Gene Ther Mol Biol Vol 5, 81-86, 2000

Characterisation of the *p53* gene in the rat CC531 colon carcinoma

Research Article

Sacha B Geutskens[1], Diana JM van den Wollenberg[1], Marjolijin M van der Eb[1,2], Hans van Ormondt[1], Aart G Jochemsen[1], Rob C Hoeben[1*]

Departments of [1]Molecular Cell Biology and

[2]Surgery, Leiden University Medical Center, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

* **Correspondence**: Rob C Hoeben, PhD, Department of Molecular Cell Biology, Leiden University Medical Center, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands; Phone: 00 31 71 5276119; Fax: 00 31 71 5276284; E-mail: r.c.hoeben@lumc.nl

Key words: colorectal cancer, mutated p53

Abbreviations: complementary DNA, (cDNA); Dulbecco's modified Eagle's medium, (DMEM); horse-radish-peroxidase, (HRP); reverse transcription-PCR, (RT-PCR); wild-type *p53*, (wtp53)

Received: 6 November 2000; accepted: 7 November 2000; electronically published: February 2004

Summary

The *p53* gene was characterised in CC531 colon carcinoma of the Wag/Rij rat, a model frequently used for the evaluation of anti-cancer treatments. The gene incurred a large in-frame deletion, with junctions in exons 4 and 8, and encodes a protein of approximately 32 kDa, lacking the entire DNA-binding domain. No wild-type *p53* allele is retained. Functional analysis shows that the mutated protein can repress the function of wtp53 protein.

I. Introduction

Colorectal cancer is one of the most common malignant tumours and a major cause of cancer death in developed countries. Over 700, 000 men and women are found to have colorectal cancer globally each year (Soussi et al, 1996).

The liver is the first vascular bed in which disseminating colorectal cancer cells are trapped. Liver metastases are detected in 20% of all patients undergoing resection of their primary colorectal tumour (Wanebo et al, 1978). Ultimately, about 75% of all colorectal cancer patients develop liver metastases. At present, the only chance of long-term survival is complete resection of these metastases, an operation that is exclusively performed on patients with no signs of irresectable extra-hepatic disease.

Animal models are indispensable in the search of new approaches for the treatment of colorectal tumour metastases. One of the few well-characterised animal models for hepatic colorectal cancer makes use of the rat CC531 cell line. The CC531 colon carcinoma cell line is derived from a 1,2-dimethyl-hydrazine-induced tumour and syngeneic with the Wag/Rij rat (Thomas et al, 1993). It is a representative model for secondary liver metastases and frequently used for studying effects of various anti-cancer treatments (Marinelli et al, 1991; Oldenburg et al, 1994; Veenhuizen et al, 1996; Griffini et al, 1997).

Two-thirds of all colorectal tumours contain mutations in the *p53* gene, illustrating the essential role of p53 in the aetiology of colorectal cancer (Soussi et al, 1996). Loss of p53 function, as frequently occurs in cancer cells, causes loss of growth control and abrogation of programmed cell death (Bellamy, 1997). Reintroduction of wild-type *p53* (wtp53) arrests cell growth and may induce apoptosis, as has been described for various tumour cell lines (Xu et al, 1997; Anderson et al, 1998). A relationship has also been established between the presence of a mutation in the *p53* gene and the clinical prognosis (Hamelin et al, 1994).

In view of the prominent role of the *p53* gene for colorectal cancer, we assessed the integrity of the *p53* gene in the CC531 cancer cell line, and discovered a homozygous in-frame deletion within the coding region of the *p53* mRNA, resulting in removal of the entire DNA-binding domain. The mutated protein is capable of inhibiting transcription activation by wtp53. Our data not

only reveal the p53 status of this model, but also provide a genetic marker that allows development of a sensitive PCR-based assay for the detection of cancer cells amid normal cells.

II. Results

A. PCR amplification and sequence analysis of the p53 mRNA and chromosomal DNA in CC531 cells

To study whether the p53 gene of the CC531 cell line contains a mutation, RT-PCR was performed to amplify the entire coding region of the p53 gene. Instead of the expected 1200-bp fragment, only a fragment of ca. 600 bp was amplified, under various conditions (**Figure 1A**). Moreover, when internal primers (annealing to exons 4 and 8) were used no amplified product was detected (**Figure 1A**), suggesting a deletion within the coding region. Indeed, sequence analysis and comparison with the published sequence of the rat p53 gene (Hulla and Schneider, 1993), revealed that a large part of the coding region was absent. This in-frame deletion encompasses part of exon 4, exons 5-7 and part of exon 8 and removes amino acids 105 to 326.

