Short Communication

The TP53 Codon 72 Polymorphism May Affect the Function of TP53 Mutations in Breast Carcinomas but not in Colorectal Carcinomas[1]

Anita Langerød, Ida R. K. Bukholm,[2] André Bregård, Per Eystein Lønning, Tone Ikdal Andersen,[3] Torleiv O. Rognum, Gunn Iren Meling, Ragnhild A. Lothe, and Anne-Lise Børresen-Dale[4]

Department of Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway [A. L., I. R. K. B., A. B., T. I. A., R. A. L., A-L. B. D.]; Department of Oncology, Haukeland University Hospital, Bergen, Norway [P. E. L.]; and Institute for Forensic Medicine, The National Hospital, Oslo, Norway [T. O. R., G. I. M.]

Abstract

An Arg/Pro polymorphism in codon 72 of the *TP53* gene was analyzed in blood samples from 390 breast and 162 colorectal cancer patients previously investigated for *TP53* mutations in their tumors. Among the breast cancer cases, 228 were homozygous for the Arg72 allele, of which, 65 (28.5%) also had a *TP53* mutation in their tumors. In contrast, of 26 cases that were homozygous for the Pro72 allele, only 1 case (3.8%) had a *TP53* mutation in the tumor ($P = 0.004$). Cloning the *TP53* gene from tumor DNA followed by sequencing was performed in 14 heterozygotes with tumor mutation, and 9 of the mutations resided on the Arg72 allele. Among the colorectal cancer cases, no difference in mutation frequency was seen between the two different homozygotes, 40 *TP53* mutations in 97 Arg72 homozygous cases (41.2%) *versus* 7 in 16 Pro72 homozygous cases (43.8%). These results suggest a selective growth advantage for cells carrying a type of *TP53* mutation seen in breast carcinomas when the mutation resides on an Arg72 allele.

Introduction

Mutations in the *TP53* gene are considered to represent the most common genetic alteration in human cancer. These mutations (mostly missense mutations) may damage the normal function of TP53 as a transcription factor, and the induction of repair or apoptosis may be abolished. Consequently, other genetic alterations may accumulate in the cell. In breast cancer, the *TP53* gene is mutated in ~20–30% of the tumors and in colorectal cancer in 50–60% [reviewed in Ref. 1].

In addition to gene mutations, several reports have focused on *TP53* polymorphisms as risk factors for malignant disease. Two of 14 known polymorphisms located in the *TP53* gene alter the amino acid (International Agency for Research on Cancer TP53 Mutation Database).[5] The alleles of the polymorphism in codon 72, exon 4, encode an arginine amino acid (CGC; Arg72) with a positive-charged basic side chain and a proline residue (CCC; Pro72) with a nonpolar-aliphatic side chain. Significant association between the codon 72 polymorphism and risk of cancer have been reported, although the results with regard to most cancer diseases, including breast (2–4) and colorectal carcinomas (5–7) remain inconclusive.

The Arg/Pro polymorphism is located in a proline-rich region (residues 64–92) of the TP53 protein, where the Pro72 amino acid constitutes one of five PXXP motifs resembling a SH3 binding domain. The region is required for the growth suppression and apoptosis mediated by TP53 but not for cell cycle arrest [reviewed in Ref. 1]. The two polymorphic variants of wild-type TP53 have been shown to have some different biochemical and biological properties (8) such as different binding to components of the transcriptional machinery and different activation of transcription, but they did not differ in their ability to bind DNA.

The TP73 protein, a homologue of the TP53 protein, is able to activate TP53-responsive promoters and induce apoptosis in TP53-deficient cells. Marin *et al.* (9) recently showed that some TP53 mutants can bind to and inactivate TP73 and that the binding of such mutants was enhanced when the mutation occurred on the Arg72 allele. They also reported a higher frequency of *TP53* mutations on the Arg72 compared with the Pro72 allele in different squamous cell cancers. These findings were supported by Tada *et al.* (10), which found an overrepresentation of mutations on the Arg72 allele in tumors from different tissues. Interestingly, they found a preferential selection of the Arg72 allele in cancers with recessive TP53 mutants (mutants that do not inactivate wild-type TP53 in a dominant negative manner). It was suggested that recessive TP53 mutants achieve a selective growth advantage by an Arg72-dependent inactivation of TP73, whereas the dominant negative TP53 mutants inactivate the remaining wild-type TP53 allele in an Arg72-independent manner.

