[CANCER RESEARCH 60, 1777–1788, April 1, 2000]

*Review*

# p53 Antibodies in the Sera of Patients with Various Types of Cancer: A Review[1]

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## Abstract

**p53 antibodies (p53-Abs) were discovered 20 years ago during the course of tumor-associated antigens screening. The discovery of p53 mutation and accumulation of p53 in human tumors shed new light on the p53 humoral response. In the present review, we have compiled more than 130 papers published in this specific field since 1992. We demonstrate that p53-Abs are found predominantly in human cancer patients with a specificity of 96%. Such antibodies are predominantly associated with *p53* gene missense mutations and p53 accumulation in the tumor, but the sensitivity of such detection is only 30%. It has been demonstrated that this immune response is due to a self-immunization process linked to the strong immunogenicity of the p53 protein. The clinical value of these antibodies remains subject to debate, but consistent results have been observed in breast, colon, oral, and gastric cancers, in which they have been associated with high-grade tumors and poor survival. The finding of p53-Abs in the sera of individuals who are at high risk of cancer, such as exposed workers or heavy smokers, indicates that they have promising potential in the early detection of cancer.**

## Introduction

The discovery of the p53 protein was the culmination of two types of studies: (*a*) the very well known virological approach, with the binding of p53 protein to oncoviral proteins (1–5); and (*b*) the more discrete serological approach with the study of TAAs[3] (6, 7). In 1979, DeLeo *et al.* (7) showed that the humoral response of mice to some methylcholanthrene-induced tumor cells such as MethA was directed to the p53 protein. This protein was found to accumulate in tumor cells of different origin but was undetectable in normal cells. It was also found that animals bearing SV40 tumors elicited an immune response specific for p53 (1, 5, 6). In 1982, Crawford *et al.* (8) first described antibodies against human p53 protein in 9% of breast cancer patient sera. No significant clinical correlation was reported, and, at that time, no information was available concerning mutations of the *p53* gene. Caron de Fromentel *et al.* (8) later found that such antibodies were present in sera of children with a wide variety of cancers. The average frequency was 12%, but this figure increased to 20% in Burkitt's lymphoma.

Those studies, performed in the early 1980s, were virtually ignored for more than 10 years because of a lack of interest in p53 during that period. In the early 1990s, it was discovered that the *p53* gene is the most common target for molecular alteration in every type of human cancer (10). This provoked considerable interest in the study of the p53 protein and its function in normal and transformed cells. It also led to the rediscovery of this humoral response, which had been found in cancer patients. Since 1992, more than 150 papers have been published on p53-Abs. This review will summarize this literature and focus on the future applications of this assay.

For a comprehensive view of the p53 discovery, the reader is referred to the excellent review published by Crawford (11). More reviews on p53 have recently been published (12–15).

## The p53 Protein

The tumor suppressor p53 is a phosphoprotein barely detectable in the nucleus of normal cells (16). On cellular stress, particularly that induced by DNA damage, p53 can arrest cell cycle progression, thus, allowing the DNA to be repaired (17) or it can lead to apoptosis (18). These functions are achieved, in part, by the transactivational properties of p53, which activate a series of genes involved in cell cycle regulation. In cancer cells that bear a mutant p53, this protein is no longer able to control cell proliferation, which results in inefficient DNA repair and the emergence of genetically unstable cells (12–15). The most common changes of p53 in human cancers are point missense mutations within the coding sequences of the gene (10, 19). Such mutations are found in all of the major histogenetic groups, including cancers of the colon, stomach, breast, lung, brain, and esophagus (20). It is estimated that *p53* mutations is the most frequent genetic event in human cancers and accounts for more than 50% of cases. More than 90% of the point mutations reported thus far are clustered between exons 4 and 10 and are localized in the DNA binding domain of the p53 protein (21). One of the most striking features of the inactive mutant p53 protein is its increased stability (half-life of several hours compared with 20 min for wild-type p53) and its accumulation in the nucleus of neoplastic cells. Positive immunostaining is usually indicative of abnormalities of the *p53* gene and its product, but it is highly dependent on the type of p53 mutation for review (22, 23).

