Pergamon

European Journal of Cancer Vol. 33, No. 8, pp. 1314–1322, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: 80959-0849(97)00118-4

Original Paper

The Status of p53 in the Metastatic Progression of Colorectal Cancer

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In order to investigate the role of *TP53* in tumour progression and metastasis, we analysed 33 liver metastases of colorectal carcinomas and 19 primary colon carcinomas from the same hospital with respect to mutational changes, loss of heterozygosity and expression of the *TP53* tumour suppressor gene. Direct sequencing of PCR products corresponding to the coding region of *TP53* revealed that 13 of 19 primary tumours (68%) and 23 of 33 liver metastases (70%) had mutations in the *TP53* gene. The distribution of mutations along the coding region of *TP53* was similar in liver metastases compared to primary tumours. Thus, codon specificity did not seem to be a relevant factor and cells carrying specific *TP53* mutations seem to have no selective advantage in the metastasising process. Comparing our data with the mutational spectra found in other countries did not reveal differences in the distribution of mutations along the coding region. Most of the metastases analysed showed loss of heterozygosity (LOH, 9 of 12 cases, 75%) and strong nuclear staining in immunohistochemistry (10 of 17 cases, 59%). Furthermore, with respect to mRNA expression levels, tumours carrying *TP53* mutations showed significantly higher p53 mRNA levels compared to those without *TP53* mutations. Thus, regulation of p53 mRNA levels seems to be subject to selection processes in tumourigenesis. © **1997 Published by Elsevier Science Ltd.**

Key words: colorectal cancer metastase, p53 mutations, immunohistochemistry, PCR, mRNA-expression, loss of heterozygosity

Eur J Cancer, Vol. 33, No. 8, pp. 1314–1322, 1997

INTRODUCTION

AMONG THE most frequent genetic lesions observed in human cancer are mutations of the *TP53* tumour suppressor gene [1, 2]. Mutations of this gene have been associated with tumours from a variety of human organs and have also been detected as germline mutations in inherited forms of cancer [3–5]. In sporadic human malignancies, *TP53* mutations can occur in both early and late phases of tumour progression depending on the tumour type [6].

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Received 5 Jul. 1996; revised 27 Jan. 1997; accepted 4 Feb. 1997.

Most of the point mutations accumulate in highly conserved regions of the *TP53* coding sequence [7]. However, mutations outside these regions have also been described in human cancers [8]. The oncogenic potential of *TP53* mutations is not only due to the loss of the tumour suppressive function but also to a dominant negative effect, which is caused by oligomerisation of wild-type and mutant p53 protein, driving the wild-type protein into the mutant conformation [9].

On the cellular level, p53 is involved in regulation of the cell cycle [10]. The p53 protein is phosphorylated in a cell cycle-dependent manner by key regulators of the cell cycle [11, 12]. At the G1/S-boundary, the p53 protein is translocated to the nucleus where it exerts its function [13].

Furthermore, loss of p53 function eliminates the growth arrest in response to DNA damage [14] and enhances the frequency of gene amplification [15, 16], suggesting a role of p53 in the control of a cell cycle checkpoint and in the maintenance of genome integrity [17]. *TP53* codes for a DNA binding protein which activates transcription *in vitro* [18]. Both DNA binding and transactivating activity get lost in most of the point-mutated p53 proteins [19, 20]

In colorectal carcinogenesis, abnormalities of the *TP53* gene have been associated with advanced cancer stages. In contrast to certain somatic alterations already detectable in adenomas (e.g. mutations of the *RAS* oncogene and of the *APC* tumour suppressor gene), point mutations of *TP53* and allelic loss of the *TP53* chromosomal locus usually occur late in colorectal carcinogenesis [21]. Several studies which investigated the mutational status of the *TP53* gene have found a significant association between the presence of a mutation and poor patient survival [22–25]. In contrast, studies investigating only expression of the p53 protein have shown contradictory results. While p53 overexpression has been correlated significantly with poor prognosis in short- and long-term survival in some studies [26, 27], other studies have been unable to detect such a correlation [25, 28].

In this study, we analysed the status of p53 in metastases of colorectal carcinomas with respect to point mutations, allelic deletions and expression levels. The goal of our study was to analyse the role of *TP53* mutations not only in the development of primary tumours but also in metastatic tissues.

