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Original Paper

Ki-*ras* Oncogene and *p53* Tumour Suppressor Gene Mutations in Colorectal Carcinomas from the European Saar–Luxembourg Region are Less Frequent than Predicted by the Classic Adenoma–Carcinoma Sequence Model

M. Pauly,[1] M. Schmitz,[1] I. Kayser,[1] Ö. Türeci,[2] P. Lagoda,[2] G. Seitz[2] and M. Dicato[1]

[1]Laboratoire de Recherche sur le Cancer et les Maladies du Sang (RCMS), Bâtiment des Sciences, Centre Universitaire de Luxembourg, Avenue de la Faïencerie 162A, L-1511 Luxembourg, Grand Duchy of Luxembourg; and [2]Uniklinik der Universität des Saarlandes, Homburg/Saar, Germany

Recent investigations of colorectal cancer (CRC) have suggested that the accumulation of specific alterations in cell-growth regulating genes trigger the stage-wise progression to malignancy and that at least some of them could be useful for prognosis. In this study, the frequency, location and type of mutations of the Ki-*ras* proto-oncogene exons 1–2 and *p53* tumour-suppressor gene exons 5–9 were analysed in colorectal carcinomas of 72 patients from the European Saar–Luxembourg region using PCR–SSCP screening and direct sequencing. The incidences of Ki-*ras* activating and *p53* inactivating point mutations in these European samples were much lower (Ki-*ras*: 5 (6.9%) and *p53*: 13 (18.1%)) than reported for both genes in American studies (40–50% at least) ($P < 1 \times 10^{-3}$). These results suggest that other genetic mechanisms than those proposed for the classic adenoma–carcinoma sequence model can frequently underlie CRC development and that Ki-*ras* and *p53* mutations should not be considered as universal markers for CRC. © **1997 Published by Elsevier Science Ltd.**

Key words: Ki-*ras* gene, *p53* gene, mutation, colorectal neoplasms, Europe, Germany, Luxembourg, Saar

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INTRODUCTION

COLORECTAL CANCER (CRC) is the third most prevalent malignancy in Europe [1]. It has also been reported to be an important cause of death from cancer in several countries, especially in Luxembourg, Ireland, Denmark and Germany [2]. From 1991 to 1992, for example, 106 and 729 new cases per year of malignant colorectal neoplasm (14.1% and 13% of the total number of annual malignant neoplasms) [3, 4] were, on average, diagnosed in Luxembourg and in the German Saar Country, respectively. Furthermore, over the same period, average mortalities of, respectively, 120 and 408 death cases per year (12.2% and 13.8% of the totality of annual cancer death cases) [5, 6] were attributed to CRC in these same countries.

Recent investigations revealed that specific genetic alterations in cell-growth regulating proto-oncogenes and tumour-suppressor genes accompanied CRC development and suggested that their accumulation triggered the stage-wise progression to malignancy [7–9]. In the classical adenoma–carcinoma sequence model [10], mutations in the Kirsten-ras (Ki-*ras*) proto-oncogene, located on chromosome 12p and a member of the *ras* gene family, would result in the histopathological evolution from class I adenomas (less than 1 cm in diameter) to class II adenomas (more than 1 cm in diameter, but without any foci of carcinoma) during the early stages of CRC. In 40–50% of class II adenomas, Ki-*ras* has been shown to carry activating point mutations mainly located in exon 1, at codons 12 and 13, and sometimes in exon 2, at codon 61 [11–13]. In the same model, deletions and mutations in the tumour-suppressor gene *p53*, located on chromosome 17p and known to be the most frequently altered gene in a wide variety of human cancers [14, 15], would correspond to the transition from class III adenomas (more than 1 cm in diameter, with foci of carcinoma) to carcinomas during the late stages of CRC. One allele of the *p53*

Correspondence to M. Pauly.
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gene has been found to be deleted in up to 75% of colorectal carcinomas, whereas the other allele harbours a point mutation in more than 90% of these cases [16]. While the first allele is usually destroyed by a large-sized deletion, giving rise to a loss of heterozygosity, the normal function of the remaining *p53* allele is mostly inactivated by a missense mutation in exons 5–8, frequently located in the five evolutionarily conserved domains of the gene [14, 17, 18]. As has been shown in several studies on various cancers, including CRC, point mutations in both the Ki-*ras* [19] and the *p53* gene [20, 21] represent an important prognosis factor.

In this study, attention was directed to analysis of the frequency, location and type of mutations in the Ki-*ras* proto-oncogene exons 1–2 and in the *p53* tumour-suppressor gene exons 5–9 in surgically resected colorectal carcinoma samples of 72 CRC patients from the European Saar–Luxembourg region. In order to perform a statistically representative and significant evaluation for the Saar–Luxembourg region, two sample series collected in two distinct areas of this region were analysed and compared: the L-series encompassed 36 colorectal carcinoma samples from the Grand Duchy of Luxembourg and the S-series 36 samples of the same stage from the German Saar Country. Genomic DNA extracted from these tumour tissue samples was systematically screened for mutations in the exons named above, using as a detection method the single-strand conformation polymorphism (SSCP) analysis of DNA fragments produced in amplifying the genomic regions of interest by the polymerase chain reaction (PCR). Mutations were then identified by DNA sequencing directly performed on the mutated PCR products, without the need for any previous cloning procedure.