Thus, the resulting p53 protein lacks the entire specific DNA-binding domain, but still contains the domain responsible for tetramerization with other p53 proteins (**Figure 1B**).

To verify that the deletion is not the result of aberrant RNA splicing, chromosomal DNA from CC531 cells was analysed.

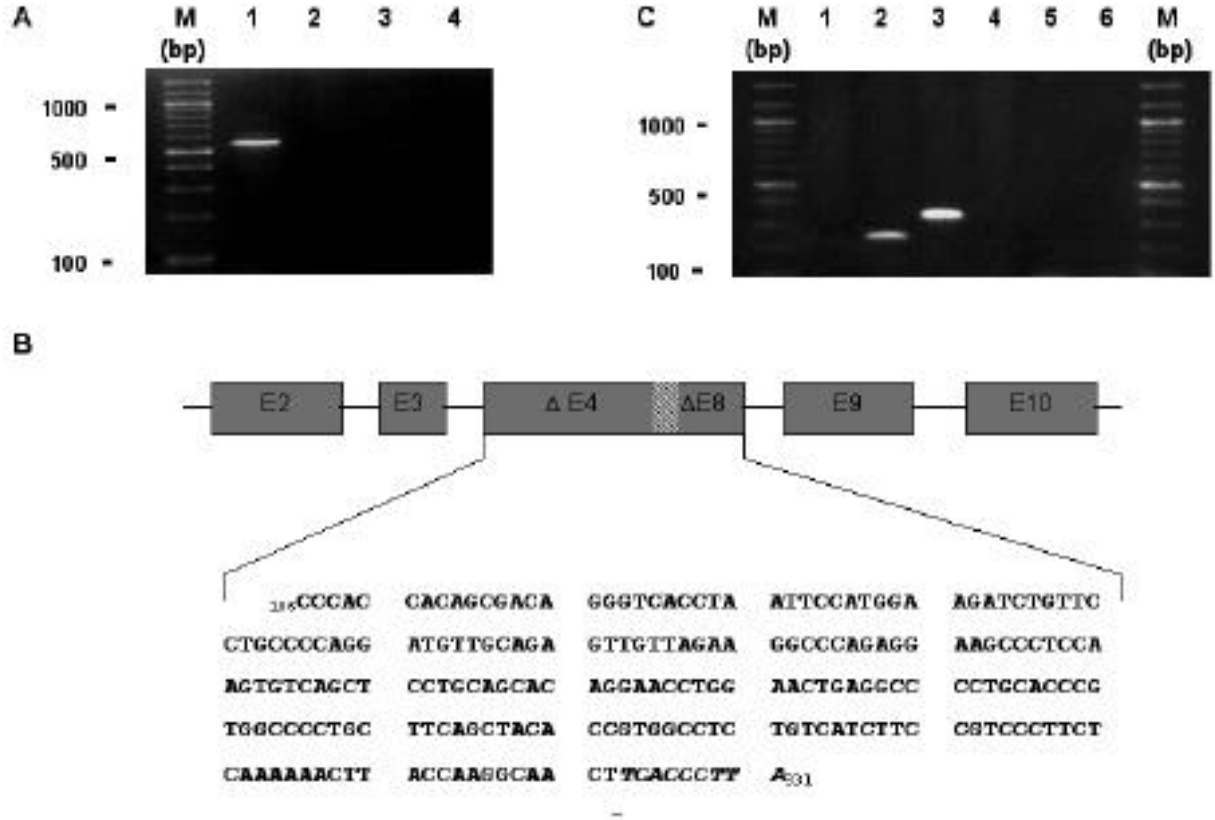

Figure 1.
A: Lane 1 shows the 600-bp p53-cDNA fragment obtained by PCR-amplification on RNA isolated from CC531 cells. In lanes 2, 3 and 4, a PCR fragment is absent if primer combinations are used spanning exon 1/exon 4, exon 8/exon 10 and exon 4/exon 8 respectively. Fragment sizes have been estimated by comparison to a 100 bp DNA marker (first lane). **B**: Schematic representation the genomic configuration of the coding region of *p53* gene in CC531 cells. The exons are indicated. Below the graph the sequence of the fused exons 4 and 8 is given. The filled triangle, below the sequence marks the junction. **C**: PCR amplification of a 200-bp DNA fragment encoding the fused exons 4 and 8 (primers F4/R8) of the *p53* gene from CC531-derived chromosomal DNA (lane 2). No fragment is detected if primers F7/R8 are used (lane 1). A 300-bp fragment containing the 5' exon 7/ 5' exon 8 (F7/R8 primers) region is amplified if chromosomal DNA from Wag/Rij rat hepatocytes is used (lane 3). No fragment is detected if this DNA is amplified with F4/R8 primers (lane 4). Lane 5 and 6 depict H_2O controls for primer pairs F7/R8 and F4/R8 respectively. Fragment sizes have been estimated by comparison to a 100-bp DNA marker (first lane).

Therefore, forward primers were developed binding in the remaining part of exon 4 (F4) and in exon 7 (F7) and one reverse primer downstream of the junction in exon 8 (R8). If a wild-type *p53* allele were present, it would be expected to yield a PCR-product of about 300-bp when F7 is combined with R8. If, on the other hand, the deletion occurs on the chromosomal DNA, a ≈200-bp product is expected if F4 is combined with R8. If the mutated allele is the only one present, no product should be seen with F7 and R8.

