, that DNA aneuploidy is already present in the adenoma precursors of adenocarcinomas, from about 30% up to 70% corresponding to small adenomas to adenomas bearing early cancer, whereas p53 mutations are rare.

A possible way to address this question is to determine the incidence of p53 mutations in DNA diploid and DNA aneuploid tumours. If p53 mutations represented the only mechanism of aneuploidization, all tumours characterized by DNA aneuploidy should be p53 mutated.

Flow cytometry based on forward and perpendicular scattering and DNA content was applied to DAPI stained suspension of nuclei to sort out DNA diploid (DI_d) and aneuploid (DI_a) nuclei. Using a PCR-based DGGE approach, flow cytometrically sorted nuclei were screened for the presence of genomic p53 mutations in exons 5–8 encompassing 4 highly conserved domains of the p53 protein. More than 90% of known p53 mutations reported so far in human tumours occur in this region [28], corresponding to the core of the protein involved in sequence specific DNA binding [14]. DNA sequencing was then performed on the DGGE variants.

Lymph node metastases, when present, were also analyzed following the same procedures.

2. Material and methods

2.1. Tumour material and histology

Twenty partial large bowel resections were used to obtain multiple samples from 20 human adenocarcinomas. An accurate sampling was first done using liquid-nitrogen frozen tumour material. Multiple hematoxylin–eosin stained cryostatic sections were used as histotopographic references. Areas with necrotic cells were identified and removed from the confining frozen material. Similarly, areas with prevalent muscle and connective tissue were discarded. Samples were obtained from superficial and deep invasive tumour margins (Fig. 1(A)). Samples to be later used for flow cytometry and sorting combined with multiple molecular genetic analyses (including K-ras2 and p53 mutations, deletions at 1p, aneusomy of various chromosomes) were stored without fixation at -80°C .

A reference sample was fixed, instead, in 10% buffered formalin for 24 h and embedded in paraffin. The paraffin blocks were handled according to customary protocols for the histological diagnosis and grading by the criteria of Astler and Coller [1] and WHO [32]. Astler–Coller stages were B1 in 2, B2 in 8, and C2 in 10 cases. Differentiation degree was moderate in 15 and poor in 5 cases. Adenocarcinomas were located in the left bowel in 12 cases and in the right bowel in 8. Patients were 8 females and 12 males, aged 35–93 years (median, 68 years).

