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Diversity of Human p53 Mutants Revealed by Complex Formation to SV40 T Antigen

J. Bártek, B. Vojtěšek and D.P. Lane

The products of the two major suppressor genes p53 and Rb interact with the oncogene products of the DNA tumour viruses. These viral–host protein interactions mimic and interfere with the normal interactions of p53 and Rb with host proteins. The Rb gene product is frequently mutated in human cancers such that it no longer binds to viral or host proteins. In contrast we find that this is not the case with p53 as some, but not all, mutant p53 proteins still bind to the SV40 T antigen. In particular the hot spot mutation found in most Chinese and African cases of hepatocellular carcinoma (HCC) retains T binding activity. The simple subdivision of different p53 mutations revealed by this analysis may have diagnostic and prognostic consequences.
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INTRODUCTION

THE ONCOGENES of the DNA tumour viruses act at least in part by physically complexing to the protein products of two tumour suppressor genes, p53 and retinoblastoma (Rb). These interactions are proposed to neutralise the growth regulatory activity of the suppressor gene products by blocking their interactions with other host proteins [1, 2] or by leading to their rapid degradation [3]. Strong support for these ideas has come from the finding that the oncogenic activity of the DNA tumour viruses is curtailed by mutations that destroy the p53 or Rb binding activity of their oncoproteins [4–6]. Furthermore the mutations of the Rb gene found in human cancer seem to be specifically localised to the sites on the Rb protein required for binding to papova virus T antigens and adenovirus E1a proteins [7–9]. The capacity of p53 to bind to the SV40 T antigen is an intrinsic property of the protein as the two purified proteins will assemble *in vitro* [10]. This activity of p53 has been conserved in evolution leading to the suggestion that the interaction mimics a normal interaction of p53 with a host protein that is essential

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Table 1. Binding of human mutant p53 proteins to SV40 T antigen

Cell line	Tumour of origin	p53 mutation* Codon No.	Aminoacid change	Conserved region	Binding to SV40 T antigen §
BT20	Breast carcinoma	132	Lys>Glu	II	–
HOS	Osteosarcoma	156	Arg>Pro	II/III	–
SK-UT-1	Leiomyosarcoma	175	Arg>His	III+IV	+
		248	Arg>Gln		
T47D	Breast carcinoma	194	Leu>Phe	III/IV	++
DLD-1	Colon carcinoma	241	Ser>Phe	IV	+
SK-LMS-1	Leiomyosarcoma	245	Gly>Ser	IV	–
Namalwa	Burkitt's lymphoma	248	Arg>Gln	IV	++
		248	Arg>Trp		
RD†	Rhabdomyosarcoma	248	Arg>Trp	IV	–
SW837†	Colon carcinoma	248	Arg>Trp	IV	–
BT549‡	Breast carcinoma	249	Arg>Ser	IV	++
MDA468	Breast carcinoma	273	Arg>His	V	–
A431	Vulval carcinoma	273	Arg>His	V	–
HT29	Colon carcinoma	273	Arg>His	V	–
SW620	Colon carcinoma	273	Arg>His	V	–
MDA-MB-231	Breast carcinoma	280	Arg>Lys	V	–
BT474	Breast carcinoma	285	Glu>Lys	V	–
SW480	Colon carcinoma	273	Arg>His	V	–
		309	Pro>Ser		

*All the lines examined are homozygous for single p53 point mutations except SW480, SK-UT-1 and Namalwa. In the SW480 cell line both mutations are on the same allele [42] in the Namalwa [43] and SK-UT-1 cell line [44] and "(W. Warren, personal communication)" they are on different alleles. References to homozygous mutants: BT-20, BT 549, MDA-MB-231 and BT474 [45], HOS [46], DLD-1, HT29 and SW620 [47], SK-LMS-1 and RD [44], SW837, T47D and MDA-468 [42] and A431 [48]. †RD and SW837 express p53 mutant at codon 248 Arg>Trp which is precisely the germ line mutation detected in two of the six Li–Fraumeni families examined [34, 35]. ‡BT 549 express p53 mutant at codon 249 Arg>Ser which is the precise mutation found as a hot spot (11/13) in HCC [32, 33]. §T antigen binding to p53 was summarised taking into account all three assays. ++ = strong binding as found in the control SV40 transformed cell line. + = detectable binding but much weaker than control. – = background or very weak binding.