MATERIALS AND METHODS

RNA isolation and sequencing was performed as described previously [29]. Fresh frozen tumour tissues from patients undergoing abdominal surgery were cryostat-sliced. Areas showing a tumour cell content of more than 70% were pooled and used for RNA extraction. Total RNA was isolated from homogenised tissue (RNAzol-kit/Cinna Biotecx Laboratories, Houston, Texas, U.S.A.), reverse transcribed into cDNA (M-MLV-Reverse Transcriptase, Gibco/BRL, Bethesda, Maryland, U.S.A.) and cDNA sequences corresponding to the coding region of *TP53* PCR-amplified (primer sequences shown below). 50 ng cDNA were amplified for 30 cycles using 1 unit Taq Polymerase (Cetus) in a total volume of 50 µl (0.5 µM Primer, 200 µM dNTP each, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% Gelatine). PCR products were purified ('Gene Clean'-kit, Bio 101, La Jolla, California, U.S.A.) and bound via biotin–streptavidin binding to magnetic particles ('Dynabeads M-280', Dynal, Hamburg, Germany). Single-stranded templates for sequencing were generated by alkaline denaturation and magnetic separation of the strands using the protocol for solid-phase sequencing of Dynal. Sequence reactions were performed using the Sequenase 2.0 kit of USB (Cleveland, Ohio, U.S.A.) based on the Sanger dideoxy-chain termination method. PCR products comprising all four hot spots were generated with the primers P15 and P18 (codons 120–311). Extending the sequence reaction from primer P16 revealed the sequence of hot spots A and B, from primer P18 the sequence of hot spots C and D. Primer pairs P19/P20 and P23/P24 were used to sequence the 5'end of the *TP53* reading frame, primer P17 and P22 to sequence the 3'end.

Loss of heterozygosity

Loss of *TP53* alleles was determined using two different methods. One based on a length polymorphism due to a variable number of tandem repeats (VNTR) located near the chromosomal marker YNZ22 (HGM locus *D17S30*). PCR amplification using primer sequences flanking this region results in products ranging from 170 bp to 870 bp, as expected for 1–11 repeat units [30]. The second method is based on a restriction fragment length polymorphism due to a BstUl restriction site in codon 72 of p53. PCR amplification using the primers P11 and P12 generates a 263 bp fragment comprising this codon which is cut by BstUl to 174 bp and 89 bp fragments.

Immunohistochemical staining

Immunohistochemical detection of p53 protein was performed using the mouse anti-p53 monoclonal antibody PAb 1801 (Dianova, Hamburg, Germany), which recognises a denaturation resistant epitope between amino acids 32 and 79 [31]. Visualisation of antibody binding sites was performed with the immunoalkaline (APAAP) technique [32]. 7 µm thick acetone fixed cryostat sections were preincubated in 0.1% Triton-X-100 in phosphate-buffered saline (PBS) and incubated for 2 h with PAb 1801. The primary antibody was detected with calf intestinal alkaline phosphatase conjugated rabbit anti-mouse immunoglobulines (Dako, Hamburg, Germany).

mRNA expression

TP53 mRNA concentration was determined using a differential PCR assay [33, 34]: cDNA sequences of the target gene *TP53* and a reference gene (β_2-microglobulin and β-actin, respectively) were coamplified in the same reaction vessel. The ratio of the intensities of the two resulting bands indicated the relative gene expression of *TP53*. Quantification of PCR products was performed via determination of the high optical density at 260 nm after separation of the PCR products by means of high-performance liquid chromatography (HPLC), using the TSK DEAE-NPR column of Perkin–Elmer Cetus. *TP53* cDNA sequences were amplified using the primers P15 and P16 which both span exon–intron boundaries to avoid amplification of contaminating genomic DNA.

Primer sequences

P11 (p53): 5'-TTGATGCTGTCCCCGGACGA-3'
P12 (p53): 5'-TCAGGGCAACTGACCGTGCA-3'
P15 (p53): 5'-GTCTGTGACTTGCACGTACT-3'
P16 (p53): 5'-CAGTCAGAGCCAACCTCAGG-3'
P17 (p53): 5'-CGACATAGTGTGGTGGTGCC-3'
P18 (p53): 5'-GTTGTTGGGCAGTGCTCGCT-3'
P19 (p53): 5'-TCTAGAGCCACCGTCCAGGGA-3'
P20 (p53): 5'-GTGCTGTGACTGCTTGTAGA-3'
P22 (p53): 5'-AGGCTGTCAGTGGGGAACA-3'
P23 (p53): 5'-AGCCAGACTGCCTTCCGGGT-3'
P24 (p53): 5'-CGTAGCTGCCCTGGTAGGTT-3'
VNTR-5': 5'-CGAAGAGTGAAGTGCACAGG-3'
VNTR-3': 5'-CACAGTCTTTATTCTTCAGCG-3'
β-actin-5': 5'-CCTTCCTGGGCATGGAGTCCT-3'
β-actin-3': 5'-GGAGCAATGATCTTGATCTTC-3'
β_2-microglobulin-5': 5'-CTTACTGAAGAATGGAGAGAGA-3'
β_2-microglobulin-3': 5'-CTTACATGTCTCGATCCCACTT-3'

TP53 and β_2-microglobulin primer sequences were designed using the *TP53* sequence published in [35] and using the β_2-microglobulin sequence published in [36]. The VNTR and β-actin primer sequences were taken from [30] and [37], respectively.

RESULTS

Detection of TP53 mutations by direct sequencing

To generate templates for sequencing, cDNA sequences corresponding to the *TP53* coding region were PCR-amplified and directly sequenced. The results are summarised in Table 1. Twenty-three of the 33 liver metastases (70%) had mutations in the *TP53* gene. All but three of the mutations were missense mutations which led to an amino acid exchange. Figure 1(a) shows an example of a G to A transition found in codon 173 in the liver metastasis and the corresponding primary tumour of the same patient. One mutation was a deletion of a complete codon (codon 237, Figure 1(b)). Mutations resulting in premature stop codons could be detected in two cases (pts 45 and 112). One (pt 45) was a frame shift mutation located at the 5'end of the *TP53* reading frame and consisted of a deletion of exon 3. This deletion of 22 bp (codons 26–32) resulted in a truncated p53 protein due to a premature translation stop at codon 43. The other was a nonsense mutation located in codon 236 (pt 112).