MATERIALS AND METHODS

Patients

Samples were obtained from 72 CRC patients who had been living during their whole life in the European Saar–Luxembourg region, either in the Grand Duchy of Luxembourg for the L-series (36 samples, Table 1) or in the German Saar Country for the S-series (36 samples, Table 2).

The male:female ratio of the CRC patients was 1:1.1 in the L-series and 2:1 in the S-series. They ranged in age from 33 to 86 years in the L-series (average age: 65 years) and from 25 to 85 years in the S-series (average age: 58 years).

The patients were classified according to tumour stage (TNM system, UICC [22]). All samples were characterised by a histological staging equal to or greater than pT1 or 2, respectively, and were therefore considered to be late carcinoma stage. In the L-series, 23 patients were grade II, 6 grade III, 2 Dukes' A, 10 Dukes' B, 7 Dukes' C and 1 Dukes' D. In the S-series, 27 patients were grade II, 9 grade III, 4 Dukes' A, 13 Dukes' B, 9 Dukes' C and 10 Dukes' D.

Tumour samples and non-tumour control samples

Colorectal tumour samples were isolated during surgical resection from the excised material, freed of surrounding normal tissue, immediately frozen in liquid nitrogen and stored at −80°C until DNA preparation. The primary site of these samples was defined as right-sided for a location in the ascending and the first two-thirds of the transverse colon or as left-sided for a location in the last third of the transverse, the descending or the sigmoid colon or the rectum. Histopathological staging (TNM system, UICC [22]) was conducted respectively at the Histopathology Department of the National Health Laboratory (Laboratoire National de Santé) of Luxembourg for the L-series or at the Pathology Institute of the Saar Country University, Homburg, for the S-series.

As non-tumour control samples, blood was freshly collected from the same patients as above in vacutainers (Becton Dickinson, Rutherford, New Jersey, U.S.A.) containing the sodium salt of EDTA (ethylenediaminetetraaceticacid) as an anticoagulant. Leucocytes were collected by centrifugation at 4000 rpm for 10 min at 4°C after lysis of the red blood cells by a 15 min wash with the same initial volume of NH_4Cl 0.1 M (E. Merck, Darmstadt, Germany) in an ice bath at 0°C (repeated three times), and stored at −80°C until DNA preparation.

DNA preparation

Cellular genomic DNA from frozen solid tumour tissues or blood (leucocytes) samples was prepared according to the salt–chloroform extraction method [23], a modification of the standard proteinase K–phenol extraction method [24].

PCR amplification

The specific primers (Genset, Paris, France) used to amplify exons 1 or 2 of the human Ki-*ras* gene were designed and used as previously described [25–27]. Those primers used to amplify each of exons 5–9 of the human *p53* gene were provided with the human p53 amplimer panel (Clontech, Palo Alto, California, U.S.A.). All were exon-flanking primers designed to detect mutations located within exons as well as in flanking consensus splicing donor and acceptor sequences. PCR conditions were as specified by the respective manufacturer. In general, genomic DNA (200 ng) was denatured for 5 min at 95°C and amplified in a 25 µl reaction mixture containing the adequate sense and antisense primer couple (10 pmol of each primer), 1 unit of thermostable Taq DNA polymerase (Perkin-Elmer, Emeryville, California, U.S.A.), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Sigma Chemical Company, St Louis, Missouri, U.S.A.), 3 µCi [α-^{32}P] dCTP (labelling grade, specific activity: 800 Ci/mM, from ICN, Costa Mesa, California, U.S.A.), 10 mM Tris–HCl (pH 8.3) (Life Technologies, Gaithersburg, Maryland, U.S.A.), 50 mM KCl (Merck), 1.0 mM (for exon 2 of Ki-*ras*) or 1.5 mM (for p53 exons) or 2.0 mM (for exon 1 of Ki-*ras*) $MgCl_2$ (Merck), for 30 thermal cycles, each comprising a denaturation step of 1 min at 95°C, a primer annealing step of 2 min at the specified annealing temperature and a primer extension step of 3 min at 72°C, followed by a post-elongation step of 10 min at 72°C.