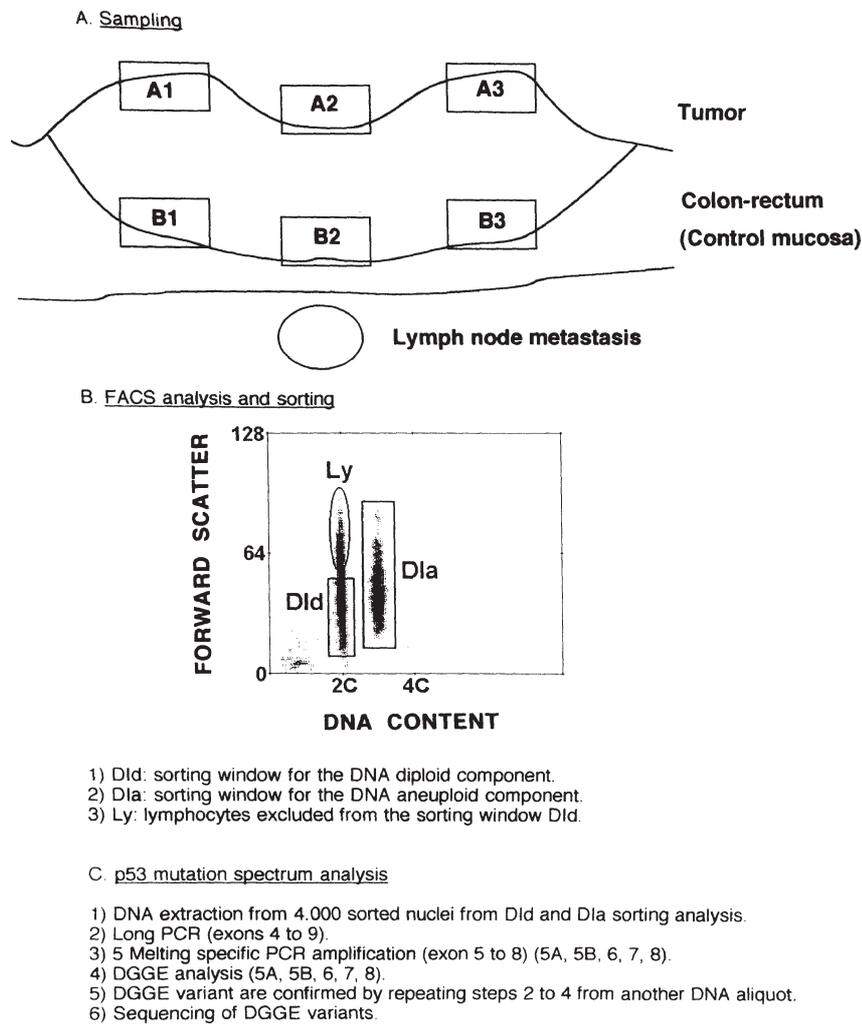


Fig. 1. Schematic outline of the experimental procedures. Samples of tissue were obtained from multiple sites of the tumour (A). Two separate analyses were performed, respectively, for superficial and deep tumour sectors. (B) shows an example of flow cytometric analysis of forward scatter versus DNA from DAPI stained nuclei suspensions. As previously shown [25], this approach was useful to separate DNA diploid (2C) inflammatory cells (Ly) from DNA diploid (DId) and aneuploid (DIa) cell components. (C) shows the main steps of the procedure for the analysis of p53 mutation spectrum using flow cytometric sorted nuclei. Separate analyses were performed for DIa and DId cell components with exclusion of inflammatory cells.

2.2. DNA fluorescence activated flow cytometry and sorting

Nuclei suspensions from fresh-frozen material were prepared and stained with 4,6-diamidino-2-phenylindole-2-hydrochloride (DAPI, Sigma Chemical Co., St. Louis, MO, USA) using multiparameter flow cytometric measurements and sorting as previously detailed [25,26]. Briefly, nuclei from the inflammatory cell component were recognized and excluded according to high values of 0° -forward-scattering and 2c-DNA content. Tumour samples were analyzed together with the 20 matching histologically proven control mucosa samples and 7 lymph node metastases. For tumours characterized by DNA aneuploid subpopulations, both DNA diploid and aneuploid nuclei were sorted (Fig. 1(B)). DNA

content of tumour nuclei was evaluated relative to DNA content of tissue-specific and individual-specific histologically proven control mucosa and tissue-infiltrating lymphocytes.

2.3. PCR conditions

Sorted nuclei were stored at -80°C . Before use, nuclei were washed 30 min at $1500 \times g$ in phosphate buffered saline, resuspended in 1X Polymerase Chain Reaction (PCR) buffer (Promega, Madison, WI, USA) and heated to 100°C for 10 min before PCR reaction [7]. Starting from about 4000 nuclei, the genomic region encompassing exons 4–9 was first amplified as described by Nigro et al. [36]. Nested PCRs were performed using 5 pairs of melting domain specific primers (exon 5 was amplified using two sets of primers, obtaining the 5' half, 5A, and the 3' half, 5B, of the exon) [8]. PCRs were performed using about 1/100th of the first PCR product (exons 4–9), 15 pmol of each primer, 250 μM each dNTP, 50 mM KCl, 10 mM Tris HCl (pH 8.3), MgCl_2 ranging from 1 to 2 mM and 1.5 units of Taq Polymerase (Promega) in a final volume of 50 μl . PCRs were performed in a MJPT-100 thermal cycler (MJ Research, Inc., Watertown, MA, USA) or in an Eppendorf Master Cycler (Germany). In the first round of PCR (exons 4–9) the 30 cycles were 1'30'' at 95°C , 1'30'' at 58°C and 2'30'' at 70°C , followed by final extension at 70°C for 7'. PCR programs for nested PCRs were as described [18]. Negative (no DNA added) and positive (genomic DNA from characterized samples [18]) controls were regularly used for each set of PCR.