Missense mutations also predominated among the primary colorectal tumours (Table 2). Thirteen of 19 primary tumours (68%) had mutations in the *TP53* gene. Only two of the mutations were frame shift mutations caused by a deletion of one nucleotide in exon 5 or 6. Thus, of all mutations leading to truncated forms of p53, only one (UPN 45, Table 1) was located outside exons 5–8. In contrast, no missense mutations were found outside exons 5–8, which is consistent with *TP53* mutation patterns of other tumours [8].

Most of the mutations which led to base substitutions were G:C to A:T transitions (27 of 34, 79%). There was no significant difference between primary tumours and metastases, with 8 of 11 (73%) and 19 of 23 (83%) belonging to this type of mutation, respectively. These results are consistent with former studies on primary colon tumours, where approximately 80% of all transitions have been reported to be G:C to A:T [1]. More than half the mutations occurred at CpG dinucleotides (19 of 34, 56%). The high mutability of CpG dinucleotides is attributed to the presence of 5-methylcytosine residues found at these dinucleotides in the mammalian genome [38]. In the *TP53* gene, complete methylation of all CpG dinucleotides has been reported recently [39].

Site specificity of TP53 mutations in liver metastases

We also analysed our data with respect to stage specific and epidemiological differences in the distribution of mutations along the *TP53* gene. There seems to be a preferential occurrence of mutations in exons 7 and 8 compared to exons 5 and 6. However, there was no significant difference in the distribution of mutations along the coding region of *TP53* between primary tumours and liver metastases. Furthermore, no significant epidemiological differences could be detected, although some studies of other coutries reported a higher incidence of mutations in codon 175 compared to our study [21, 24].

Loss of heterozygosity at the p53 locus

Since, in most cases, no wild-type base was visible at the mutant position on the sequence gel (not shown), there had to be a functional homozygosity for the mutation at the ex-

Table 1. TP53 mutations in liver metastases of colorectal carcinomas

UPN	Tumour grade	Codon	Exon	Mutation		CpG	Alleles
1	2–3	266	8	G → A	Gly → Arg	–	ND
2	2	266	8	G → A	Gly → Arg	–	2
Ly3	2	248	7	C → T	Arg → Trp	+	1
4	1	248	7	G → A	Arg → Gln	+	ND
19	2	257	7	T → C	Leu → Pro	–	ND
26	3	173	5	G → A	Val → Met	–	ND
34	2	245	7	G → A	Gly → Ser	+	NI
35	1	237	7	Del	Met		1
43	1–2	248	7	G → A	Arg → Gln	+	1
45	3	26–32	3	Del	STOP	–	ND
50	1–2	273	8	G → A	Arg → His	+	ND
51	1–2	175	5	G → A	Arg → His	+	1
S51	1–2	175	5	G → A	Arg → His	+	1
64	2	248	7	C → T	Arg → Trp	+	1
66	2–3	245	7	G → A	Gly → Ser	+	1
72	2	158	5	C → T	Arg → Cys	+	1
88	2–3	275	8	G → A	Cys → Tyr	–	1
92	2	236	7	T → A	Tyr → Asn	–	NI
103		244	7	G → A	Gly → Asp	–	ND
104		179	5	A → G	His → Arg	–	ND
105		248	7	G → A	Arg → Gln	+	ND
106		248	7	C → T	Arg → Trp	+	ND
108		174	5	G → A	Arg → Lys	–	ND
112		236	7	C → A	Tyr → STOP	–	ND
123		161	5	G → A	Ala → Thr	–	ND

Ly, lymph node metastasis; S, skin metastasis; UPN, unit patient number; ND, not determined; NI, not informative; Del, deletion.