for p53 function [11, 12]. This idea has been strengthened by the recent discovery of host proteins that bind Rb in a "T antigen like" manner [13–15] and the capacity of E1a to displace Rb from a cellular transcription complex that contains the E2F DNA binding factor and the cyclin A protein [16–20]. The binding of p53 to T antigen has a profound effect on T antigen function. The p53-T complex is defective in SV40 replication activity reflected in a loss of DNA polymerase α [21, 22] binding activity and a loss of DNA helicase activity [10, 23]. Point mutations in the p53 gene are a frequent feature of human cancer [24]. Because these mutations are so frequent their simple occurrence may be of limited prognostic significance. Some preliminary comparisons suggest that different mutations may have different effects on the biological activity of p53 in transformation assays [25]. Although it has been sometimes assumed that all mutations in p53 found in human tumours abolish T antigen binding [24] we decided to re-examine this question as a potential method for subclassifying the properties of p53 mutations in human cancer.

MATERIALS AND METHODS

Cell culture

The origin and names of the 17 human tumour-derived cell lines used in the present study are listed in Table 1. The breast cancer cell line BT-20 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 μg/ml bovine insulin (Sigma). All remaining cell lines were grown in DMEM with 10% FBS. For some experiments, the exponentially growing cells were infected with SV40 virus at 37°C and cultured for a further 60 h before harvesting.

Antibodies

Polyclonal antisera to SV40 large T antigen and p53 protein were prepared by immunising rabbits with pure T antigen [26] and bacterially expressed full length human p53 [27], respectively. Mouse monoclonal antibodies PAb421 and PAb419 [28] were originally provided by E. Harlow, the PAb 1005 antibody to human p53 [29] was obtained from A. Levine.

Immunoprecipitation and immunoblotting

Cells were lysed in 150 mmol/l NaCl, 50 mmol/l Tris pH 8.0, 5 mmol/l EDTA, 1% NP40, 1 mmol/l polymethanesulphonyl fluoride for 30 min on ice. The cell extract was centrifuged at 15000 *g* for 30 min and the pellet discarded. The extract was preabsorbed with Protein-G sepharose (Pharmacia), pure monoclonal antibody was added and the mixture was left overnight at 4°C on a rotating wheel. Protein G beads were added and the incubation was continued for 1 h. The beads were then washed four times in lysis buffer. Denaturing polyacrylamide

gel electrophoresis and immunoblotting were performed as described by Harlow and Lane [30]. Protein from 10 μl of beads immunoprecipitated as above was loaded per track. Blots were blocked in 0.1% Tween 20 in phosphate buffered saline (PBS), probed overnight at 4°C with either rabbit anti-p53 or rabbit anti-T antiserum diluted 1:100 and washed in 0.1% Tween 20 in PBS. The blots were then incubated for 2–3 h at room temperature in Dako peroxidase-conjugated swine anti-rabbit Ig diluted 1:100 and washed again in 0.1% Tween 20 in PBS. The peroxidase activity was visualised with chloronaphthol (Sigma).