Amplification of chromosomal DNA derived from CC531 cells yielded a 200-bp product with the F4/R8 primers and no product with the F7/R8 primers. In contrast, PCR amplification of chromosomal DNA isolated from Wag/Rij rat hepatocytes yielded no product with F4/R8 primers, but a fragment of ca.300-bp when F7/R8 primers were used (**Figure 1C**). These data demonstrate that the deletion is present on the chromosomal DNA level. Moreover, the CC531 cell line is homozygous for the mutated *p53* allele.

B. Analysis and function of the mutated p53 protein

Because the deletion does retain the p53 open reading frame, a p53 protein lacking the central domain be synthesised. To investigate this possibility, the CC531 p53-encoding cDNA was cloned in an expression vector and transfected into the human Hep3B (no p53) and HepG2 (wtp53) cell lines. After 48 h, these cells and, as controls, rat CC531 and BxC22 cells, were analysed for presence of human and rat p53. Indeed, a smaller p53-protein of ca. 32 kDa was detected in CC531 cells, which co-migrates with a band seen in the Hep3B and HepG2 cells transfected with pCMVCC53 (**Figure 2**). Since the CC531-derived p53 protein lacks the DNA-binding domain, but still contains the tetramerization part, it was hypothesized that the protein might act as a dominant-negative mutant. To investigate whether the 32-kDa p53 protein can affect transcription activation of wtp53, the pCMVCC53 vector was transfected into HepG2 cells, together with a p53-responsive luciferase-reporter plasmid. Transfection of pOLXALuc, which contains a p53-responsive element (Steegenga et al, 1995) yields a higher luciferase activity than transfection with pXALuc, which lacks a p53-reponsive element (Peltenburg and Schrier, 1994) (**Figure 3**).

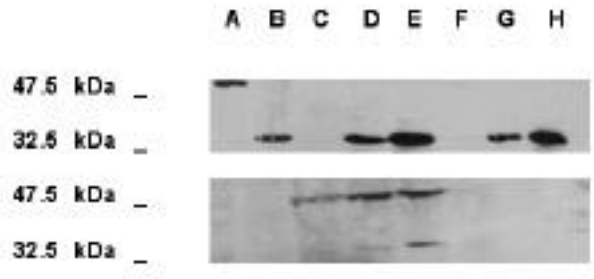

Figure 2. Immunoblot analysis on cell lysates of BxC22 (lane A; rat wtp53), CC531 (lane B, rat mutp53), HepG2 (lane C, human wtp53) and Hep3B (lane F, human, no p53). HepG2 and Hep3B cells transfected with 10 μg (lane D and G) or 20 μg (lane E and H) of pCMVCC53.Blots have been incubated with PAb122, recognising the C terminus of rat and human wtp53 (blot A) or with DO-1, recognising human wtp53 (blot B). Both BxC22 and HepG2 express normal-size (ca. 53 kDa) wtp53. CC531-derived cell lysates and those transfected with the CC53-construct express a shorter p53 protein of ca. 32 kDa. Protein sizes have been estimated by comparison to a broad-range protein marker.