2.4. DGGE conditions

The PCR products obtained with the GC-clamped ampliprimers were analyzed by DGGE [18]. The introduction of a GC-clamp into the amplified fragment allows a nearly 100% detection rate of any base change within the fragment [39]. The DGGE apparatus was from CBS Scientific Co. (Del Mar, CA, USA). Parallel denaturing gradient gels were prepared with 8% acrylamide-bisacrylamide (37 : 1) in TAE (40 mM Tris acetate/1 mM EDTA, pH 8.0) and by varying denaturant concentrations (100% denaturant corresponds to 7 M urea and 40% formamide). The range of the denaturing gradient was different and specific for each amplified fragment [18]. Gels were run in TAE at 60°C , 50 V for 16–20 h. After electrophoresis, the gels were stained for 20 min in 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide, visualized using a UV transilluminator and photographed using Polaroid type 55 films. Once a DGGE variant was observed, a new 4000 nuclei aliquot was used through all the procedure to confirm the presence of the DGGE variant and to avoid misclassification of PCR artefacts.

2.5. DNA sequencing

PCR products for sequencing were obtained starting from either genomic DNA or eluted DGGE mutant homoduplex bands. The PCR products, obtained using the same primers for DGGE analysis but without "GC-clamp", were cloned into the plasmid pGEM-T (Promega, Madison, WI, USA) according to the manufacturer's instructions. The inserts of a series of independent clones were characterized by PCR-DGGE analysis. Three to 5 independent clones, showing the same mutant homoduplex band pattern as the one obtained starting from genomic DNA, were then sequenced using CircumVent(exo-) kit (New England Biolabs, Beverly, MA, USA) in the presence of $[\alpha^{35}\text{S}]\text{dATP}$. Sequencing reaction products were electrophoresed on 7% polyacrylamide–7 M urea gels in TBE (45 mM Tris borate/1 mM EDTA, pH 8.0) for 2 h at 50 mA. Gels were then fixed for 15 min in 10% methanol and 10% acetic acid, dried, and exposed to β -MAX films (Amersham, Milan, Italy).

2.6. Loss of heterozygosity

Loss of heterozygosity (LOH) in proximity of the p53 locus was evaluated by PCR using the highly polymorphic marker YNZ22 (band 17p13.3). Primers and PCR conditions were those described by Bata-nian et al. [2]. Lysate from 4000 nuclei of each component of the tumour and from the control mucosa sectors, were separately used as template for PCR amplification. The primers used allow amplification of a region with a variable number of tandem repeats (VNTR). PCR products were separated on a 2% agarose gel, stained with 0.5 $\mu\text{g/ml}$ ethidium bromide and visualized using a UV transilluminator. LOH was considered to have occurred when one of the 2 alleles present in PCR products obtained with control mucosa was missing from PCR products obtained from the biopsy DNA.

2.7. Statistical analysis

Association of p53 genetic with DNA ploidy was tested according to contingency tables by means of the Fisher's exact test [23].

3. Results

Figure 1 illustrates the multiple methodological steps performed for studying the presence of p53 mutations in superficial and deep infiltrating tumour sectors and in DNA diploid and aneuploid nuclei (respectively indicated by DI_d and DI_a) obtained from 20 human colorectal adenocarcinomas.

Table 1 shows that 5 tumours had only DI_d nuclei while 15 tumours comprised both DI_a and DI_d cells, the latter very likely being normal non tumour cells. Overall, DNA aneuploidy incidence was 75% for the primary tumours, 60% for the lymph node metastases (Table 2), and absent in the control mucosa (not shown). Table 1 also shows that in three out of 15 DNA aneuploid adenocarcinomas, the DI_a component was only present in the superficial sectors and not in the invasive margins.