We have investigated whether somatic *TP53* mutations exist in combination with a specific constitutional allele variant of the codon 72 polymorphism (Arg72 or Pro72) in a series of breast carcinomas and a series of colorectal carcinomas, which are known to have different *TP53* mutation spectrum.

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[2] Present address: Department of Surgery, Akershus University Hospital, 1474 Nordbyhagen, Norway.
[3] Present address: Department of Oncology, Ullevål University Hospital, 0407 Oslo, Norway.
[4] To whom requests for reprints should be addressed, at Department of Genetics, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway. Phone: 47-22-93-44-19; Fax: 47-22-93-44-40; E-mail: a.l.b.dale@labmed.uio.no.

[5] Internet address: www.iarc.fr/P53/index.html.

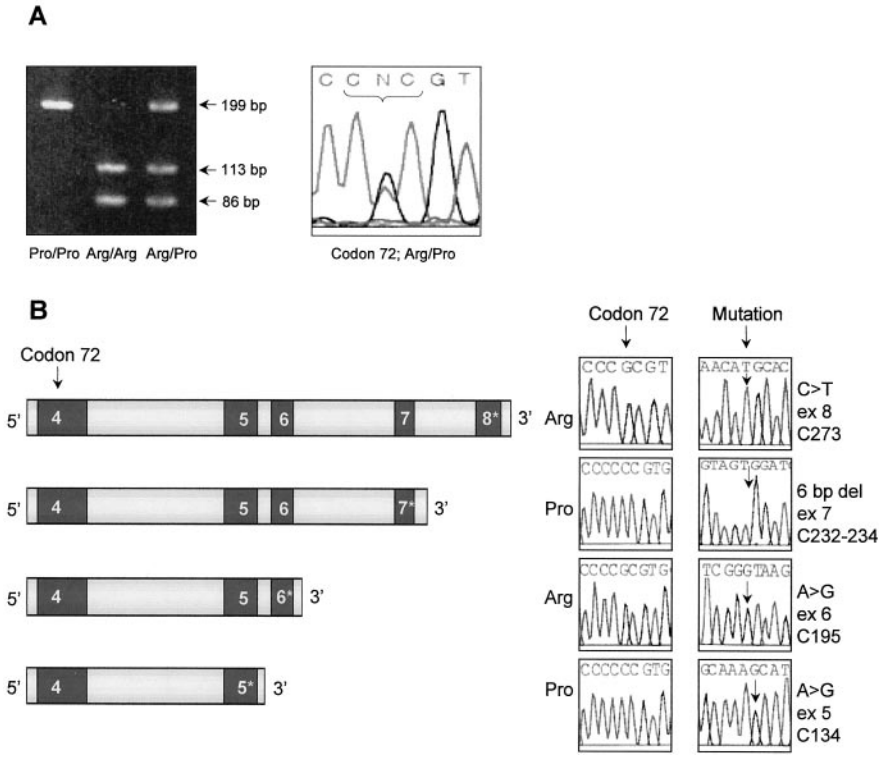

Fig. 1. *A*, the *TP53* codon 72 polymorphism was analyzed in DNA from blood using RFLP. Electrophoresis in 7.5% acrylamide gel gave an allele pattern of the two alleles of 113 bp +86 bp (Arg) and 199 bp (Pro). The gel shows three patients with different genotypes, along with the sequence of a heterozygous sample. *B*, from heterozygous samples (Arg72/Pro72) with a *TP53* mutation in their tumor, the *TP53* gene was cloned and sequenced to determine which allele the mutation resided on. Four different fragments were designed for cloning to include the polymorphism in exon 4 as well as the respective mutation (*, mutations located in exons 5–8). The part of the DNA sequence with the polymorphism and mutation are shown (mutations sequenced in reverse direction).