SSCP analysis

SSCP analysis of PCR products amplified as described above was mainly performed as previously specified [27]. Labelled PCR products were mixed to an equal volume of a dye loading solution (95% deionised formamide, standard TBE (Tris-borate-EDTA buffer 1x concentrated [24]), 0.2% (w/v) each of xylene cyanol and bromophenol blue), denatured for 5 min at 95°C, chilled on ice and immediately loaded on to a 0.4 mm thick non-denaturing 6% polyacrylamide gel (mono/bis-acrylamide ratio: 29/1) containing 10% glycerol in TBE buffer 1x. While renaturing, base-specific single-stranded conformers were separated by electrophoresis carried out on a FeatherVolt 3000 V power supply/Stratatherm Cold temperature controller combo (Stratagene,

Table 1. Clinical and genetic features of the L-series CRC patients

Patient number	Sex/age	Location	Histological grading	Ki-*ras* gene	*p53* gene
			TNM/grade/Dukes'	Exon/codon/mutation	Exon/codon/mutation
L1	M/48	R (C)	NA	—	—
L2	M/81	R(C)	T4N0Mx/II/B	—	6/213/CGA→CGG (Arg→Arg)
L3	F/59	L (R)	NA/NA/A	—	5/135/TGC→TAC (Cys→Tyr) 7/235/AAC→AGC (Asn→Ser)
L4	M/73	L (S)	T2N2Mx/II/C	—	—
L5	M/66	L (RS)	T2N0M0/II/NA	1/12/GGT→GTT (Gly→Val)	—
L6	M/47	L (R)	NA	—	—
L7	M/52	L (C)	T3N0Mx/II/B	—	—
L8	F/60	L (S)	T3N0Mx/II/B	—	5/NA
L9	M/70	L (R)	NA	—	—
L10	M/63	L (C)	T2N0Mx/II/B	—	5/163/TAC→TGC (Tyr→Cys)
L11	M/62	R (C)	T4N2Mx/III/NA	—	—
L12	M/79	L (S)	T3N0Mx/II/NA	—	—
L13	F/77	L (R)	T3NxM1/II/NA	—	—
L14	F/47	R (C)	T3N0Mx/II/B	—	5/137/CTG→CAG (Leu→Gln)
L15	M/77	L (RS)	T3N0Mx/II/B	—	5/176/TGC→TTC (Cys→Phe)
L16	F/80	L (S)	NA/NA/D	—	5/154/GGC→GGG (Gly→Gly) 6/213/CGA→CGG (Arg→Arg)
L17	F/63	L (S)	T3NxM1/III/NA	—	6/192/CAG→TAG (Gln→stop)
L18	M/65	R (C)	T4N2Mx/III/NA	—	—
L19	F/76	L (R)	T3N0Mx/II/NA	—	—
L20	M/56	R (C)	T3N0Mx/II/NA	—	—
L21	F/74	R (C)	T3N1Mx/III/C	—	—
L22	F/86	R (C)	T3N0Mx/II/B	—	5/154/GGC→AGC (Gly→Ser) 6/213/CGA→CGG (Arg→Arg) 8/282/CGG→TGG (Arg→Trp)
L23	M/48	L (R)	T4N0Mx/II/NA	—	—
L24	M/54	L (S)	NA	—	5/NA 6/213/CGA→CGG (Arg→Arg)
L25	F/65	L (S)	T4N2M1/NA/NA	1/12/GGT→AGT (Gly→Ser)	5/NA
L26	M/33	R (C)	T3N0Mx/II/NA	—	—
L27	F/69	L (S)	T4N3Mx/II/C	—	—
L28	F/64	L (R)	T3N2M1/III/C	—	—
L29	F/73	L (RS)	T4N2Mx/II/C	—	—
L30	F/76	L (C)	T3N0Mx/II/B	1/12/GGT→GTT (Gly→Val)	—
L31	F/69	L (R)	T3N0Mx/II/NA	—	—
L32	F/72	R (C)	T3N1Mx/III/B	—	—
L33	F/58	L (RS)	T1N0Mx/II/A	—	9/NA
L34	F/75	L (RS)	T4N1Mx/II/C	—	—
L35	F/52	L (RS)	T4N2Mx/II/C	—	5/175/CGC→CAC (Arg→His) 6/213/CGA→CGG (Arg→ARG)*
L36	M/86	L (S)	T3N0Mx/II/B	—	—

F, female; L, left-sided; M, male; NA, not available; R, right-sided; (R), rectum; S; sigmoid colon; C, colon.
*Found by TaqI restriction analysis. Codon numbering of the Ki-*ras* proto-oncogene is according to Ref. [11] and that of the *p53* tumour-suppressor gene to Ref. [40].

Heidelberg, Germany) for 4 h at a constant power of 50 W and a constant temperature of 20°C in a 4°C cold room. The gel was finally dried in an oven for 1 h at 80°C and exposed for autoradiography to a Hyperfilm MP (Amersham, Little Chalfont, Buckinghamshire, U.K.) for at least 15 h at room temperature.