Plate immunoassays

A sandwich immunoassay for quantitation of the binding of mutant p53 proteins to SV40 T antigen was performed essentially as described by Gannon *et al.* [31], using monoclonal anti-p53 antibody PAb1005 (to measure the levels of p53 and p53-T complexes), monoclonal anti-T antibody PAb419 (for T antigen levels) or a control antibody as solid phase reagents and polyclonal rabbit antisera to either p53 or T to detect the captured proteins. For the solid phase, affinity purified monoclonal antibodies were used at a concentration of 20 μg/ml to coat the wells of 96 well microtitre plates. After coating overnight, plates were blocked by 4 h incubation in a 5% solution of bovine serum albumin (BSA) in PBS and rinsed in PBS, followed by sequential incubations in the wells of serially diluted cell extracts of known protein concentration (overnight at 4°C), polyclonal rabbit anti-T or anti-p53 antisera (diluted 1:1000, 2 h at 4°C) and peroxidase-conjugated swine anti-rabbit immunoglobulin antisera (Dako, diluted 1:1000, 2 h at 4°C). All washes between incubations used PBS with 0.1% NP40. The enzymatic reaction was visualised with tetramethylbenzidine and the results monitored in a Flow enzyme linked immunosorbent assay (ELISA) reader. Assay points were determined in duplicate. Independent infection, extraction and ELISA experiments were performed at least twice with each line to verify reproducibility. For the *in vitro* association experiments, 1 μg of pure T antigen was mixed with 0.4 ml cell extract (protein concentration of 20 μg/ml) and incubated on ice for 3 h before serially diluting the extract for sandwich ELISA.

Immunocytochemistry

Monolayers of cells were fixed directly in tissue culture dishes for 5 min in 50% acetone in methanol and air dried. Indirect immunoperoxidase staining for p53 or SV40 T antigen was performed as described in Harlow and Lane [30] using hybridoma supernatants followed by peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dako) and diaminobenzidine as chromogen.

RESULTS

Formation of the T-p53 complex in SV40 virus infected cells

To directly examine the effect of different mutations in p53 on interaction with SV40 T antigen we infected a range of different human tumour cell lines containing known p53 mutations with SV40 virus. At 60 h after infection we used an immunoprecipitation and immunoblotting assay to examine the expression of large T, p53 and the p53-T complex in the infected cells. Immunoprecipitation with the anti-T monoclonal antibody PAb 419 followed by immunoblotting of the immunoprecipitates showed that large T was expressed in all the infected cell lines. Control immunoprecipitations demonstrated the specificity of the detection system (Fig. 1a). When the same immunoprecipitates were analysed on a separate gel by immunoblotting with an anti-p53 antibody we detected dramatic differences in the amount of p53 present in the anti-T immunoprecipitate. In some cell lines a strong band of p53 was present indicating the efficient formation of a p53-T complex by certain mutant p53 proteins. In other cell lines no such complex was apparent (Fig. 1b). To extend this result and to determine if the absence of complex was due to an absence of p53 we reversed the procedure and used an anti-p53 antibody for the immunoprecipitation. High levels of p53 were present in all the cell lines but again very clear differences were apparent in their capacity to bind to T antigen. For example in the RD cell line p53 was clearly present in the immunoprecipitate but no T was present while in the BT549 cell line the p53-T complex was readily detected. The SK-UT-1 line showed an intermediate phenotype as only small amounts of T were found in the complex (Fig. 1c).