Materials and Methods

Materials. The study included 390 Norwegian breast cancer cases. These were from two different consecutive series (129 and 130 samples, respectively) previously described (11, 12) and from two series of advanced breast cancer cases (84 and 47, respectively), of which, one has been described previously (13). One hundred sixty-two Norwegian colorectal cancer cases previously analyzed for *TP53* mutations in their tumors were also included in this study (14). DNA had been isolated from both blood cells and tumor tissue using chloroform/phenol extraction followed by ethanol precipitation (Nucleic Acid Extractor 340A; Applied Biosystems) according to standard procedure.

Genotyping. DNA from blood samples was analyzed for the genetic variation in codon 72 in exon 4 of the *TP53* gene using Restriction Fragment Length Polymorphism analysis (15). Genomic DNA (50 ng) was amplified in 25 μl of PCR reactions (Eppendorf Mastercycler Gradient), containing 12.5 pmol of each primer (F: 5′-TTGCCGTCCCAAGCAATGGATGA-3′, R: 5′-TCTGGGAAGGGACAGAAGATGAC-3′), 2.5 μl of 10× buffer (Gene Amp from Applied Biosystems, containing 100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% W/v gelatin), 10 mM deoxynucleotide triphosphate, and 0.75 units of AmpliTaq DNA Polymerase (Applied Biosystems). A 199-bp fragment was amplified using a PCR program starting with denaturation for 3 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 68°C, and 30 s at 72°C. Restriction analysis was performed mixing 8 μl of PCR product, 9 μl of H_2O, 2 μl of 1× NEBuffer 2, 1 μl of (10 units/μl) *Bst*UI (New England BioLabs), and incubated for 3 h at 60°C. Electrophoresis in 7.5% acryl amide gel gave an allele pattern of the two alleles of 113 bp +86 bp (Arg) and 199 bp (Pro), respectively (Fig. 1*A*).

Mutation Analysis. *TP53* mutation detection in tumor DNA was performed using Constant Denaturing Gradient Gel Electrophoresis (16) or Temporal Temperature Gradient Gel Electrophoresis (17). The samples from the two advanced breast cancer series (131 cases) have been screened for mutations in exons 2–11 of the *TP53* gene (13). One of the consecutive breast cancer series (130 cases) has been reanalyzed to include exons 2–11 (analysis of exons 5–8 reported in Ref. 12). The samples from the other consecutive breast cancer series (129 cases; Ref. 11), as well as the colon cancer cases (162 cases; Ref. 14) have been screened for *TP53* mutations in exons 5–8.

Table 1 Primers used for cloned inserts

Fragment	Primer
Exon 4–5 (1319 bp)	4F: 5′-gctgggggggctgaggacc-3′
	5R: 5′-gcaatcagtgaggaatcaga-3′
Exon 4–6 (1527 bp)	6R: 5′-ccactgacaaccaccctt-3′
Exon 4–7 (2217 bp)	7R: 5′-aggggtcagcggcaagcaga-3′
Exon 4–8 (2679 bp)	8R: 5′-aggcataactgcacccttgg-3′

Cloning and Sequencing. The cloning of the *TP53* gene from tumor DNA was performed using the TOPO TA Cloning kit (Invitrogen). Four different fragments were designed to comprise the polymorphism in exon 4, as well as the respective mutation (Fig. 1*B*). DNA (<50 ng) was amplified in 25 μl of PCR reactions (Eppendorf Mastercycler Gradient), containing 12.5 pmol of each primer (for primer sequences see Table 1), 2.5 μl of 10× buffer (Gene Amp; Applied Biosystems) giving a concentration of 1.5 mM Mg^{2+}, 10 mM deoxynucleotide triphosphate, and 0.75 units of AmpliTaq DNA Polymerase (Applied Biosystems). The PCR program started with denaturation for 2 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 63°C, 60 s at 72°C, and finally 10 min at 72°C. The PCR product was analyzed by gel electrophoresis (7.5% acrylamide)

Table 2 Overview of *TP53* mutations found

Characterization and distribution of *TP53* mutations in breast carcinomas with respect to genotype of the codon 72 polymorphism. The heterozygote samples that were cloned and sequenced to determine which allele of the codon 72 the mutation resided on are shown in bold and the respective allele underlined. (The sample IDs are designated MT and ULL for the two consecutive series, and LB and FU for the advanced breast cancer series.)