## The Specificity of p53 Antibodies in Cancer Patients

**Dosage of p53 Antibodies.** The initial work on p53-Abs used either immunoprecipitation or Western blot as the detection method (8, 9). Later, several ELISAs were developed to handle large numbers of specimens (24, 25), and some of them are now commercially available. The diversity of all of these assays could account for the variation in the frequency of p53-Abs observed in the literature. One of the most important parameters seems to be the antigen used for these assays. It has been shown that p53-Ab recognized immunodominant epitopes localized in the $NH_2$ and COOH termini of the protein (see below). It is thus essential to use the entire p53 protein as antigen. Several attempts to develop an ELISA with synthetic peptides corresponding to these immunodominant epitopes have been unsuccessful, because they lead to a high level of false-negative results (26).

p53 is heavily phosphorylated at the $NH_2$ and COOH termini. Such phosphorylation can have an important influence on the reactivity of p53-Abs toward the protein, which suggests that p53 expressed in mammalian cell is a better antigen than those expressed in *Escherichia coli*. Recently, it has been shown that IgA p53-Abs are found

Received 10/25/99; accepted 2/16/00.

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[1] Supported by Ligue Nationale contre le Cancer (Comité de Paris), the Association pour la Recherche contre le Cancer (ARC), the Mutuelle Générale de l'Éducation Nationale (MGEN), Direction de la Recherche Clinique (DRC) de l'Assistance Publique-Hôpitaux de Paris Contract Grant AOA94084.

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[3] The abbreviations used are: p53-Ab, p53 antibody; TAA, tumor-associated antigen; LSH, loop-sheet-helix (motif); SCLC, small cell lung cancer; NSCLC, non-SCLC; ADC, adenocarcinoma.

as the only isotype in some patients with head and neck cancers. The fact that a majority of ELISAs use a secondary antibody specific for IgG may eventually lead to false-negative sera.[4]

p53-Abs have also been found in other body fluids such as the ascites of women with ovarian cancer, the pleural effusions of patients with pancreatic, colon, and lung tumors, and the saliva of patients with oral cancer (27–29). Such antibodies are correlated with serum p53-Abs (27, 28).

**p53 Antibodies and p53 Mutation Frequency.** Eighty serological analyses of p53 status in 18 cancer types that were published before August 1999 have been compiled (Table 1). Control experiments were performed in 36 studies, involving 2404 individuals who were either healthy or had nonmalignant diseases. Studies involving 9489 patients with various types of cancer were analyzed. Statistical analysis demonstrated that p53 antibodies are a specific marker of patients with neoplasia ($P < 10^{-4}$; Table 2). An analysis of p53-Abs in each type of cancer also showed a significant correlation with malignant diseases for most types of cancer except for testicular carcinoma, melanoma, hepatoma, and glioma, in which the frequency of p53-Abs was very similar to that of the control group (Table 2). Fig. 1 shows the correlation between the frequency of p53-Abs and the frequency of p53 mutations published in the literature. There is a very strong correlation between the two rates, again arguing that p53 mutations are involved in the appearance of these antibodies. Apart from the healthy control, it is striking to observe that three cancers, well-known to be devoid of p53 mutations, hepatoma (30), testicular carcinoma (31, 32), and melanoma (33, 34), are also negative for p53-Abs. However, cancers such as esophageal carcinoma and oral squamous cell carcinoma, which have a high rate of p53 mutations, also have a high frequency of p53-Abs. The only exception is glioma, with a very low rate of p53-Abs (35, 36) despite a high frequency of p53 mutations (37). In a complete study involving both molecular and serological analyses, Rainov *et al.* (35) found p53 mutations in 24 of 60 glioblastomas, but none of these patients had p53-Abs. Several explanations can account for the lack of a p53 immune response in this type of cancer. It is possible that the immune privilege of the brain does not allow the induction of this humoral response. It is also possible that there is inefficient antigen presentation if p53 cannot cross the brain barrier, or if no immune response can be elicited in the brain. Furthermore, we should keep in mind that, in one series, 70% of the patients were treated with dexamethasone before serum collection (35), which can induce perturbation in the immune response. In the other studies, such information was not available.