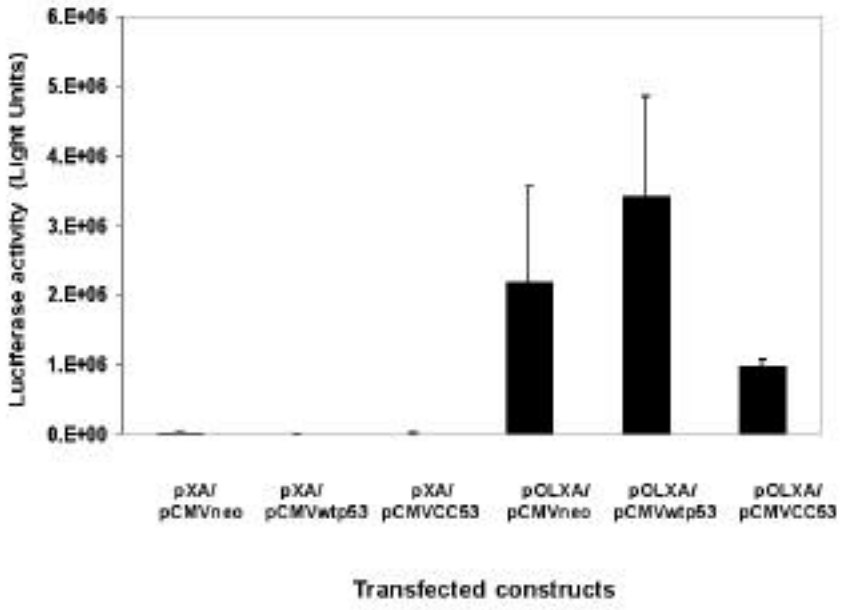

Figure 3. Luciferase activity in HepG2 cell lysates. Lysates were made 48 h after transfection of HepG2 cells with the various constructs. Cells ($1x10^5$/well) were transfected with the calcium-phosphate precipitation method, with 1.5 μg pXAluc or pOLXALuc, 20 ng of either pCMVneo, pCMVwtp53 or pCMVCC53, and 20 ng pCMVlacZ as indicated. Precipitates were made in duplicate and the experiment was performed three times. Luciferase activities depicted are corrected for differences in transfection efficiencies determined with the β-galactosidase assay.

.

Co-transfection of a plasmid encoding human wtp53 further increased expression of pOLXALuc, but not that of pXALuc, indicating the validity of the approach. The activity of pOLXALuc was clearly reduced upon co-transfection with pCMVCC53, while pXALuc expression was not affected. This suggests that the 32-kDa p53 protein acts as a dominant inhibitor of wtp53 function. Expression of a non-p53-regulated reporter, lacZ, which is driven by the CMV promoter, did not show manifest differences between various co-transfections.

III. Discussion

Genetic alteration of the tumour-suppressor gene p53 is frequently found in cancer, especially in the DNA-binding domain that spans exons 5 through 8 (Tullo et al, 1999; Veldhoen et al, 1999). In many studies, only this region is screened for the presence of mutations in clinical samples. The rat colon carcinoma studied here, showed a large deletion, removing amino acids 105 to 326. A smaller p53-protein of ca.32 kDa is translated which is recognized by a human and rat-p53 specific antibody, PAb 122, that is known to bind to the C-terminus of the wild-type protein. Although the DNA-binding domain is deleted, the 32-kDa p53 protein can impair transcriptional activation by regulation of wtp53. Such a dominant-negative effect has been described earlier (Chen, 1998; Roemer, 1999) and probably results from disruption of DNA binding of wtp53 by forming hetero-tetramers with the mutant p53 (Deb et al, 1999). This type of mutants is also thought to exhibit a gain of function by generating genomic instability, increasing oncogenic transformation (Gualberto et al, 1998).