Sample ID	Genotype / Codon 72	Mutation / Exon	Codon	Codon change	Base change	Aminoacid	Type
ULL-T-271	Arg/Arg	3	29	1bp ins	ins A		Frameshift
ULL-T-177	Arg/Arg	4	110	CGT > CCT	G > C	Arg > Pro	Missense
LB 105 A	Arg/Arg	<5	1 bp upstream	Intronic	G > A		Splice
ULL-T-096	Arg/Arg	5		18 bp ins			In frame
ULL-T-038	Arg/Arg	5	138	GCC > GTC	C > T	Ala > Val	Missense
LB 101 A	Arg/Arg	5	151	CCC > TCC	C > T	Pro > Ser	Missense
MT 064	Arg/Arg	5	156	CGC > CCC	G > C	Arg > Pro	Missense
ULL-T-106	Arg/Arg	5	159	GCC > GAC	C > A	Ala > Asp	Missense
LB 703 B	Arg/Arg	5	168	CAC > CCC	A > C	His > Pro	Missense
ULL-T-099	Arg/Arg	5	173	GTG > CTG	G > C	Val > Leu	Missense
MT 193	Arg/Arg	5	174–180	17 bp del			Frameshift
FU M312	Arg/Arg	5	175	CGC > CAC	G > A	Arg > His	Missense
MT 083	Arg/Arg	5	175	CGC > CAC	G > A	Arg > His	Missense
ULL-T-171	Arg/Arg	5	175	CGC > CAC	G > A	Arg > His	Missense
LB 205 A	Arg/Arg	5	176	TGC > TTC	G > T	Cys > Phe	Missense
MT 101	Arg/Arg	5	181	CGC > CAC	G > A	Arg > His	Missense
FU 07	Arg/Arg	<6	3 bp upstream	Intronic	T > G		Splice
LB 307 B	Arg/Arg	6	190	CCT > CTT	C > T	Pro > Leu	Missense
MT 120	Arg/Arg	6	194	CTT > CGT	T > G	Leu > Arg	Missense
FU 23	Arg/Arg	6	195	ATC > ACC	T > C	Ile > Thr	Missense
ULL-T-179	Arg/Arg	6	196	22 bp del			Frameshift
FU M307	Arg/Arg	6	197–199	6 bp del			In frame
LB 111 A	Arg/Arg	6	204	GAG > TAG	G > T	Glu > Stop	Nonsense
MT 078	Arg/Arg	6	204	GAG > TAG	G > T	Glu > Stop	Nonsense
LB 805 A	Arg/Arg	6	213	CGA > TGA	C > T	Arg > Stop	Nonsense
ULL-T-188	Arg/Arg	6	216	GTG > ATG	G > A	Val > Met	Missense
FU M326	Arg/Arg	6	217	GTG > ATG	G > A	Val > Met	Missense
LB 123 A	Arg/Arg	6	217–221	14 bp del			Frameshift
MT 106	Arg/Arg	7	238	TGT > TTT	G > T	Cys > Phe	Missense
LB 305 A	Arg/Arg	7	239–242	11 bp del			Frameshift
MT 050	Arg/Arg	7	242	TGC > TTC	G > T	Cys > Phe	Missense
MT 359	Arg/Arg	7	242	TGC > TAC	G > A	Cys > Tyr	Missense