Direct DNA sequencing

DNA fragments corresponding to aberrant or extra bands after autoradiography and showing a mobility shift when compared to the respective wild-type (non-mutated) control in SSCP analysis were considered as mutated. They were cut and electroeluted out of the gel in a model 1750 sample concentrator (Isco, Lincoln, Nevada, U.S.A.) for 3 h at 1 W. TBE 0.1x containing 0.005% (w/v) sodium dodecylsulphate (SDS) (Sigma) and TBE 1x were used as an electro-elution buffer in the inner and the outer compartment, respectively. After phenol–chloroform extraction and ethanol precipitation, the collected DNA fragments were reamplified by PCR as above, but without labelling and while applying 40 thermocycles. PCR products were then separated from primer–dimer contaminants by electrophoresis on a 1.5% agarose gel (Biogel) and purified on Glassfog, both provided with the Mermaid kit (Bio 101, La Jolla, California, U.S.A.). Direct DNA sequencing of these PCR products was performed on single-stranded templates produced by asymmetric hot-start PCR after 30 thermocycles of 30 s at 95°C for denaturation and 30 s at 70°C for annealing–extension, while using only one 5′-[^{33}P]-end labelled primer (specific activity: 3000 Ci/mmol) of each couple specified above, chain-terminating

Table 2. Clinical and genetic features of the S-series CRC patients

Patient number	Sex/age	Location	Histological grading	Ki-*ras* gene	*p53* gene
			TNM/grade/Dukes'	Exon/codon/mutation	Exon/codon/mutation
S1	M/65	L (C)	T2N0M1/II/A	—	—
S2	M/83	L (R)	T3N0M0/II/B	—	6/NA
S3	M/49	R (C)	T3N0M0/III/B	—	—
S4	F/80	L (R)	T4N0M0/II/B	—	—
S5	M/70	L (R)	T2N0M0/II/A	—	7/248/CGG→TGG (Arg→Trp)
S6	F/41	L (R)	T3N1M0/II/C	—	—
S7	M/61	L (C)	T3N0M0/II/B	1/12/GGT→GTT (Gly→Val)	—
S8	F/58	L (R)	T3N3M1/II/D	—	—
S9	M/69	L (C)	T3N0M0/III/B	—	—
S10	M/58	L (R)	T3N3M1/II/D	—	—
S11	F/75	L (S)	T3N3M0/II/C	—	—
S12	M/33	L (C)	T3N1M1/II/D	—	—
S13	F/34	L (R)	T1N1M0/II/C	—	—
S14	F/63	L (S)	T3N0M0/II/B	—	6/213/CGA→CGG (Arg→Arg)*
S15	M/49	L (C)	T3N1M0/II/C	—	—
S16	M/49	R (C)	T3N2M1/III/D	—	—
S17	M/44	L (S)	T3N0M0/II/B	—	—
S18	M/25	L (R)	T2N0M0/II/A	—	—
S19	M/66	L (S)	T3N1M0/II/C	—	—
S20	M/55	L (R)	T3N1M0/II/C	—	—
S21	M/65	L (NA)	T3N0M0/II/B	—	—
S22	F/34	L (C)	T2N0M0/II/A	—	—
S23	M/64	L (R)	T3N0M1/III/D	1/12/GGT→GTT (Gly→Val)	9/317/CAG→TAG (Gln→stop)
S24	M/46	L (S)	T4N0M0/II/B	—	8/273/CGT→CAT (Arg→His)
S25	M/59	L (S)	T3N3M0/II/C	—	—
S26	F/85	L (R)	T4N1M0/II/C	—	—
S27	M/54	R (C)	T3N2M1/II/D	1/13/GGC→GGG (Gly→Gly)	intron 6/T→C
S28	F/51	L (C)	T4N3M1/III/D	—	5/175/CGC→CAC (Arg→His)
S29	F/40	R (C)	T3N2M1/II/D	—	—
S30	F/47	L (C)	T4N3M1/III/D	—	—
S31	M/78	L (R)	T3N0M0/II/B	—	—
S32	F/68	R (C)	T4N0M0/III/B	—	—
S33	M/82	L (NA)	T3N2Mx/III/C	—	—
S34	M/57	L (R)	T3N1M1/II/D	—	—
S35	M/63	R (C)	T2N0M0/II/B	—	—
S36	M/55	L (R)	T3N0M0/III/B	—	—

F, female; L, left-sided; M, male; NA, not available; R, right-sided; (R), rectum; S, sigmoid colon; C, colon.
*Found by TaqI restriction analysis. Codon numbering was as in Table 1.

dideoxynucleotides and the thermostable Taq DNA polymerase (sequencing grade) at high temperature with the fmol DNA sequencing system (Promega, Madison, Wisconsin, U.S.A.), as previously described [28].

Statistical data evaluation

Statistical data were evaluated using the χ^2 test with Yate's correction using either the Epi Info software, version 5.01 (Dean J, Dean A, Burton A, Dicker R, 1990, Centers for Disease Control, Epidemiology Program Office, Atlanta, Georgia, U.S.A. and World Health Organization, Geneva, Switzerland), or the BMDP statistical software from the Biomathematics Department (University of California, Los Angeles, California, U.S.A.).