CC531 is a valuable model for secondary liver metastases in the rat and often used for studying the therapeutic effect of various anti-neoplastic agents (Marinelli et al, 1991; Oldenburg et al, 1994; Veenhuizen et al, 1996). The p53 protein is essential in the induction of apoptosis by several anti-cancer therapeutics (Tishler and Lamppu, 1996; Hagopian et al, 1997; Anderson et al, 1998). Mutations in the *p53* gene are associated with drug resistance, so the p53 status of this model is highly important. The *p53* deletion characterised here might explain the resistance of CC531 to cisplatinum treatment (Gheuens et al, 1993) that activates p53-dependent apoptotic pathways. Despite the dominant negative activity of the CC531 p53 protein, transfer of wtp53 rendered the CC531 colon carcinoma susceptible for apoptosis (Van der Eb et al, *manuscript in preparation*). Combining chemotherapeutics with the transfer of wtp53 might give a higher efficacy of anti-tumour treatment than with the anti-neoplastic agent alone.

In addition, the characterisation of the junction of this deletion has allowed the differentiation of CC531 tumour and non-tumour cells via PCR. This technique permits detection and quantification of minute amounts of tumour cells in extra-hepatic tissues, such as lymph nodes and lungs and it will make the CC531 model even more valuable for exploration of new avenues for treatment of colorectal cancer.

IV. Materials and Methods

A. Tissue culture and cell lines

The CC531 cell line is a moderately differentiated adenocarcinoma of the colon, syngeneic with Wag/Rij rats (Thomas et al, 1993). BxC22 is a wtp53-expressing Ad5 E1-transformed Wag/Rij baby rat kidney cell line. HepG2 and Hep3B are human hepatoma cell lines expressing wtp53 (Hosono et al, 1991) or lacking p53 expression (Farshid and Tabor, 1992) respectively. All cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal-calf serum (GIBCO Laboratories, Grand Island, NY, USA), at 37 °C/5% CO_2.

B. Polymerase chain reaction (PCR)

For reverse transcription-PCR (RT-PCR), RNA was isolated directly from tissue culture *with* RNAzolB according to the manufacturers protocol (Campro Scientific, Veenendaal, The Netherlands). RNA was treated with DNase I (Roche Diagnostics, Almere, The Netherlands) to degrade contaminating DNA. First-strand-complementary DNA (cDNA) of 1 µg of RNA was synthesized with SuperscriptII RNase H** reverse transcriptase (Gibco/Life Technologies, Breda, The Netherlands) and an Oligo-dT primer. The cDNA (1 µg) was amplified with primers complementary to the 5' and 3' ends of the coding region of wild-type rat *p53* (forward primer: 5' GTG GAT CCT GAA GAC TGG ATA ACT GTC 3'; reverse primer: 5' AGT CGA CAG GAT GCA GAG GCT G 3') (Van den Heuvel et al, 1990), with Pfu polymerase (Stratagene, Amsterdam, The Netherlands) in buffer supplied by the manufacturer. Primers complementary to internal sequences of the p53 coding region (forward primer: 5' TAC CAC TAT CCA CTA CAA GTA CAT G 3'; reverse primer: 5' TTT CTT CCT CTG TCC GAC GGT CTC 3') were used as a control. The amplified 600-bp fragment was sequenced by BaseClear (Leiden, The Netherlands).

Chromosomal DNA was isolated with the NP40 protocol (Maniatis et al, 1989). DNase was heat-inactivated and RNase (Merck, Darmstadt, Germany) was added to degrade RNA. One microgram of DNA was amplified with primers annealing to exon 8 (R8): 5' AAT CCA ATA ATA ACC TTG GTA CCT T 3', exon 7 (F7): 5' TGT GCC TCC TCT TGT CCC 3' or exon 4 (F4): 5' CGA CAG GGT CAC CTA ATT CC 3' of wild-type rat p53 chromosomal DNA with Taq polymerase (Roche Diagnostics, Almere, The Netherlands).

C. Western immunoblotting

Cells (total of $3x10^6$) were lysed in 750 µl NP40/SDS (2%/0.2%) buffer (25 mM Tris pH 7.4, 50 mM NaCl, 0.5% deoxycholate). Protein lysates (40 µl) were size-fractionated by gel electrophoresis in 10% SDS-polyacrylamide. Proteins were transferred to Immobilon-P (0.45 µm, Millipore Corporation, Bedford, USA) and incubated with an antibody specifically recognizing human wtp53, DO-1, or with PAb 122, recognizing both human and rat p53 (Schmieg and Simmons,1984). As a second antibody horse-radish-peroxidase (HRP)-conjugated antibody, GαM-IgG (Jackson Immunoresearch Laboratories,

Westgrave, USA) was used. The blots were visualized by exposion to Kodak XAR-films. The broad-range protein marker was used as a standard (BioRad laboratories, Veenendaal, The Netherlands).