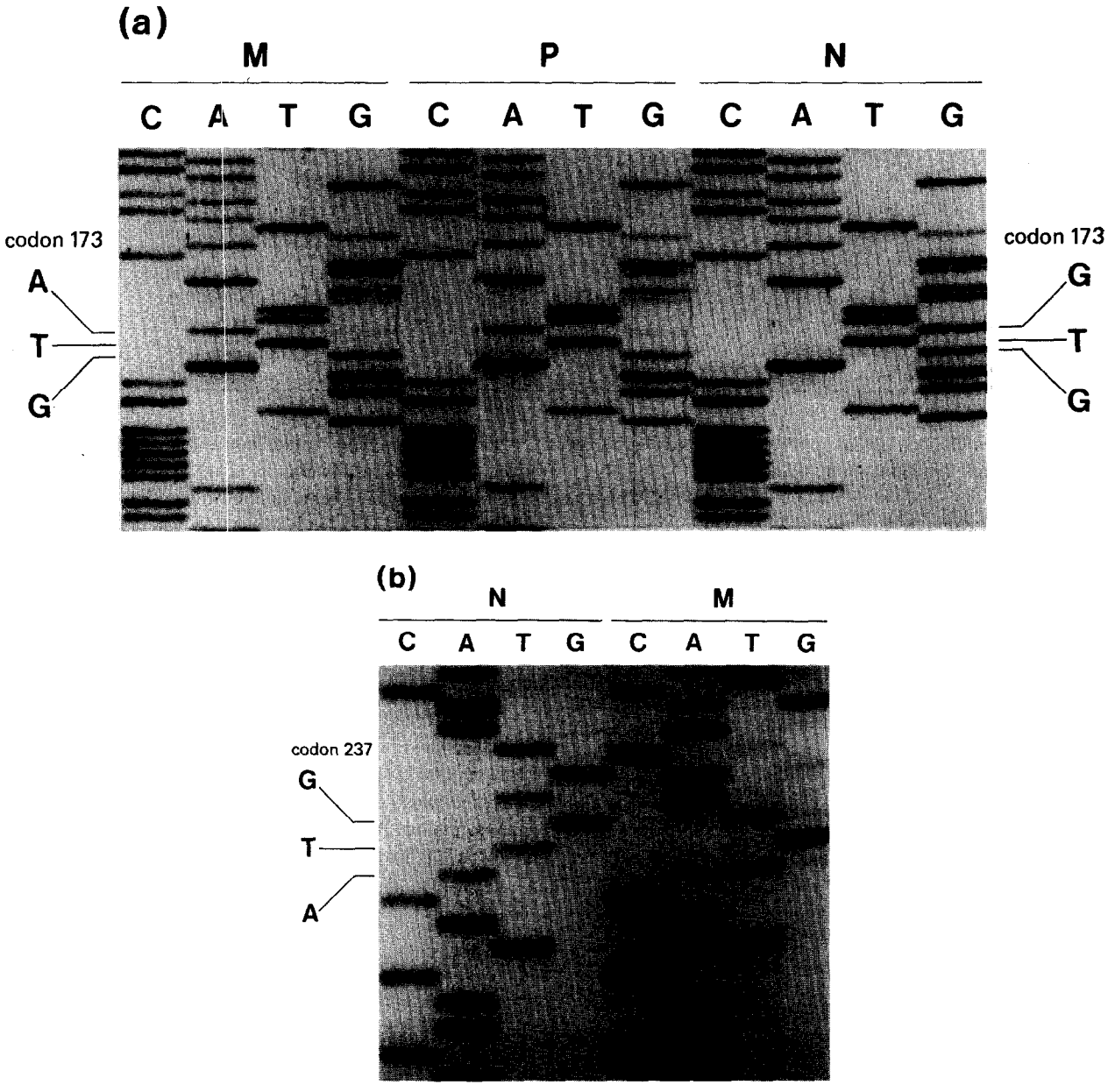

Figure 1. Sequence of *TP53* cDNA corresponding to exon 5 (a) and exon 7 (b) derived from normal (N) and metastatic (M) liver tissue and from the corresponding primary colon tumour (P) of two patients. (a) The same G to A transition in codon 173 is seen in both the primary and the metastatic tissue of patient number 26. (b) A deletion of codon 237 in the metastasis of patient number 35 is shown.

pression level. This could be due either to overexpression of mutant p53 which would mask expression of the wild-type allele or due to loss of heterozygosity of the nonmutated allele. To check the second possibility, we determined the number of *TP53* alleles in the metastases using two different methods. Figure 2 shows an example of a length polymorphism based on a VNTR region located near the *TP53* locus. Loss of heterozygosity was clearly evident in patients 3, 35 and 64. The minor band seen in metastatic tissue of patient number 64 was probably caused by contamination of the tissue sample with surrounding nonmalignant tissue. Data for patient number 34 was not informative with this method. The minor band in patient 46 was due to an amplification artefact that appeared in different samples with this PCR method.

The second method which was used to detect allelic loss was based on a RFLP polymorphism. An example is shown in Figure 3. Loss of one *TP53* allele was clearly evident in patients 43 and 66 where only the allele without a restriction site was left in the tumour. Data for patients 32 and 34 were not informative with this method. Patient 2 did not show loss of heterozygosity.

In summary, with one exception, allelic loss at the *TP53* locus was detectable only in those metastases where a mutation in the *TP53* gene was found (Table 1). Nine of 12 informative cases (75%) showed loss of heterozygosity. Only one tumour was detected which carried a *TP53* mutation and retained both alleles (UPN2). Our results are consistent with the data published for primary colon cancer, which