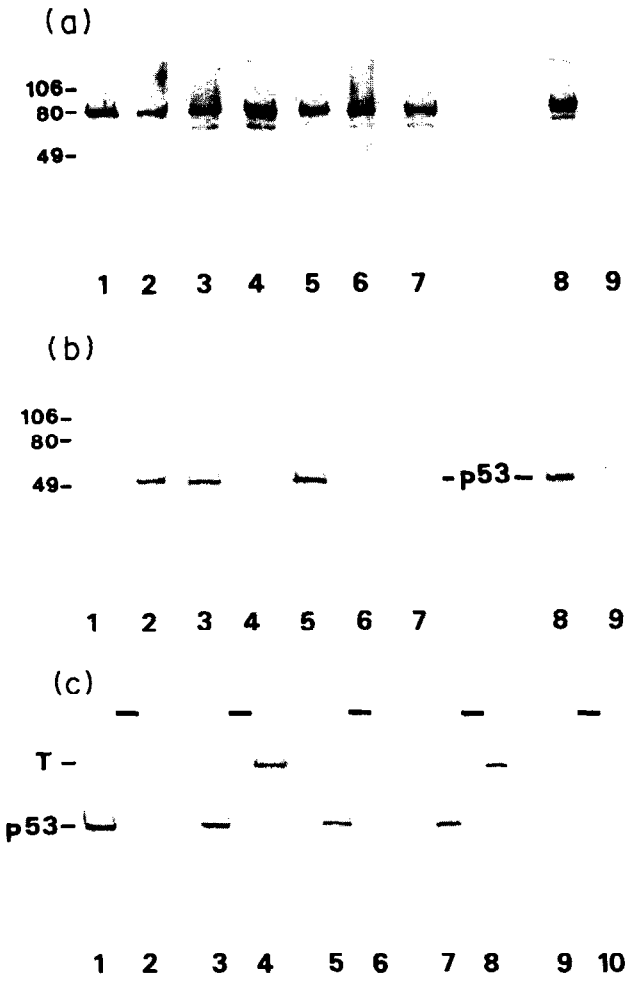

Fig. 1. Immunoblot analysis of mutant p53/T complexes. (a) Immunoprecipitation of T antigen with PAb 419 from cell extracts of: BT20 (lane 1), T47D (lane 2), Namalwa (lane 3), SW837 (lane 4), BT549 (lane 5), A431 (lane 6), MDA-MB-231 (lane 7) and SVK14 (a control SV40-transformed human cell line, lane 8). Lane 9, negative control (immunoprecipitation with an anti-B galactosidase monoclonal antibody of SVK14 extract, probed with rabbit anti T serum). (b), As (a) but probed with rabbit anti-p53 serum. (c) p53 was immunoprecipitated from cell lines SK-UT-1 (lanes 1, 2), BT549 (lanes 3, 4), RD (lanes 5, 6), SVK14 (positive control, lanes 7, 8) and uninfected human foreskin fibroblasts (negative control, lanes 9, 10) with the anti-p53 monoclonal antibody PAb1005 (1H5) (lanes 1–10) and the blots probed with rabbit anti-p53 serum (lanes 1, 3, 5, 7, 9) or rabbit anti-T serum (lanes 2, 4, 6, 8, 10).