RESULTS

*Detection and identification of mutations in the Ki-*ras *gene*

36 colorectal carcinoma samples of CRC patients from the Grand Duchy of Luxembourg (L-series, Table 1) and 36 samples of the same stage from the German Saar Country (S-series, Table 2) were analysed for mutations in exons 1–2 of the Ki-*ras* gene using PCR–SSCP analysis for mutation detection and direct sequencing for mutation identification as described above. The PCR–SSCP analysis technique represents a rapid and sensitive strategy allowing a large number of samples to be screened quickly for mutations in specific genes. In the case of the Ki-*ras* proto-oncogene, for which codons other than 12 and 13 within exon 1 have sometimes been found to be mutated [30], we explored exon 1 completely using this technique rather than dot-blot hybridisation with oligonucleotide probes targeted to a restricted region of exon 1 only. Direct dideoxynucleotide sequencing of the mutated DNA fragments was coupled to asymmetric PCR and carried out at high temperature: this strategy not only yields higher amounts of chain-terminated products, but also prevents rehybridisation of the complementary strands of the initial PCR product, which, at lower temperatures, often induces artificial elongation stops. To exclude fortuitous mutations caused by Taq polymerase infidelity artefacts, PCR–SSCP analysis and sequencing were confirmed by two independent experiments for each sample. Moreover, direct sequencing was performed on both strands. Typical results

obtained by these techniques during the present study are shown in Figure 1.

Non-silent point mutations in the Ki-*ras* gene were found in 3 out of 36 cases (8.3%) in the L-series (samples L5, L25, L30, Table 1) and in 2 out of 36 cases (5.6%) in the S-series (S7, S23, Table 2): they were all located in exon 1, codon 12 and able to induce an amino acid substitution in the p21 protein encoded by the Ki-*ras* proto-oncogene [13]. In the case of S27, however, the point mutation located in codon 13 was a silent, neutral third-position base substitution without any coding change (Table 2). These mutations comprised two transversions and one transition in the L-series (GGT to GTT: L5, L30 and GGT to AGT: L25) and three transversions in the S-series (GGT to GTT: S7, S23; GGC to GGG: S27).