Table 2. TP53 mutations in primary colorectal carcinomas

UPN	Stage	Grade	Codon	Exon	Mutation		CpG
KP1	pT3, pN0, pM1	G2-3	148/149	5	T deleted	STOP 169	
KP4	pT4, N1, M1	G3	248	7	G → A	Arg → Glu	+
KP6	pT4, No, M0		248	7	G → A	Arg → Glu	+
KP7	pT3, N0	G2	189/190	6	C deleted	STOP 246	
KP9	pT3, N0	G2	273	8	GT → AG	Arg → Gln	+
KP10	pT4, N1	G3	245	7	G → A	Gly → Ser	+
KP11	pT3, N2, M1	G3	282	8	C → T	Arg → Trp	+
KP16	pT3, N1, M1	G3	248	7	G → A	Arg → Glu	+
KP17	pT4, N0	G2	163	5	T → C	Tyr → His	–
KP19a			257	7	T → C	Leu → Pro	–
KP20	pT3, N0	G3	179	5	C → T	His → Tyr	–
KP26a			173	5	G → A	Val → Met	–
KP27	pT3, N1	G3	248	7	C → T	Arg → Trp	+

have also shown that deletions at the *TP53* locus occur in combination with *TP53* mutations [21].

Immunohistochemical detection of the p53 protein

Mutant p53 proteins are usually far more stable than wild-type p53 protein, which displays a half-life of only 20–30 min [40]. Thus, mutant p53 protein is preferentially detectable upon immunohistochemistry, while staining of wild-type p53 remains generally below the detection level. We analysed whether protein stabilisation of p53 was also involved in the metastases and used the monoclonal antibody PAb 1801 for detection. Table 3 summarises the results. Nuclear staining (intense/intermediate) was evident in 10 of 17 liver metastases analysed (59%) and was only restricted to tumour tissues of those metastases which had point-mutated *TP53* genes. Tumours with *TP53* mutations resulting in truncated p53 proteins (UPN 45) were negative for p53 protein. The results are consistent with immunochemical studies published in lung cancer where two expression groups have been defined depending on the type of *TP53* mutation [41]. High expressors all had missense mutations in exons 5–8, while low expressors harboured mainly those mutations leading to truncated forms of p53.

Weak to moderate staining of the cytoplasmatic compartment was also observed in many of the positive tumour tissues. Of six metastases which showed predominantly cytoplasmic staining, four did not carry mutated *TP53* genes. However, not all tumours which harboured *TP53* mutations were positive for p53 (UPN 72). Interestingly, *TP53* mutations which have been described to be normally expressed when mutated in the germline [3], showed strong overexpression at the protein level when mutated in metastases (UPN 3). Thus, the actual level of p53 protein in a

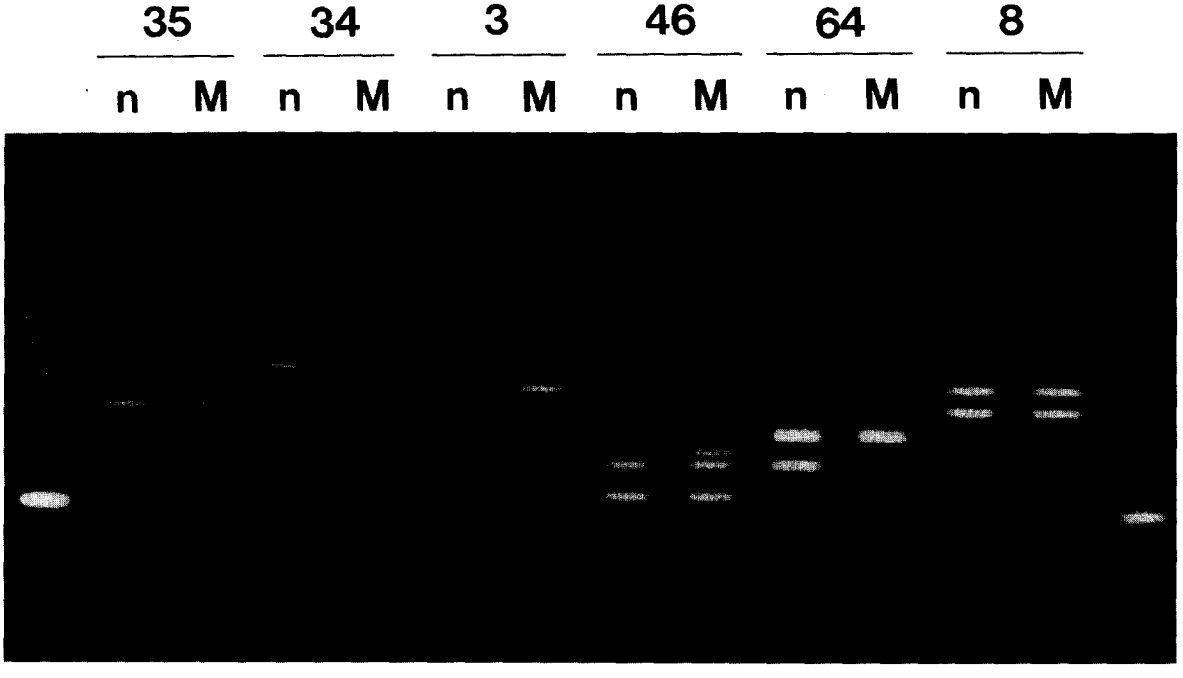

Figure 2. Loss of heterozygosity at the p53 locus detected by a length polymorphism due to a variable number of tandem repeats (VNTR) located in the vicinity of the chromosomal marker YNZ22. Gel electrophoresis of PCR products after amplification using primers flanking this VNTR region is shown. PCR products of 10 ng genomic DNA extracted from non-metastatic (n) and metastatic (M) tissue of the same patient are loaded alternately (unit patient number of Table 1). Left and right lane molecular weight marker (123 bp ladder).