PCR-based DGGE analysis of exons 5–8 of the p53 gene was performed separately for the different components as indicated in Tables 1 and 2. This screening allowed the identification of DGGE variants which were further characterized by DNA sequencing.

Ten out of 15 DI_a and none of the DI_d tumours showed a single p53 DGGE variant in at least one of the different components analyzed (Table 1). In one case (tumour no. 4), the control mucosa showed exactly the same variant observed in the tumour component, suggesting either a germinal mutation or a silent polymorphism. DNA sequencing of this DGGE variant indicated an AT > GC transition in intron 6, 31 base pairs downstream from the 3' end of the exon 6. This mutation was not predicted to create an alternative splice site and may represent a new polymorphism [10].

In all tumours but one (no. 3), superficial and deep infiltrating tumour margins shared the same DGGE variant (Table 1). In tumour no. 3, the deep infiltrating margins but not the superficial sectors showed a DGGE variant.

DGGE variants, when present, were observed in both DI_d and DI_a cell components in all primary tumours but one (no. 13), and in the 4 metastases that showed both DI_d and DI_a cell nuclei (Table 2). In tumour no. 13, the DGGE variant was present only in the DI_a component of both superficial and deep infiltrating margin sectors.

Among the 7 lymph node metastases analyzed, 6 showed the presence of a single DGGE variant, 5 of which were identical to the one observed in the matching primary tumour and one had no mutant counterpart in the primary tumour (Tables 1 and 2).

Table 1

Presence of p53 mutations in DNA diploid and DNA aneuploid cell components of superficial and deep infiltrating margins of human colorectal adenocarcinomas

Case number	Tumour ¹ sector	p53 DGGE variants ²	
		DId	DIIa
<i>Primary tumour with metastasis</i>			
1	A	Mut (exon 6)	Mut (exon 6)
	B	Mut (exon 6)	Mut (exon 6)
	M	Mut (exon 6)	Mut (exon 6)
5	A	wt	wt
	B	wt	wt
	M	wt	_ ³
6	A	Mut (exon 6)	Mut (exon 6)
	B	Mut (exon 6)	Mut (exon 6)
	M	Mut (exon 6)	Mut (exon 6)
8	A	Mut (exon 5)	Mut (exon 5)
	B	Mut (exon 5)	_ ³
	M	Mut (exon 5)	_ ³
9	A	Mut (exon 8)	Mut (exon 8)
	B	Mut (exon 8)	Mut (exon 8)
	M	Mut (exon 8)	Mut (exon 8)
11	A	Mut (exon 8)	Mut (exon 8)
	B	Mut (exon 8)	Mut (exon 8)
	M	Mut (exon 8)	Mut (exon 8)
20	A	wt	_ ³
	B	wt	_ ³
	M	Mut (exon 5)	_ ³
<i>Primary tumour without metastasis</i>			
2	A	Mut (exon 5)	Mut (exon 5)
	B	Mut (exon 5)	_ ³
3	A	wt	wt
	B	Mut (exon 7)	_ ³
4	A	Pol (inton 6)	Pol (inton 6)
	B	Pol (inton 6)	Pol (inton 6)
7	A	wt	wt
	B	wt	wt
10	A	wt	wt
	B	wt	wt
12	A	Mut (exon 7)	Mut (exon 7)
	B	Mut (exon 7)	Mut (exon 7)
13	A	wt	Mut (exon 6)
	B	wt	Mut (exon 6)
14	A	w	wt
	B	wt	wt
15	A	wt	wt
	B	wt	wt
16	A	wt	_ ³
	B	wt	_ ³

Table 1
(Continued)

Case number	Tumour ¹ sector	p53 DGGE variants ²	
		DId	DIA
17	A	wt	_ ³
	B	wt	_ ³
18	A	wt	_ ³
	B	wt	_ ³
19	A	wt	_ ³
	B	wt	_ ³

¹A is for superficial tumour sectors; B is for deep infiltrating tumour margins; M is lymph node metastasis.