MT 135	Arg/Arg	7	244–247	8 bp del			Frameshift
MT 161	Arg/Arg	7	245	GGC > AGC	G > A	Gly > Ser	Missense
LB 309 A	Arg/Arg	7	248	CGG > CAG	G > A	Arg > Gln	Missense
MT 024	Arg/Arg	7	248	CGG > CAG	G > A	Arg > Gln	Missense
ULL-T-250	Arg/Arg	7	248	CGG > CAG	G > A	Arg > Gln	Missense
MT 160	Arg/Arg	7	248	CGG > TGG	C > T	Arg > Trp	Missense
LB 111 B	Arg/Arg	7	249	AGG > GGG	A > G	Arg > Gly	Missense
MT 071	Arg/Arg	7	251	ATC	del C		Frameshift
FU 27	Arg/Arg	7	256	12 bp ins			In frame
FU M327	Arg/Arg	8	261–269	24 bp del			In frame
LB 120 A	Arg/Arg	8	266	GGA > AGA	G > A	Gly > Arg	Missense
FU 26	Arg/Arg	8	273	CGT > CCT	G > T	Arg > Pro	Missense
LB 107 B	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
LB 208 A	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
LB 405 A	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
FU M310	Arg/Arg	8	273	CGT > CAT	G > LA	Arg > His	Missense
FU M321	Arg/Arg	8	273	CGT > TGT	C > T	Arg > Cys	Missense
MT 029	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
MT 240	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
ULL-T-164	Arg/Arg	8	273	CGT > CAT	G > A	Arg > His	Missense
ULL-T-071	Arg/Arg	8	273	CGT > GGT	C > G	Arg > Gly	Missense
LB 706 A	Arg/Arg	8	277	TGT > TAT	G > A	Cys > Tyr	Missense
ULL-T-215	Arg/Arg	8	278	CCT > CTT	C > T	Pro > Leu	Missense
MT 119	Arg/Arg	8	281	GAC > GGC	A > G	Asp > Gly	Missense
FU 12	Arg/Arg	8	282	CGG > GGG	C > G	Arg > Gly	Missense
MT 181	Arg/Arg	8	282	CGG > CAG	G > A	Arg > Gln	Missense
ULL-T-154	Arg/Arg	8	282	CGG > TGG	C > T	Arg > Trp	Missense
MT 003	Arg/Arg	8	285	GAG > AAG	G > A	Glu > Lys	Missense
LB 404 B	Arg/Arg	8	286	GAA > AAA	G > A	Glu > Cys	Missense
LB 406 A	Arg/Arg	<9	2 bp upstream	Intronic	A > G		Splice
FU M317	Arg/Arg	<9	2 bp upstream	Intronic	A > G		Splice
FU 04	Arg/Arg	>9	1 bp downstream	Intronic	G > C		Splice