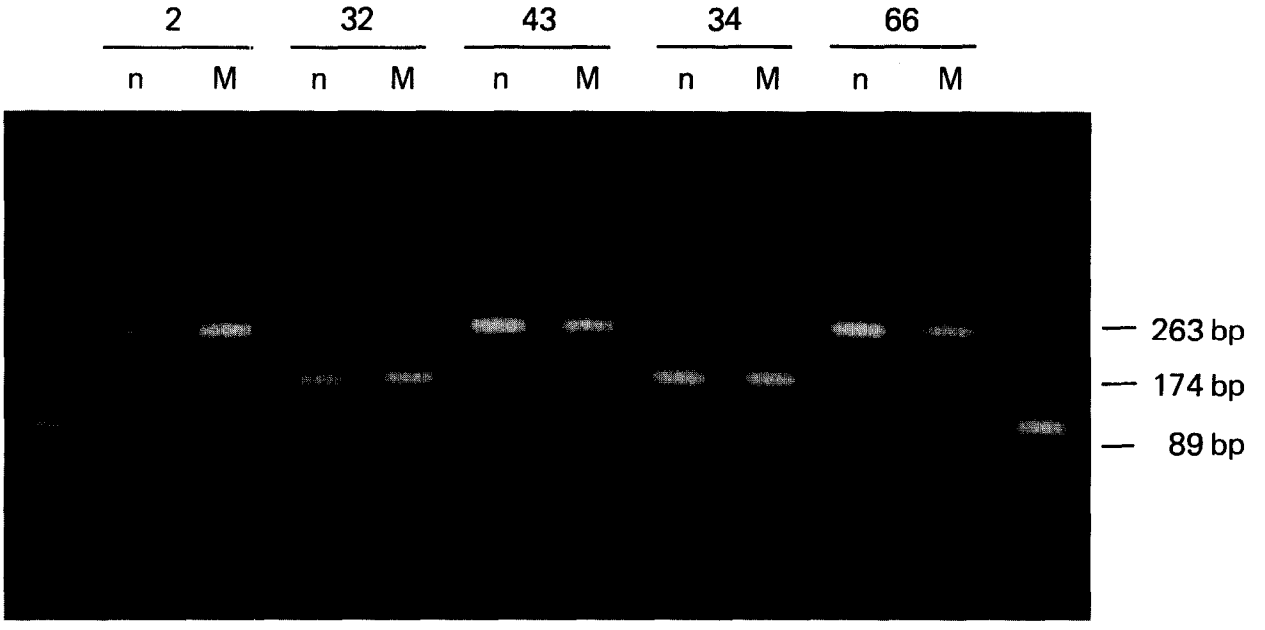

Figure 3. Loss of heterozygosity of *TP53* alleles detected by a restriction fragment length polymorphism (RFLP) due to a BstUl restriction site in codon 72 of *TP53*. Gel electrophoresis of PCR products after amplification using primers P11 and P12 flanking this restriction site is shown. PCR products of 10 ng genomic DNA extracted from non-metastatic (n) and metastatic (M) tissue of the same patient are loaded alternately (unit patient number of Table 1). The 263 bp fragment is cut by BstUl to 174 bp and 89 bp fragments. Left and right lane molecular weight marker (123 bp ladder).

tumour cell depends not only on the *TP53* mutation itself but may be subject to other regulatory processes.

p53 mRNA expression

To test whether immunohistochemical staining could be due to enhanced mRNA transcription levels, we determined the amount of p53 mRNA transcripts with the aid of a differential PCR assay [33, 34]: cDNA sequences of the target gene *TP53* and a reference gene (β_2-microglobulin) were coamplified in the same reaction vessel; the ratio of the intensities of the two resulting bands indicated the relative gene expression of p53 (Figure 4). PCR products were quantified using HPLC as described previously (42); the resulting values are summarised in Table 3. Interestingly, tumours with point-mutated *TP53* genes showed significantly higher expression levels when compared to tumours without *TP53* mutations ($P < 0.01$). These results were confirmed with expression assays using β-actin as a reference gene (data not shown). Furthermore, high mRNA expression levels were correlated with strong immunohisto-