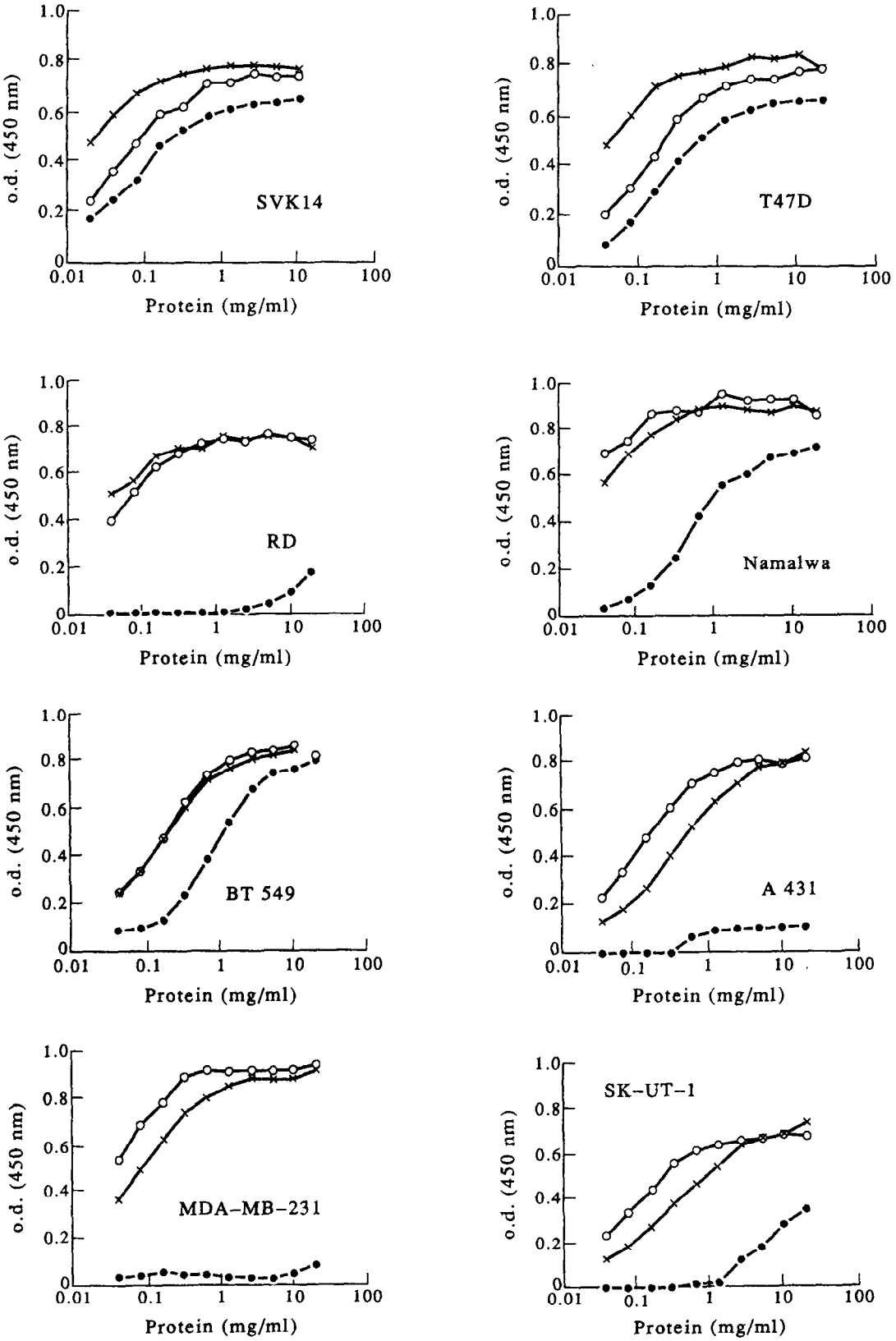

Fig. 2. Quantitation of the binding of mutant p53 proteins to SV40 T antigen in SV40-infected tumour cell lines by two site immunoassay. The level of p53 (-○-), T antigen (-x-) and p53-T complex (-●-).

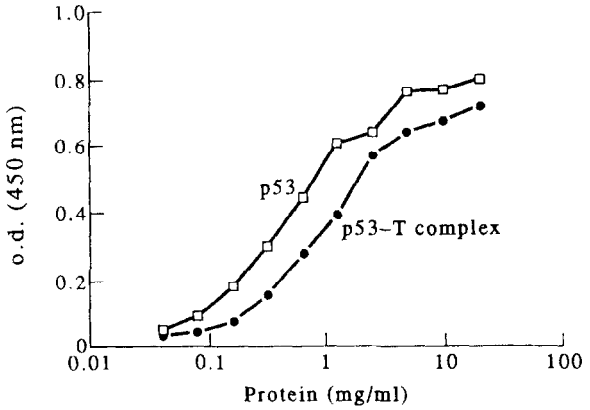

Fig. 3. An example of a p53-T complex formation and p53 level in extract of uninfected tumour cell line BT549 preincubated with pure T antigen *in vitro* and estimated using a sandwich immunoassay.

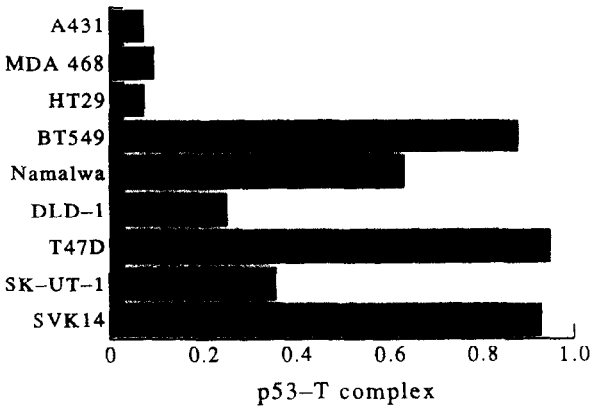

Fig. 4. A summary graph of mutant p53 proteins in complex with pure T antigen, expressed as a ratio of the p53-T complex (optical density at 450 nm)/p53 level (o.d. at 450 nm) at a protein concentration of 5 μg/ml and indicated as black bars in the graph.