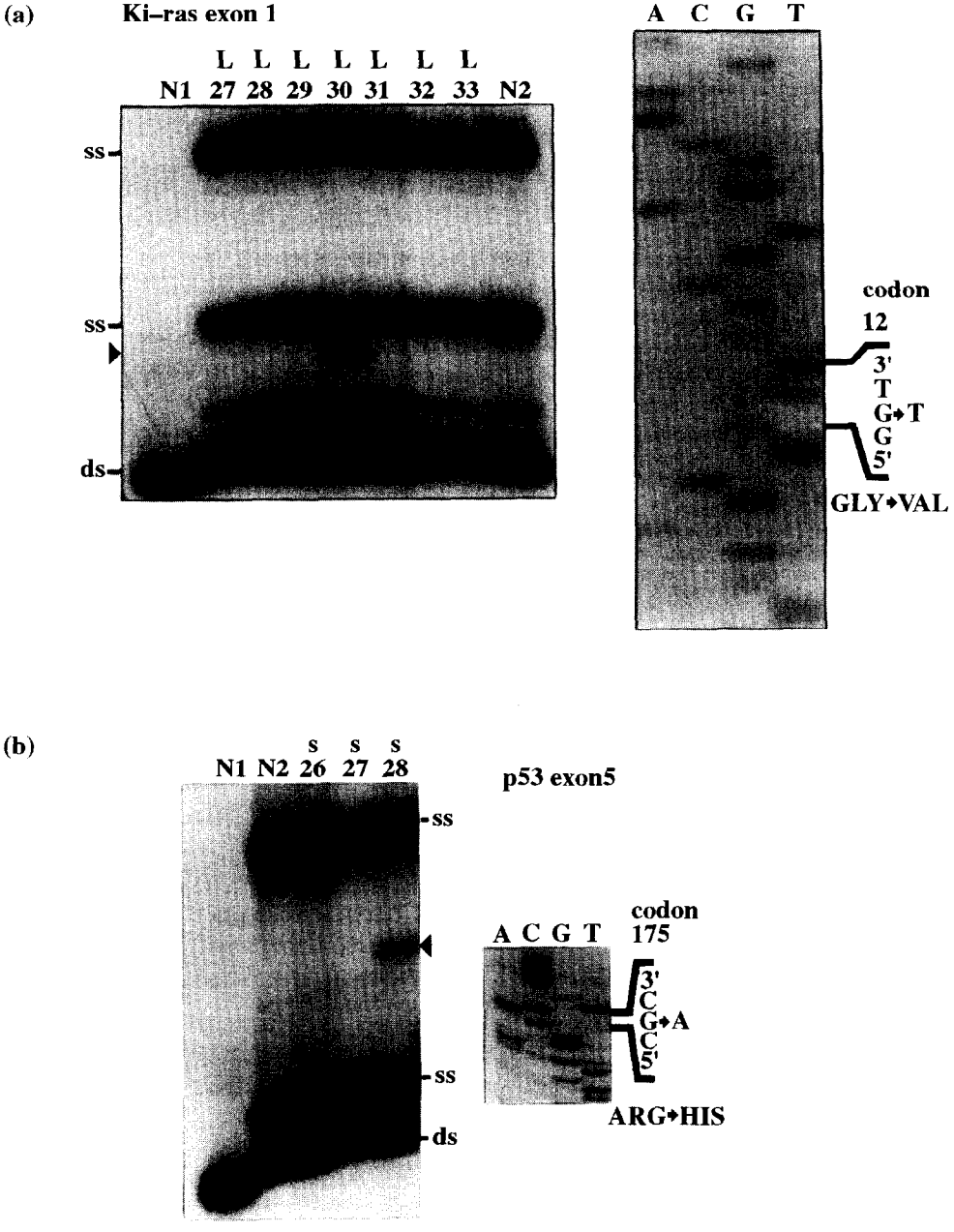

Figure 1. Representative PCR-SSCP analysis and direct sequencing of mutations of the (a) Ki-*ras* and (b) *p53* genes in colorectal tissue samples. Mutations in PCR-amplified DNA fragments of Ki-*ras* exon 1 and *p53* exon 5 were detected by SSCP analysis and identified by direct sequencing as described in Patients and Methods. Autoradiographs of typical electrophoresis gels as performed during PCR-SSCP analysis and direct sequencing are shown on the left and right of each figure, respectively. In the case of PCR-SSCP analysis, sample numbers are indicated at the top of each gel and the position of the two denatured single-stranded (ss) and the residual double-stranded (ds) DNA fragments at the margins. An arrowhead points towards the aberrant bands of mutated fragments showing a mobility shift when compared to the wild type non-denatured (N1) or denatured (N2) control sample of a normal human individual. The wild type bands also appeared in all tumour samples, due to contaminations by normal tissue. In the case of direct sequencing, reactions with chain-terminating dideoxynucleotides were performed on the mutated DNA fragments as indicated at the top of each gel. The deduced sequence and the identified mutations are shown together with the induced amino acid substitutions in the case of samples L30 (Table 1) and S28 (Table 2) at the right margin.

All mutations were somatic and not hereditary, since none simultaneously occurred in the blood (leucocytes), control samples of the same patients being prepared as indicated above. The differences in the incidence of Ki-*ras* activating mutations between Luxembourg and the German Saar were not significant ($P = 0.643$). Together, they provided a total incidence for both areas of 5/72 cases (6.9%), which we considered as representative of a non-silent Ki-*ras* mutation incidence in the European Saar–Luxembourg region.

Detection and identification of mutations in the p53 *gene*

The colorectal carcinoma samples of the L- and S-series were also analysed as above for mutations in exons 5–9 of the *p53* gene (Figure 1).

Non-silent somatic point mutations in the *p53* gene were found in 9 out of 36 cases (25%) in the L-series (samples L3, L10, L14, L15, L17, L22, L35, Tables 1 and 3) and in 4 out of 36 cases (11.1%) in the S-series (S5, S23, S24, S28, Tables 2 and 3). Multiple mutations were discovered in samples L3 and L22. They were located in several exons of the *p53* gene, mainly within exons 5–8. Respectively 67% and 75% of the non-silent *p53* mutations in the L- and S-series samples occurred in the evolutionarily conserved domains II–V. 67% of these mutations were clustered in exon 5 in the L-series, but were scattered over a broader area, including exon 9, in the S-series (Figure 2). Some mutations fell in the defined mutational hot-spots [14] in codons 154 (L22), 175 (L35, S28), 176 (L15), 248 (S5), 273 (S24) and 282 (L22). All these mutations were mis-sense mutations, except in the case of L17 and S23, which harboured a non-sense mutation. The mis-sense mutations were able to induce an amino acid substitution in the p53 protein and the non-sense mutations to cause a premature stop in protein synthesis with a truncation of the p53 protein [14, 17, 18]. In the case of L16, however, the point mutation was again a silent, neutral third-position base substitution, and in the case of S27, it was most probably also silent (cf. Discussion).

The *p53* mutations found in the L- and S-series comprised only substitutions, but no deletions nor insertions (Table 3).

In the L-series, there were seven transitions (70% of the total number of mutations): five (50%) were G:C to A:T transitions with two (20%) at non-CpG sites (codon 135: TGC to TAC: L3, codon 192: CAG to TAG (antisense): L17), and 3 (30%) at CpG sites (codon 154: GGC to AGC: L22, codon 175: CGC to CAC: L35, codon 282: CGG to TGG (antisense): L22). There were also two (20%) A:T to G:C transitions (codon 163: TAC to TGC: L10, codon 235: AAC to AGC: L3). Furthermore, there were three (30% of the totality of mutations) transversions (codon 137: CTG to CAG: L14, codon 154: GGC to GGG: L16, codon 176: TGC to TTC: L15).