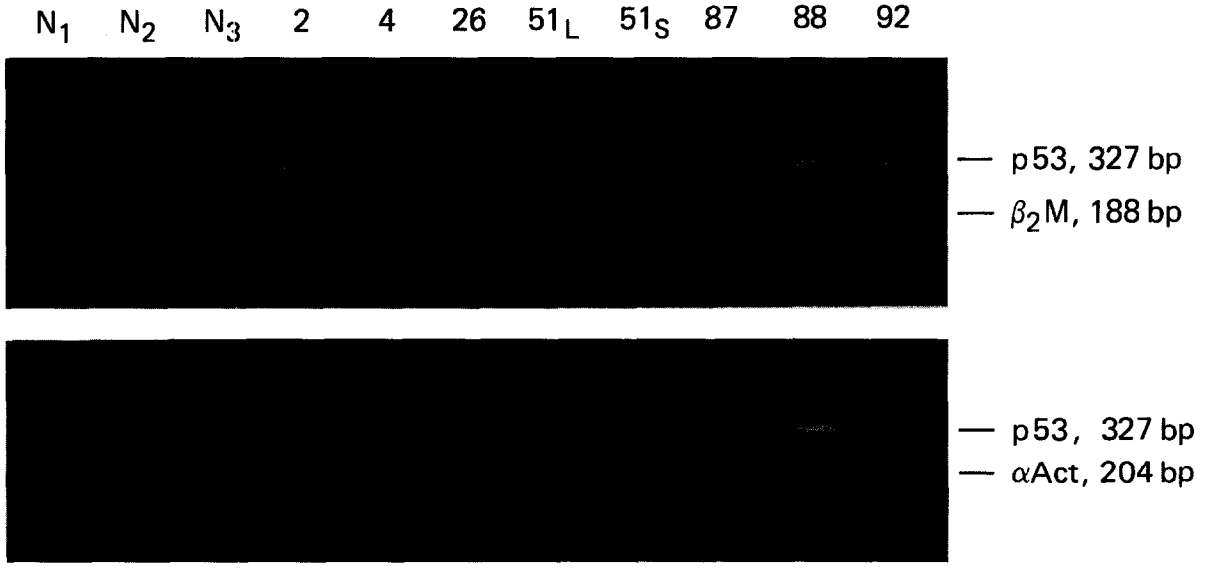

Figure 4. Relative mRNA expression of p53. The cDNA sequences of the target gene *TP53* and a reference gene (β_2-microglobulin and βactin, respectively) were coamplified in the same reaction vessel. The ratio of the intensities of the two resulting bands indicates the relative gene expression of *TP53*. N_1–N_3, normal colon mucosa of 3 patients with primary colorectal cancer; 2–92, unit patient numbers used in Tables 1 and 2; 51_L, 51_S, liver metastasis and skin metastasis of UPN 51, respectively. Compared to normal colon mucosa, overexpression of p53 was evident in patients 2, 4, 26, 51, 88 and 92, all of which had point mutated *TP53* genes.

Table 3. p53 expression in metastases of colorectal cancer

UPN	Tissue	Mut*	PAb1801†	β2M‡
1	L	266	++ n/c	5.2
2	L	266	+ + + n	5.0
3	Ly	248	+ + + n	0.7
4	L	248	++ n/c	3.1
8	L	–	+c	0.9
19	L	257	ND	3.0
21	L	–	ND	3.7
26a	Co	–	ND	2.5
26b	Co	173	ND	4.1
26	L	173	ND	2.4
27	L	–	ND	0.6
28	Lu	–	++ c	0.6
32	L	–	+c	0.8
34	L	245	ND	2.1
35	L	237	+c/n	3.1
42	Om	–	+c	0.3
43	L	248	+c	3.0
45	L	STOP	–	2.4
50	L	273	++ n/c	4.2
51	L	175	+ + + n	1.7
51	Sk	175	++ c	1.9
64	L	248	+ + + n	1.5
65	L	–	–	1.0
66	L	245	+ + + n	2.3
72	L	158	–	1.1
86	Co	–	–	0.3
87	L	–	–	0.8
88	L	275	+ + + n	2.9
92	L	236	++ n/c	3.1

*Mutated *TP53* codons taken from Table 1.

†Evaluation of immunohistochemical staining using anti-p53 monoclonal antibody PAb1801. Cytoplasmatic (c) and nuclear (n) staining is indicated. Staining was considered to be strong (+ + +) when intense staining could be observed in most cells, intermediate (++) when staining was less intense or when strong staining could be observed in approximately 50–70% of the cells, and weak (+) when there was slight staining in all cells or parts of them.

‡Relative mRNA expression levels of *TP53* with β_2-microglobulin (β2M) as a reference gene. PCR products of cDNA sequences were quantified photometrically via HPLC; the resulting values were related to normal colon mucosa.

L, liver metastasis; Ly, lymph node metastasis; Co, primary colon carcinoma; Lu, lung metastasis; Om, omentum metastasis; Sk, skin metastasis; UPN, unit patient number; ND, not determined

chemical staining (Spearman rank order correlation, $P = 0.015$).

DISCUSSION

In this report, we describe the status of *TP53* in liver metastases and primaries of colorectal carcinomas with respect to point mutations, allelic deletions and expression levels. Direct sequencing of the whole *TP53* coding region revealed mutation frequencies of 70% in hepatic metastases and 68% in primary colorectal tumours. Thus, according to our study the overall incidence of *TP53* mutations does not significantly change during haematogenous dissemination. Our data on primary colorectal tumours are consistent with those of other studies, where an incidence of 50–70% has been reported [21, 22, 43, 44]. The majority of mutations consisted of G:C to A:T transitions and more than half of these occurred at CpG dinucleotides which is also consistent with the data available on primary colon cancer.