²When tumour and control mucosa samples showed identical DGGE variant, the mutation was considered of germinal origin (polymorphism, indicates as Pol); when the variant was present exclusively in the tumour the mutation was of somatic origin (indicated as Mut). DId: DNA diploid cell component, DIA: DNA aneuploid cell component.

³DNA aneuploid component was not present.

Note: wt = wild type.

Table 2

p53 mutations in DNA diploid and aneuploid cell components of lymph node metastases from human colorectal adenocarcinomas*

Case ¹ number	p53 DGGE variants ²	
	DId	DIA
1 (1)	Mut (exon 6)	Mut (exon 6)
2 (5)	wt	_ ³
3 (6)	Mut (exon 6)	Mut (exon 6)
4 (8)	Mut (exon 5)	_ ³
5 (9)	Mut (exon 8)	Mut (exon 8)
6 (11)	Mut (exon 8)	Mut (exon 8)
7 (20)	Mut (exon 5)	_ ³

*See details also in the legend of Fig. 1.

¹Number in parenthesis refers to primary tumour number as listed Table 1.

²DId: DNA diploid cell component, DIA: DNA aneuploid cell component.

³DNA aneuploid component was not present.

Typical results of the PCR based DGGE analysis on p53 exons 8 and 7 are shown in Fig. 2 for tumours nos 11 and 12 (panels A and B, respectively). For both tumours, a robust band corresponding to the wild-type allele was visible in the DId component, along with very faint heteroduplex bands. On the contrary, the band corresponding to the mutant allele was almost the only one visible in the DIA component. A similar pattern was also observed in the DId and DIA components of the invaded lymph nodes (Fig. 2, panel A). These results indicate that only a small fraction of the alleles (< 10%) in the cell population of the DId component are variant alleles and suggest that LOH could have occurred in the DIA cell component. This hypothesis was confirmed for tumours 6 and 8, by LOH analysis at the highly polymorphic marker YNZ22 which maps close to the p53 gene (band 17p13.3). In both cases two alleles were present in the control mucosa and in the DId tumour cell component, while only one allele was present in the matching DIA tumour cell component (not shown).