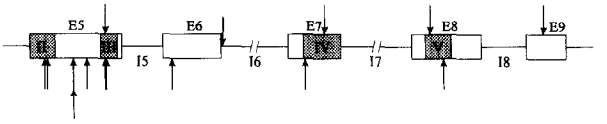

Figure 2. Distribution of *p53* tumour-suppressor gene mutations in colorectal carcinomas from the European Saar–Luxembourg region. Exons (E) are represented by boxes, introns (I) by lines. The roman numbers refer to the evolutionarily conserved domains (shaded boxes) of the *p53* gene [14]. The locations of the *p53* mutations found in colorectal carcinoma samples from Luxembourg (L-series, Table 1) and from the German Saar Country (S-series, Table 2) are symbolised by vertical arrows below and above the figure, respectively.

In the S-series, there were only five transitions: 1 (20%) G:C to A:T transition at a non-CpG site (codon 317: CAG to TAG (antisense): S23) and three (60%) transitions of the same type at CpG sites (codon 175: CGC to CAC: S28, codon 248: CGG to TGG (antisense): S5, codon 273: CGT to CAT: S24). There was also 1 (20%) A:T to G:C transition (donor splice site, position +2 from the 5′-start (+1) of intron 6: T to C (antisense): S27).

Again, as above for the Ki-*ras* mutations, the differences in the incidence of non-silent *p53* mutations between Luxembourg and the German Saar were not significant ($P = 0.126$). Overall, there was a non-silent *p53* mutation incidence for the European Saar-Lux region of 13/72 cases (18.1%). There were 9/15 (60% of all *p53* mutations) G:C to A:T transitions, with 6/15 (40%) at CpG sites, 3/15 (20%) A:T to G:C transitions and 3/15 (20%) transversions (Table 3).

In the case of samples L2, L16, L22, L24, L35 and S14, a silent transition in *p53* exon 6, codon 213 (CGA to CGG), was also detected in the control blood samples from the same patients and represented a naturally occurring, hereditary germ-line polymorphism. All these samples were heterozygous in that one *p53* allele was normal, whilst the other allele had the A:T to G:C transition, which also destroyed a Taq I restriction site located at this site. PCR–SSCP analysis data regarding this *p53* dimorphism could therefore be confirmed by Taq I restriction analysis as described elsewhere ([29], results not shown). In the case of samples L35 and S14, PCR–SSCP analysis was unable to reveal the presence of the mutated allele, which was only found by restriction analysis. The mutated allele was thus present in 5 out of 72 chromosomes from the 36 patients of the L-series (frequency: 6.9%), and in 1 chromosome out of 72 from the 36 patients of the S-series (frequency: 1.4%). These alterations were not taken into account in the statistical evaluations of the mutation incidences in this study. Aberrant bands were observed in PCR–SSCP analysis for exon 5 (L8, L24, L25), exon 6 (S2) and for exon 9 (L33), but the presence of a mutation could

Table 3. Types and frequencies of non-silent mutations of the p53 *gene in colorectal carcinomas from Luxembourg and the German Saar Country*

Geographic area	Non-silent mutations/ total cases	G:C→A:T transitions/ total mutations	G:C→A:T transitions at CpG sites/total mutations	A:T→G:C transitions/ total mutations	Transversions/ total mutations
Luxembourg	9/36 (25%)	5/10 (50%)	3/10 (30%)	2/10 (20%)	3/10 (30%)
German Saar	4/36 (11.1%)	4/5 (80%)	3/5 (60%)	1/5 (20%)	0/5 (0%)
Both areas	13/72 (18.1%)	9/15 (60%)	6/15 (40%)	3/15 (20%)	3/15 (20%)

not be confirmed nor identified by direct DNA sequencing. In the present study, only mutations which could be identified by sequencing were considered for statistical evaluation.

DISCUSSION

Most of the mutations found in colorectal carcinoma samples from the European Saar–Luxembourg region as analysed in this study were non-silent point mutations located in codon 12, exon 1 of the Ki-*ras* proto-oncogene or in the evolutionarily conserved domains in exons 5–8 of the *p53* tumour-suppressor gene. Such mutations are likely to provide a tumour cell with further growth advantages and thus to favour the progression to malignancy.

Non-silent mutations in codon 12 of the Ki-*ras* proto-oncogene are able to convert this proto-oncogene to an active oncogene while inducing amino acid substitutions at critical sites of the p21 protein, a membrane receptor with GTPase activity, which, after a conformational change, is considered to stimulate cell growth in a constitutive manner [11, 13]. Non-silent point mutations of the *p53* tumour-suppressor gene, especially those falling into the five evolutionarily conserved domains, are likely either to cause an amino acid substitution in relevant functional regions of the p53 protein (mis-sense mutation) or a premature abortion of translation and the synthesis of a truncated p53 protein (non-sense mutation), thus disrupting the normal p53 cell growth-inhibiting function. This function of the nuclear phosphoprotein p53 has been shown to be related to its activation of the gene encoding the p21 cyclin-dependent kinase (CDK) regulator, which in turn interacts with a cyclin-dependent protein kinase, a cyclin and the proliferating-cell nuclear antigen PCNA, thus blocking the pathway leading to DNA synthesis and cell-cycle progression [31].