ELISA analysis of the T-p53 complex

To extend and quantitate these results we used two site sandwich ELISA assays. Microtitre plates were coated with anti-T (PAb419) or anti-p53 (PAb1005) monoclonal antibodies and bound proteins and complexes detected with polyclonal anti-T or anti-p53 antibodies. These assays allowed us to measure p53 levels, T antigen levels, as well as the T-p53 complex level. The assays were also run on uninfected cells. This confirmed the specificity of the assay as no T or T-p53 complex was detected in these control cells. The assays also confirmed the high levels of p53 in these tumour cell lines. The results extended and confirmed the immunoprecipitation assay. T-p53 complexes were clearly present in the control SV40 transformed SVK14 cell line (Fig. 2) and in some of the infected cells, for example T47D and BT549 but while T and p53 were clearly detected in others no complex was present (for example RD and MDA-MB-231 cells) (Fig. 2). The ELISA was easily able to detect intermediate levels of complex formation (for example SK-UT-1) and provides an easy and rapid method for comparing different mutant proteins.

Formation of mutant p53-T antigen complexes in vitro

Because it may not always be possible or convenient to infect tumour cells with SV40 we also developed an *in vitro* test in which lysates of uninfected cells were mixed with known amounts of pure T and the assembly of the p53-T complex was measured. The results obtained from this approach were in all cases in complete agreement with the results obtained with infected cells (Figs 3 and 4). Furthermore this *in vitro* approach allowed us to analyse an extra four cell lines that were partially resistant to infection (DLD-1, HT29, MDA 468 and SW620). It will also allow the test to be carried out directly on human tumour cell extracts. In total 17 different cell lines and 13 separate mutations were examined (Table 1) representing mutations associated with several different cancer types and including the hepatocellular carcinoma (HCC) [32, 33] and Li–Fraumeni [34, 35] hot spots. Every point mutation in conserved block five which includes the somatic hot spot at aminoacid 273 reduced T antigen binding to background or near background levels. The mutations in block four, which include those germ line mutations associated with the Li–Fraumeni syndrome and the HCC hot spot, showed large variations. Thus the HCC hot spot mutant protein bound T antigen very well while the protein containing one of the most frequent germ line mutations (codon 248 Arg>Trp) found in the Li–Fraumeni syndrome did not. Mutations in conserved block four at adjacent aminoacids gave completely different results emphasising the importance of examining the effect of individual mutations. Mutations in blocks two and three, consistent with data from the murine system also inactivated T antigen binding [36]. The interpretation of the results in the heterozygous cell lines containing two different mutant p53 proteins is of course more complex. In the case of the Namalwa cell line, since the Arg>Trp 248 mutation is known to be inactive in the homozygous state (Table 1) it is reasonable to assume that it is the Arg>Gln 248 mutant protein that binds to T. This mutation is also present in the other heterozygous line SK-UT-1. This line shows an intermediate level of T binding. On the basis of this phenotype the Arg>His mutant at 175 almost certainly inactivates T binding. This is consistent with data from other studies (A. Levine personal communication).