Table 2 Continued

Sample ID	Genotype Codon 72	Mutation Exon	Codon	Codon change	Base change	Aminoacid	Type
FU M314	Arg/Arg	10	342	CGA > TGA	CST	Arg >Stop	Nonsense
ULL-T-263	Arg/Pro	4	89	11 bp ins			Frameshift
ULL-T-113	Arg/Pro	4	113	TTC > GTC	T > G	Phe > Val	Missense
MT 059	Arg/Pro	5	134	TTT > CTT	T > C	Phe > Leu	Missense
LB 709 B	Arg/Pro	5	136	CAA > TAA	C > T	Gln >Stop	Nonsense
FU 11	Arg/Pro	5	140–143	10 bp del		Stop169	Frameshift
FU M315	Arg/Pro	5	142	CCT	del C	Stop169	Frameshift
MT 052	Arg/Pro	5	156	CGC > CCC	G > C	Arg > Pro	Missense
LB 115 B	Arg/Pro	5	163	TAC > TGC	A > G	Tyr > Cys	Missense
LB 708 B	Arg/Pro	5	165	CAG > TAG	C > T	Gln >Stop	Nonsense
FU M304	Arg/Pro	5	167	CAG > TAG	C > T	Gln >Stop	Nonsense
MT 022	Arg/Pro	5	172	GTT	del T		Frameshift
MT 020	Arg/Pro	5	174–180	17 bp del			Frameshift
ULL-T-155	Arg/Pro	5	175	CGC > CAC	G > A	Arg > His	Missense
MT 208	Arg/Pro	5	179	CAT > TAT	C > T	His > Tyr	Missense
FU 05	Arg/Pro	6	195	ATC > ACC	T > C	Ile > Thr	Missense
MT 111	Arg/Pro	6	195	ATC > ACC	T > C	Ile > Thr	Missense
ULL-T-007	Arg/Pro	6	195	ATC > ACC	T > C	Ile > Thr	Missense
FU M316	Arg/Pro	6	220	TAT > TGT	A > G	Tyr > Cys	Missense
ULL-T-226	Arg/Pro	6	220	TAT > TGT	A > G	Tyr > Cys	Missense
LB 303 B	Arg/Pro	7	232–234	6 bp del			In frame
LB 206 A	Arg/Pro	7	237	ATG > ATT	G > T	Met > Ile	Missense
MT 104	Arg/Pro	7	239	AAC > ACC	A > C	Asn > Thr	Missense
FU M303	Arg/Pro	7	248	CGG > TGG	C > T	Arg > Trp	Missense
MT 112	Arg/Pro	7	248	CGG > TGG	C > T	Arg > Trp	Missense
MT 132	Arg/Pro	7	248	CGG > CAG	G > A	Arg > Gln	Missense
MT 016	Arg/Pro	8	273	CGT > CAT	G > A	Arg > His	Missense
MT 065	Arg/Pro	8	273	CGT > CAT	G > A	Arg > His	Missense
FU M301	Arg/Pro	8	282	CGG > GGG	C > G	Arg > Gly	Missense
MT 318	Arg/Pro	8	282	CGG > TGG	C > T	Arg > Trp	Missense
FU 06	Arg/Pro	8	298	GAG > TAG	G > T	Glu >Stop	Nonsense
LB 122 A	Pro/Pro	<5	1 bp upstream	Intronic	G > A		Splice

for quality check, then cloned into the pCR 2.1-TOPO vector and transformed into *Escherichia coli* according to standard protocols. The plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen), and the complete insert was sequenced (ABI 3100; Applied Biosystems) in overlapping fragments.

Statistics. Deviations from Hardy-Weinberg equilibrium of the codon 72 polymorphism were determined using χ^2 test. Cross-tabulation and χ^2 test were performed when studying the polymorphism's association with *TP53* mutations. Pearson χ^2 test or Fisher's exact test (when appropriate) was used, and statistical significance level was set to $P \leq 0.05$. Computations were performed using Excel (Microsoft Excel 97) and SPSS (version 8.0).

Results and Discussion

Among the 390 breast cancer cases genotyped, the allele frequencies were 0.76 and 0.24 for the Arg72 and Pro72 allele, respectively, and the polymorphism was shown to be in Hardy-Weinberg equilibrium. The *TP53* mutation frequencies in the different series, where screening of exons 2–11 were performed, were 14.6% (19 of 130) in the consecutive series and 28.6% (24 of 84) and 46.8% (22 of 47) in the two series of advanced breast cancer cases, reflecting the different distribution of tumor size and stage of disease between these series. Of the 228 cases that were homozygous for the Arg72, 65 (28.5%) also carried a *TP53* mutation in their tumors. In contrast, of the 26 cases that were homozygous for the Pro72 allele, only 1 case (3.8%) had a *TP53* mutation (Table 2). Thus, the occurrence of a *TP53* mutation was significantly more often found on the Arg72 allele than the Pro72 allele ($P = 0.004$). This skewed distribution was seen in all series, although each of them was too small to give significant results by their own. When limited to *TP53* mutations residing in exons 5–8, the same significant biased distribution was seen ($P = 0.007$). Only 8 of 65 mutations (12.3%) were located outside exons 5–8, of which, 6 were found in Arg72 homozygous and 2 in heterozygous (Table 2). The same skewed distribution with respect to genotype was also seen when considering only missense mutations. Of the 228 homozygotes for the Arg72 allele, 45 (19.7%) missense mutations were found, whereas none of the 26 homozygotes for the Pro72 allele carried a missense mutation ($P = 0.006$).