According to the adenoma–carcinoma sequence, these mechanisms may explain, in the case of such mutations, tumour progression from the benign adenoma stage to the malignant carcinoma stage (cf. Introduction).

While silent mutations in the Ki-*ras* and *p53* genes (L16, S27) could still have favoured tumour growth by creating pause sites that affected transcription, mRNA stability or translation [30], the A:T to G:C mutation in the donor splice site in *p53* intron 6 (S27) is unlikely to influence this growth to a larger extent in changing the splicing efficiency of the *p53* premessenger RNA, since spliceosomes recognise exon-flanking sequences with a low stringency.

Multiple point mutations of the *p53* gene as in samples L3 and L22 are generally rare events [12, 30]. It is impossible to determine in such cases whether the two mutations are present on the same or on different alleles, in the same or in different tumour cell populations, since they are located on different PCR products [30].

G:C to A:T transitions have been reported in 63% of samples, representing the main type of identified *p53* point mutations in CRC and 47% of them occurring at CpG sites [14]. With a frequency of 60%, the mutations of this type were also predominent in colorectal carcinomas from the European Saar–Luxembourg region and 40% of them were also located in CpG sites. Since the type of somatic *p53* mutations most probably reflects the action of specific environmental carcinogens [20] and since the CRC incidence could be correlated with dietary habits [9], this type of base substitution, both in the Ki-*ras* and *p53* genes, could have resulted from alkylation damage to guanines by carcinogenous *N*-nitroso compounds formed in the gastrointestinal tract after higher nitrite and protein intake, as in industrialised populations [30].

Furthermore, Ki-*ras* and *p53* point mutations had a higher frequency in colorectal carcinomas from a left-sided (71.4%, 15/21) than a right-sided primary location, following previous suggestions that the left-sided part of the large bowel is the most influenced by environmental mutagenic factors [9]. According to one hypothesis, the higher rate of alimentary lipids, for example, influences the concentration of neutral and acid sterols in the colon and changes the composition of the intestinal microflora, which in turn becomes unable to metabolise primary biliary acids, which have strong mutagenic activities, into secondary biliary acids [33].

p53 codon 213 polymorphism was higher in Luxembourg (frequency of the G-allele: 6.9%) than in the German Saar (1.4%) and reflected the ethnic differences between both countries. As reported in previous studies [29, 34], the polymorphism in Latin countries, such as Italy and South America, is characterised by a frequency of the G-allele of approximately 10% and that found in Northern countries such as North America by a frequency of 0–3%. The value of 6.9% found in this study for the samples from Luxembourg appeared close to that of Latin countries and that of 1.4% observed for the samples from the German Saar was within the polymorphism range of Northern countries. This fact is consistent with the strong ethnical representation of Latin populations in Luxembourg, as already mentioned in a study of betaglobinopathy incidences in Luxembourg [35].

The incidences of Ki-*ras* and *p53* non-silent point mutations in the colorectal carcinoma samples from the European Saar–Luxembourg region are significantly lower (Ki-*ras* 6.9%; *p53*: 18.1%) than those reported for both genes in American studies (Ki-*ras* 40–50% [10–13]; *p53*: >50% [10, 16, 32]; $P < 0.001$).

At least in the case of the *p53* gene, low mutation frequencies similar to ours have also been observed in colorectal carcinomas from some other European countries, as for example from Belgium (17.1%) (Dr N. Ravelingien, Dr T. Velu, Hôpital Erasme, Brussels) and from Great Britain (22%) [36]. We are confident that the possibility of a too low sensitivity range of the PCR–SSCP analysis can be excluded, since it has been shown that by this technique, at least 90% of the *p53* gene mutations could be detected [37] and that unidentified aberrant bands, like those found for samples L8, L24, L25, L33 and S2, might rather be attributed to an artefact due to the interaction between residual PCR primers and single-strand DNAs [38] than to the presence of a mutation. Moreover, in a few cases only (S23, S27), mutations were found simultaneously in the Ki-*ras* and the *p53* genes and this observation is contradictory to a developmental mechanism in which several genetic alterations accumulate, providing a tumour cell subpopulation with a further growth advantage at each step and thus promoting the stage-wise progression to malignancy.

Taken together and as also proposed elsewhere [39], the results of this study suggest that, at least in some European regions, other genetic mechanisms with pathways different from those of the classical adenoma–carcinoma sequence can frequently underlie CRC development and that Ki-*ras* and *p53* mutations should not be considered as universal genetic markers for CRC.

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