DISCUSSION

The Rb gene and the p53 gene are both frequent targets for mutation in human cancer [1, 2, 24]. In transfection studies both of these genes can act as suppressors of malignant growth. Mutations of these genes abolish this activity. Since these proteins are physically complexed by the products of three diverse sets of tumour viruses the concept has arisen that binding to the tumour virus oncogenes inhibits their suppressor function [1, 2]. This idea is supported by the observation that the E6 protein targets p53 for rapid degradation [3] and by the selective binding of T to the supposed active, unphosphorylated, form of Rb [37]. Consistent with the concept that the oncogene binding blocks the effector function of Rb is the frequent loss of T binding activity on mutation of Rb [7, 8]. We have now shown that eight out of 13 point mutations in p53 found in tumour cells abolish T antigen binding and that a further mutation (Ser>Phe 241) greatly reduces the affinity of p53 for T antigen. Among the mutations that inactivate T binding is one found as a germ line mutation (Arg>Trp 248) in two Li–Fraumeni families [34, 35]. Interestingly three mutations (Leu>Phe 194, Arg>Gln 248, and Arg>Ser 249) clearly did not inactivate the T binding function of p53. It is apparent from the codon 248/249 mutations that not only the precise position of the mutation but also the precise substitution at a given codon can dramatically affect T binding. The Arg>Ser 249 mutation which is in conserved box IV, is of special interest because it is the same

mutation as occurs with astonishing frequency in HCC [32, 33]. Our results strongly suggest that these proteins will have different biological activities to the other mutant p53 proteins. The HCC data imply that this particular type of mutation may be important for the genesis of HCC. The results of this study emphasise that different point mutations have different effects on p53 function. Differences have also been demonstrated for a small sample of mutants in HSP70 binding and in transfection assays that measure the suppressor activity and transforming activity of human p53 [25, 38]. Recently differences have also been found in the capacity of mutant p53 proteins to act as transcriptional transactivators when fused to the GAL4 protein [39, 40]. We have found that expression of high levels of p53 is associated with poor prognosis in certain tumours [41]. The ability to subdivide the properties of mutant p53 proteins in tumour cell extracts on the basis of T antigen binding activity may provide important further diagnostic criteria.

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Trends in Mortality from Malignant Cutaneous Melanoma in The Netherlands, 1950–1988

P.J. Nelemans, L.A.L.M. Kiemeney, F.H.J. Rampen, H. Straatman and A.L.M. Verbeek

This paper presents an analysis of trends in mortality from malignant melanoma of the skin in The Netherlands, 1950–1988. Statistical analyses show that time period effects are needed to describe the mortality trends in The Netherlands. Because this contrasts with reports from other countries, in which the trends were ascribed to a cohort effect only, log-linear models including the three factors age, time period and birth cohort, were fitted to the data. To be able to separate time period effects from birth cohort effects we assumed a mathematical function for the mortality rates in relation to age. The results obtained in this way indicate that time period effects increased up to 1970. An increase of birth cohort effects is seen for cohorts born between 1900 and 1955. For cohorts born after 1955 the mortality from melanoma seems to decrease. The most plausible explanation for the time period effect probably is improvement in death certification.
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INTRODUCTION

A RAPID RISE of incidence and of mortality from cutaneous malignant melanoma is reported from many countries in the world [1]. A doubling of incidence every 10–14 years is observed [2]. The increase in mortality is less than the rise in incidence. Mortality rates from the United States, England and Wales, and Canada studied by Lee, showed an annual increase of about 3% [3].

An international comparison of incidence rates (Fig. 1) shows that the Dutch population is at intermediate risk of getting a malignant melanoma of the skin [4]. Within the European Community The Netherlands belong to the countries in which the highest melanoma risk is seen [5]. Nationwide data about Dutch incidence of cancer over a longer period of time are not available. However, mortality data were published from 1950 onwards and can be studied for trends.

Time trends can be produced by two mechanisms, a time period effect and/or a birth cohort effect. In many countries a so-called age-cohort pattern was observed in both sexes. This means that starting with some specific birth cohort the mortality is increasing for successive birth cohorts (with a similar age profile) rather than for successive time periods. This observation of a birth cohort effect supports the idea that the rise of mortality and incidence of cutaneous malignant melanoma is real and not the result of better registration techniques.

This paper presents an analysis of the trends in melanoma mortality in The Netherlands, 1950–1988, using statistical methods described later.

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DATA AND METHODS

Mortality data

Numbers of persons with malignant melanoma of the skin as underlying cause of death from 1950 through 1988 were derived from annual publications of the Central Bureau of Statistics (CBS) [6]. Population information was also available from this