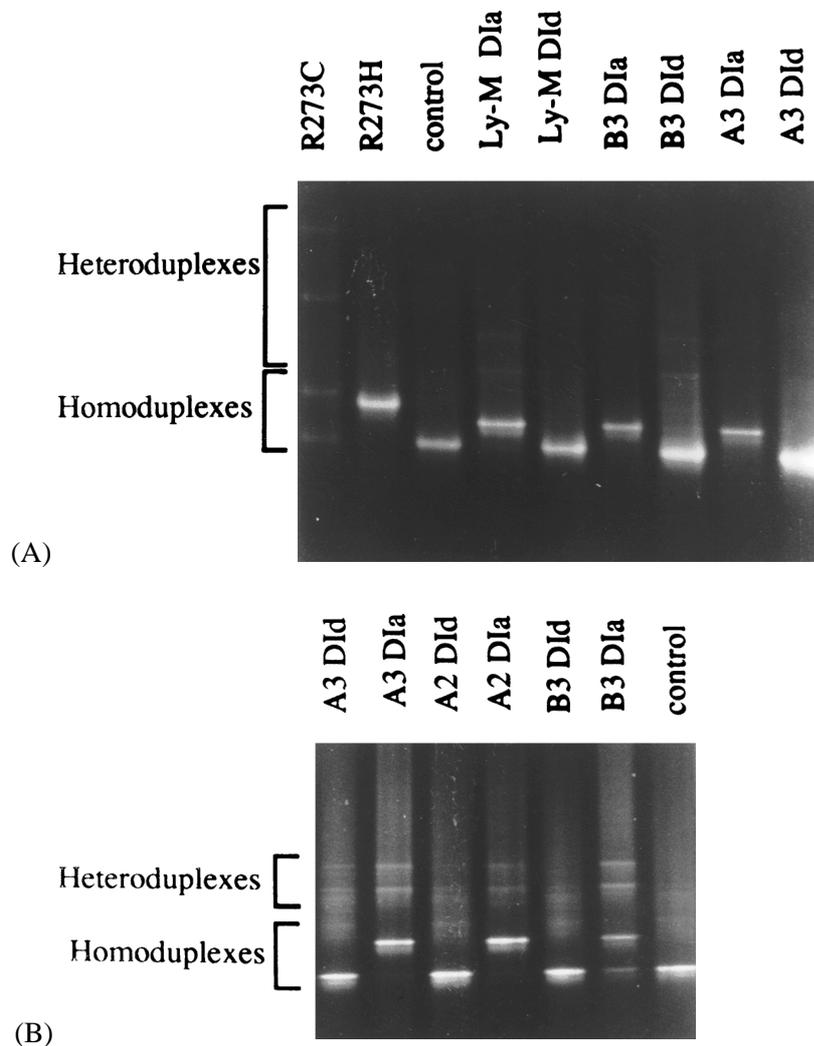


Fig. 2. PCR-based DGGE analysis of p53 gene. Panel A: analysis of exon 8 DGGE variants in tumour no. 11. R273C positive control (hemizygote for an Arg273 > Cys); R273H, positive control (homozygote for an Arg273 > His); control mucosa; lymph node metastasis (Ly-M); superficial (A) and deep (B) tumour sectors from which DIa and DIId tumour cell components were sorted. The corresponding mutation is reported in Tables 2 and 3 for primary tumour and lymph node metastasis, respectively. Panel B: analysis of exon 7 in sample 12. Superficial (A) and deep (B) tumour sectors from which DNA diploid or aneuploid nuclei were sorted; control mucosa. The corresponding mutations are reported in Table 3.

Each DGGE variant was reconfirmed from a new aliquot of nuclei, and then characterized at the molecular level by DNA sequencing (Table 3, Fig. 3). Sequencing results for tumour no. 11 (GC > AT, Arg282 > Trp, right panel) and tumour no. 12 (GC > AT, Arg248 > Gln, left panel) are shown in Fig. 3. Eight out of ten p53 changes were GC > AT transitions, the majority of which (6/8) involved CpG sites. The features of the mutations observed are consistent with those reported by others [27,28] and suggest a major role for mutagenic events occurring at CpG sites. Spontaneous or enzymatic deamination of 5-methylcytosine at CpG sites occurs frequently in the genome and the repair of the resulting G/T mismatch is relatively inefficient, as recently shown by Schmutte et al. [38].

Table 3

Molecular characterization of the p53 gene mutations observed in this study*

Tumour ¹	Exon	Mutation ²	Aminoacid change	CpG site
<i>Polymorphysm</i>				
4	6	GGA > GGG	I 6: +31 ³	
<i>Somatic mutations</i>				
1	6	CGA > TGA	Arg196 > Stop	+
2	5	CAT > CAG	His179 > Gln	-
3	7	TGC > TAC	Cys242 > Tyr	-
6	6	CGA > TGA	Arg213 > Stop	+
8	5	CGC > CAC	Arg175 > His	+
9	8	CGT > TGT	Arg273 > Cys	+
11	8	CGG > TGG	Arg282 > Trp	+
12	7	CGG > CAG	Arg248 > Gln	+
13	6	GTG > ATG	Val216 > Met	-
20	5	GAT > CAT	Asp184 > His	-

*See details also in Fig. 1.

¹Number of sample.

²Underlined: mutated base.

³Mutation in intron 6, 31 base pairs downstream from the 3' end of exon 6, is not predicted to create an alternative splice site and may represent a new polymorphism (Campomenosi et al. [10]).