D. Luciferase reporter assay

For the expression of the mutant p53 cDNA, a vector containing the p53-coding region of CC531 under the regulation of the CMV promoter (pCMVCC53) was made by digesting the purified RT-PCR fragment with BamHI and SalI and subsequent ligation into pcDNA3.1+ (Invitrogen, Leek, The Netherlands) digested with BamHI and XhoI. Plasmid constructs expressing the neomycin resistance gene (pCMVneo), the E.coli β-galactosidase gene (pCMVlacZ), or the human wtp53 cDNA (pCMVwtp53) were used and have been described earlier (Steegenga et al, 1995). The luciferase reporter constructs, pXALuc and pOLXALuc (containing the p53-consensus binding sequence) have also been described before (Peltenburg and Schrier, 1994; Steegenga et al, 1995).

Cells ($1x10^5$/well) were transfected with the calcium-phosphate precipitation method (van der Eb and Graham, 1980), with 1.5 µg pXAluc or pOLXALuc, 20 ng of either pCMVneo, pCMVwtp53 or pCMVCC53, and 20 ng pCMVlacZ. Precipitates were made in duplicate and the experiment was performed three times.

After 48 h, cells were lysed in 250 µl of cell-culture lysis reagent (Promega, Madison, WI, USA) and luciferase activity of 100 µl of lysate was determined as described before (Steegenga et al, 1995). The β-galactosidase activity resulting from co-transfection of the control plasmid pCMVlacZ was determined in a β-galactosidase assay (Maniatis et al, 1989) and served as an internal control to correct for variations in transfection efficiency. In none of the experiments, β-gal activity varied more than 20%.

Acknowledgements

The sequence of the CC531 p53 cDNA is deposited in Genbank (accession number AY009504).

References

Anderson SC, Johnson DE, Harris MP, Engler H, Hancock W, Huang W, Wills KN, Gregory RJ, Sutjipto S, Fen Wen S, Lofgren S, Shepard HM, Maneval DC (**1998**) p53 Gene therapy in a rat model of hepatocellular carcinoma: Intra-arterial delivery of a recombinant adnovirus. **Clin Cancer Res** 4, 1649-1659

Bellamy CO (**1997**) p53 and apoptosis. **Br Med Bul** 53, 522-523

Chen P (**1998**) In vitro analysis of the dominant negative effect of p53 mutants. **J Mol Biol** 281, 205-209

Deb D, Chakraborti AS, Lanyi A, Troyer DA, Deb S (**1999**) Disruption of functions of wild-type p53 by hetero-oligomerization. **Int J Oncol** 15, 413-422

Farshid M, Tabor E (**1992**) Expression of oncogenes and tumor suppressor genes in human hepatocellular carcinoma and hepatoblastoma cell lines. **J Med Virol** 38, 235-239

Gheuens E, Van der Heyden S, Elst H, Eggermont A, Van Oosterom A, De Bruijn E (**1993**) Multidrug resistance in rat colon carcinoma cell lines CC531, CC531mdr+ and CC531rev. **Jpn J Cancer Res** 84, 1201-1208

Griffini P, Smorenburg SM, Verbeek FJ, Van Noorden CJ (**1997**) Three dimensional reconstruction of colon carcinoma metastases in liver. **J Microsc** 187, 12-21

Gualberto A, Aldape K, Kozakiewicz K, Tlsty TD (**1998**) An oncogenic form of p53 confers a dominant, gain-of-function phenotype that disrupts spindle checkpoint control. **Proc Natl Acad Sci USA** 95, 5166-5171

Hagopian GS, Mills GB, Khokhar AR, Bast RC jr, Siddik ZH (**1999**) Expression of p53 in cisplatin-resistant ovarian cancer cell lines: modulation with the novel platinum analogue (1R,2R-diamino cyclohecane) (trans-diacetato) (dichloro)-platinum. **Clin Cancer Res** 5, 655-666