In two cases where we could analyse the metastasis and the corresponding primary tumour of the same patient, the same *TP53* mutation was found in both tissues. Thus, in these tumours *TP53* mutations must have occurred before the metastatic spread. The same phenomenon has been described in ovarian and prostate carcinoma [45, 46]. However, in a recent study, it was reported that an increased incidence of *TP53* mutations was associated with hepatic metastasis in colorectal tumour progression [47]. These authors found that one third of 18 hepatic metastases excised coincidentally with the corresponding primary tumour of the same patient carried *TP53* mutations only in the liver metastasis. From these data, the authors draw the conclusion that *TP53* mutations must have occurred during or after metastatic spread. However, tumour progression is thought to be the result of clonal expansion of small subpopulations of tumour cells which have acquired additional genetic lesions. Thus, it seems possible that *TP53* mutations found in metastases are present only in minor subpopulations of cells in the primary tumours. Therefore, these cells might be undetectable in whole amount preparations as has been described in cervical cancer and soft tissue sarcomas [48, 39]. In contrast, since the majority of primaries analysed in our study were in advanced stages (T3, T4, M1), the incidence of 68% *TP53* mutations, which is at the upper limit of reported mutation rates [21, 24, 25], could be the result of clonal overgrowth with *TP53* mutant cell populations. This would explain the same incidence of *TP53* mutations in primary tumours and metastases. Thus, advanced tumour stages could be highly enriched in cells having an increased potential to invade due to a *TP53* mutation. Therefore, our data did not rule out the possibility that *TP53* might be a critical factor in initial steps of the metastatic process. However, subsequent dissemination and settlement of the cell in the liver may be dependent on local factors rather than *TP53* mutations.

Selection of certain mutations in the course of tumour progression might be important for metastasis formation, which should be reflected in different mutation spectra of primary tumours and metastases. However, in our study, there was no significant difference in the distribution of mutations along the coding region of *TP53* between primary tumours and liver metastases selected from the same hospital. Furthermore, no significant epidemiological differences could be detected when comparing the mutation spectra of primary colorectal tumours in different countries. However, some studies of other countries reported a higher incidence of mutations in codon 175 than we did [21, 24]. In one of these studies, mutations at this codon have been shown to correlate significantly with poor survival [24] which would predict a higher incidence of codon 175 mutations in metastases compared to primaries. However, colorectal tumours are probably induced not only by one but rather by a wide spectrum of metabolic carcinogens. Therefore, it may be difficult to find epidemiological differences since we have to deal with overlapping mutation spectra of numerous carcinogens. The very specific Aflatoxin and Hepatitis B virus induced *TP53*-mutation spectra in liver cancer epidemiology may represent a rare case in this context.

Immunohistochemistry has been widely used to reveal accumulation of the p53 protein in a wide spectrum of human malignancies [50]. In primary colorectal cancer, immunohistochemical staining has been detected with a frequency of

40–70% [26, 27, 51, 52]. In the metastases we analysed, strong or moderate staining was evident in 59% and therefore not enhanced in comparison to primary colorectal cancer. However, it is interesting to notice that *TP53* mutations which have been described to be normally expressed when mutated in the germline [3] showed overexpression at the protein level when mutated in metastases. Thus, it appears that the actual level of p53 protein is not only determined by the *TP53* mutation itself but also by other factors which might influence the expression level or metabolic stability. Our data indicate that transcriptional downregulation of wild-type p53 might be important in tumours without *TP53* mutations. Therefore, regulation of the p53 mRNA level is likely to be subject to selection in the process of tumorigenesis. As a consequence, malignant clones overexpressing mutant p53 mRNA might be selected because the oncogenic potential of the mutation will be enhanced. Clones underexpressing wild-type p53-mRNA in tumours without *TP53* mutations might be selected because the tumour-suppressing function of p53 might be reduced.

Dysregulation of p53 mRNA expression might be due to interaction of the *TP53* promoter with other oncogenes. Recently, we showed that expression of p53 and c-myc were positively correlated in hepatic metastases carrying a *TP53* mutation only, but not in those with wild-type p53 [53]. From these experiments we conclude that c-myc might induce p53 expression and that wild-type but not mutant p53 might be involved in a negative feedback regulation of c-myc expression. Thus, elevated p53 mRNA levels might be due to defects in transacting control mechanisms of p53 expression.

In conclusion, our data indicate a mutational rate of 68% in advanced primary colorectal tumours and of 70% in distant hepatic metastases. The data do not directly support a prominent role of *TP53* in late stages of colorectal tumour dissemination. However, the primaries analysed in this study were predominantly in advanced tumour stages, where clonal overgrowth and cell survival may also be a selective factor. Taken together, *TP53* mutations are frequent events in the progression of colorectal cancer and may enhance the development of distant metastases. Furthermore, transcriptional downregulation of p53 might be a relevant factor in tumours without *TP53* mutations.

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Acknowledgements—This work was supported by the Wilhelm-Sander Stiftung, Neustadt a. d. Donau, Germany and by the Berliner Krebshilfe.