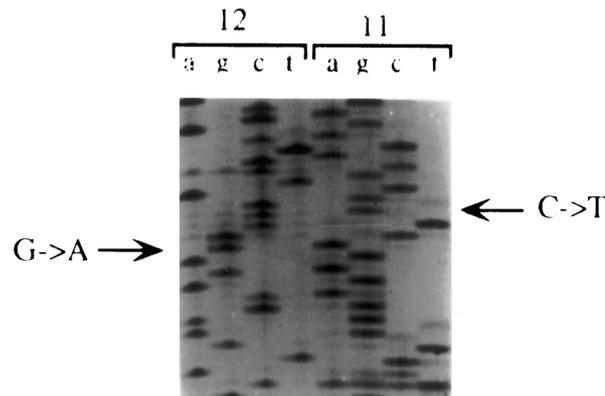


Fig. 3. Sequence analysis of DGGE variants from tumour no. 12 (left) showing a GC > AT transition at codon 248 (CGG > CAG), and from tumour no. 11 (right) showing a CG > TA transition at codon 282 (CGG > TGG).

4. Discussion

Aneuploid cells are commonly observed in human solid tumours. This observation suggests that cellular functions that control the fidelity of chromosome segregation are lost during tumour progression. In the present study, we investigated if loss of p53, a gene known to be involved in the maintenance of genome stability, plays a role in the origin of aneuploid cells in colorectal adenocarcinomas. To this purpose DId and DIa colorectal adenocarcinomas were analyzed for the presence of p53 mutations. Cell

subpopulations with an abnormal DNA content were detected by flow cytometry in 15 out of 20 carcinomas (75%). None of the DNA diploid and 60% of the DNA aneuploid tumours showed p53 mutations in the region under study (exons 5–8). Therefore, p53 mutations occurred significantly more often in DNA aneuploid than in DNA diploid tumours ($p < 0.04$, Fisher's exact test). A different situation has been reported for colorectal adenomas where p53 mutations are rare [42,43] while aneuploidy is observed in 30% of cases. Leaving aside the possibility that p53 mutations were underestimated in early adenomas, an explanation may be that different factors play a role in the generation of aneuploidy in early and late stages of colorectal carcinogenesis. In this respect it is interesting to note that cell lines transfected with human mutated K-ras2 showed chromosome instability [37], suggesting that K-ras may play a role in the generation of aneuploidy. Moreover, the high incidence and correlation with aneuploidy of 1p deletions among colorectal adenomas has suggested a possible role of gene(s) mapping to this chromosome locus in the control of chromosome stability [19,20] also prior to p53 mutations. Therefore, it seems reasonable to envisage that both p53 dependent and p53 independent mechanisms may originate aneuploid cells during colorectal cancerogenesis [24]. In case of p53 independent mechanisms, p53 mutations could play a role in DNA aneuploid subclone selection and expansion rather than in the generation of aneuploidy.

DNA aneuploid tumours, for which we have also separately analyzed the DI_d cell component, showed that both DI_a and DI_d cell components had the same p53 mutations although the relative proportion of the mutant and the wild-type alleles, as estimated by the intensity of the DGGE bands, were clearly different. Whereas the mutated allele represented the major band in the DNA aneuploid components, only very faint bands corresponding to heteroduplexes were visible in the matching DI_d components. Although many morphological and technical (FACS) strategies were used to enrich for epithelial tumour cells, we cannot exclude that the DI_d cell component found in DNA aneuploid tumour is in fact formed by normal non tumour cells. Furthermore, it is very likely that DI_a nuclei cross-contaminate the DI_d component and viceversa. Therefore, the very faint heteroduplex bands observed in DGGE for the DI_d component of DNA aneuploid tumours are most probably due to cross-contamination.

In the present study, we have also partly addressed the problem of the intratumour homogeneity of p53 mutations [9]. We are well aware that this question would require the separate analysis of many more samples than we have done (one mixed sample for superficial sites and one for deep infiltrating sites). The present series of cases suggests that there is no difference in the incidences of p53 mutations between superficial and invasive tumour margins.

In conclusion, we showed that p53 mutations occur more frequently in DNA aneuploid than in DNA diploid tumours and that cells of the DI_a cell component showed the presence of a single mutant allele, apparently generated by two hits: p53 mutation and LOH. The finding that the same mutation was observed in lymph node metastases indicates that proximal lymph node metastases are clonal with respect to the primary tumour and may be originated by the p53^{-/-} aneuploid cells, at least in the cases presently under study.

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