Hamelin R, Laurent-Puig P, Olschwang S, Jego N, Asselain B, Remvikos Y, Girodet J, Salmon RJ, Thomas G (**1994**) Association of p53 mutations with short survival in colorectal cancer. **Gastroenterology** 106, 42-48

Hosono S, Lee CS, Chou MJ, Yang CS, Shih CH (**1991**) Molecular analysis of the p53 alleles in primary hepatocellular carcinomas and cell lines. **Oncogene** 6: 237-243

Hulla JE and Schneider RP Sr. (**1993**) Structure of the rat p53 tumor suppressor gene. **Nucleic Acids Res** 21, 713-717

Maniatis T, Sambrook J, Fritch EF (**1989**) *Molecular Cloning: a Laboratory Manual, 2nd edition.* CSHL Press, Cold Spring Harbor, NY.

Marinelli A, Van Dierendonck JH, Van Brakel GM, Irth H, Kuppen PJ, Tjaden UR, Van de Velde CJ (**1991**) Increasing the effective concentration of melphalan in experimental rat liver tumours: comparison of isolated liver perfusion and hepatic artery infusion. **Br J Cancer** 64, 1069-1075

Oldenburg J, Begg AC, Van Vugt MJ, Reuvekamp M, Schornagel JH, Pinedo HM, Los G (**1994**) Characterization of resistance mechanisms to cisdiamminedichloroplatinum (II) in three sublines of the CC531 colon adenocarcinoma cell line in vitro. **Cancer Res** 54, 487-493

Peltenburg LTC, Schrier PI (**1994**) Transcriptional suppression of HLA-B expression by c-myc is mediated through the core promotor elements. **Immunogenetics** 40: 54-61

Roemer K (**1999**) Mutant p53: gain-of-function oncoproteins and wild-type p53 inactivators. **Biol Chem** 380, 879-887

Schmieg FI, Simmons DT (**1984**) Intracellular location and kinetics of complex formation between simian virus 40 T antigen and cellular protein p53. **J Virol** 52, 350-355

Soussi T (**1996**) The p53 tumour suppressor gene: a model for molecular epidemiology of human cancer. **Mol Med Today** 2, 32-37

Steegenga WT, Van Laar T, Terleth C, Van der Eb AJ, Jochemsen AG (**1995**) Distinct modulation of p53 activity in transcription and cell-cycle regulation by the large (54 kDA) and small (21 kDA) adenovirus E1B proteins. **Virology** 212, 543-554

Thomas C, Nijenhuis AM, Timens W, Kuppen PJ, Daemen T, Scherphof GL (**1993**) Liver metastasis model of colon cancer in the rat: immunohistochemical characterization. **Invas Metas** 13, 102-112

Tishler RB, Lamppu DM (**1996**) The interaction of taxol and vinblastine with radiation induction of p53 and p21 WAF1/CIP. **Br J Cancer** 27, S82-85

Tullo A, D'Erchia AM, Honda K, Mitry RR, Kelly MD, Habib NA, Saccone C, Sbisa E (**1999**) Characterization of p53 mutations in colorectal liver metastases and correlation with clinical parameters. **Clin Cancer Res** 5, 3523-3528

Van den Heuvel SJ, Van Laar T, Kast WM, Melief CJ, Zantema A, Van der Eb AJ (**1990**) Association between the cellular p53 and the adenovirus 5 E1B-55kd proteins reduces the oncogenicity of Ad-transformed cells. **EMBO J** 9, 2621-2629

Van der Eb AJ, Graham FL (**1980**) Assay of transforming activity of tumor virus DNA. **Methods Enzymol** 65, 826-839

Veenhuizen RB, Marijnissen JP, Kenemans P, Reuvekamp-Helmers MC, 't Mannetje LW, Helmerhorst TJ, Stewart FA (**1996**) Intraperitoneal photodynamic therapy of the rat CC531 adenocarcinoma. **Br J Cancer** 73, 1387-1392

Xu M, Kumar D, Srinivas S, Detolla LJ, Yu SF, Stass SA, Mixson AJ (**1997**) Parental gene therapy with p53 inhibits human breast tumors *in vivo* through a bystander mechanism without evidence of toxicity. **Hum Gene Ther** 8, 177-185

.