From 14 heterozygotes (Arg72/Pro72) with a *TP53* mutation in their tumor and where enough tumor DNA still was available, the *TP53* gene was cloned and sequenced to determine which allele the mutation resided on (Fig. 1*B*). In 9 cases, the mutations (5 missense, 2 nonsense, and 2 frameshifts) were located on the arginine allele, and in the 5 remaining samples, the mutations (2 missense, 1 frameshift, and 1 deletion) resided on the Pro allele supporting the findings in the homozygous samples (Table 2).

The observed skewed occurrence of somatic *TP53* mutations on the Arg72 allele in breast carcinomas suggests that this combination gives breast epithelial cells a growth advantage, which may increase the risk of malignant transformation and development of cancer. The coexistence of the Arg72 with a mutation may modify the TP53 protein structure in a way that

interferes either with the protein's ability to achieve sequence-specific binding to DNA or with the interaction and recruitment of the transcription machinery, causing an altered transcription pattern (18). Another possibility is that the Arg72 may modify the mutant TP53 protein's ability to bind to and interact with other proteins such as, for example, TP73. Interaction between tumor-derived TP53 mutants and TP73 has been observed (19), and the codon 72 polymorphism has been reported to be a modifier of such an interaction (9), which may interfere with TP73-induced apoptosis.

The same level of skewed distribution of mutations residing on the Arg72 as seen for all type of mutations was also seen for missense mutations, giving no evidence for a stronger effect of such mutations. However, missense mutations are of many different types, and classifications according to structure or function in different cell types in larger series may give other results. The more severe changes like deletion, insertion, nonsense, and splice mutations may lead to a truncated protein or lack of protein where a codon 72 polymorphism has no modifying impact. Analyzing nonmissense mutations as one group with respect to the codon 72 homozygotes gave no skewed distribution ($P = 0.710$). The number is, however, small, and even truncated TP53 proteins may have an impact through mechanisms like inactivating other proteins (*e.g.*, TP73) if their interacting domain is intact and the protein is stable. It cannot be excluded that the two polymorphic variants may have different effects also on such mutants.

Genotyping of the 162 colorectal cancer cases revealed allele frequencies of the Arg72 and Pro72 alleles of 0.75 and 0.25, respectively. The *TP53* mutation frequency in this cohort was 48.1%. In contrast to the breast cancer cases, no difference in the frequency of mutations between the two different homozygotes was found in the colorectal cancer cases, with 40 *TP53* mutations in 97 Arg72 homozygous cases (41.2%) *versus* 7 *TP53* mutations in 16 Pro72 homozygous cases (43.8%). The spectrum of mutations is different between these two tumor types,[6] partly attributable to tissue-specific differences in carcinogen exposure and in metabolism (reviewed in Ref. 1). Breast cancer is reported to have a high level of insertions, deletions, and nonsense mutations, and GC:AT transitions are the most frequent change, equally distributed between CpG and non-CpG areas, whereas colorectal cancer has a high frequency of CpG transitions leading to mutants with a presumable dominant negative effect (1). A recent report divided the mutations into two groups according to their predicted dominant negative or recessive characteristics based on the results of a transactivation assay (9), and the authors proposed that it was only the recessive mutations that preferentially was located on the Arg72 allele (10). The dominant negative mutants were suggested to be independent of the codon 72 polymorphism. Using the same criteria for classifying the mutations as proposed by Tada *et al.* (10) on our series, the frequency of dominant negative mutants were higher in the colorectal cancer cases (83.3%, 30 of 36) than in the breast cancer cases (61.1%, 22 of 36; $P = 0.064$). Although only a minor fraction of our mutants (72 of 175) could be classified according to these categories, these results nevertheless support the hypothesis that a tumorigenic effect of the Arg72 allele only occurs when combined with a somatic mutation of the type seen in breast carcinomas. Additional studies, including functional assays, are warranted to explore the effects of the different combined variants and their role in tumorigenesis in different tissues.

[6] Internet address: www.iarc.fr/P53/index.html.

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