## Relationship between p53 Antibodies, p53 Mutations, and p53 Accumulation

There is generally a very good correlation between the presence of p53-Abs and p53 accumulation and/or mutation in the tumor. Winter *et al.* (38) have shown that only missense mutations can lead to a p53 humoral response. As shown in Table 1, this finding was confirmed by numerous studies.

Only a subset of patients with p53 mutations will have p53 antibodies in their tumor. As shown in Fig. 1, about 20–40% of patients with a p53 mutation will have p53-Abs in their sera. These results are not due to a lack of sensitivity of the current methods of detection but to a real absence of p53-Abs. It had been suggested that only p53 mutations that are localized in exons 5 and 6 with an altered protein conformation and that bind to hsp 70 are associated with p53-Abs (39, 40). These analyses, performed on a small number of patients, were not confirmed in larger series of patients. Indeed, a compilation of all of the serological analyses performed in conjunction with molecular analyses indicates that the repartition of p53 mutations in patients with p53-Abs is similar to that in patients without antibodies (Fig. 2). Several studies have shown that, despite similar types of cancer, identical p53 mutation, and p53 accumulation, some patients could be either positive or negative for p53-Abs (41–44). These observations demonstrate that other factors contribute to this humoral response. Examination of p53-Abs during follow-up of patients during therapy indicates that the level of p53-Abs can vary depending on the tumor burden (see below). Follow-up of patients who were devoid of p53-Abs at the time of diagnosis failed to detect any *de novo* antibody production after therapy failure or relapse despite p53 accumulation in the tumor and a long follow-up. This suggests that the capacity to elicit this humoral response is linked to the biological background of the patients. It is possible that, for an identical mutation, the immune response is dependent on the specific combination of MHC class I and II molecules expressed by each individual.

There may be several explanations for the presence of p53-Abs in tumors with a wild-type p53. Technical failure cannot be neglected and is difficult to assess, nor can we exclude the possibility that the tumor is composed of heterogeneous tissue and that the fragment analyzed does not bear p53 alteration. We should also keep in mind that serological analysis is a global assay that does not depend on sampling. An undetectable metastasis with p53 alteration might be associated with a p53-negative primary tumor. As discussed below, the mechanism that leads to the formation of these antibodies is poorly understood. Accumulation may be an important component in the development of this immune response, but we cannot exclude the fact that modified processing of the mutated protein can also lead to such a response.

## Specificity of p53 Antibodies

As stated above, mutant p53 accumulation is an important component of this humoral response. Thus, it is possible that such antibodies are specific to mutant p53 or at least to a mutant p53 conformation. Several works have shown that these antibodies recognize both wild-type and mutant p53 (38, 45, 46). Using a set of overlapping synthetic peptides corresponding to human p53, precise mapping of the epitopes of p53 protein recognized by p53-Abs was performed (47). This method is very powerful and very rapid for the analysis of a large number of sample. Nevertheless, we should bear in mind that it only works with antibodies that recognize small linear epitopes. Analysis of more than 200 sera from various types of cancer unambiguously demonstrated that these antibodies recognize immunodominant epitopes localized in the $NH_2$ terminus and, to a lesser extent, in the COOH terminus of human p53 (26, 47, 48). Only a few antibodies recognize the central region of the p53 protein that harbors the mutations. We know that the identification of such epitopes is not due to a technical bias based on the use of short synthetic peptides because immunoprecipitation and immunoblot analysis of truncated p53 have led to the same observation (45, 48).

Such a finding is totally in accordance with the work performed in mice on the production of p53 monoclonal antibodies. Immunization of mice with murine, xenopus, or human wild-type p53 led to the production of monoclonal antibodies directed to linear epitopes localized in the $NH_2$ and COOH termini of p53 (49–52). The analysis of mouse sera immunized with these proteins indicates that it is due to a specific immune response of the mouse toward this region of the protein and not to a bias in the selection of the hybridoma (49). Monoclonal antibodies specific for the central region of the protein

[4] M. Tavassoli and T. Soussi. Expression of p53 in oral squamous cell carcinoma is associated with the presence of p53 autoantibodies in sera and saliva, manuscript in preparation.

Table 1 *Frequency of p53-Abs in various types of cancer: a survey of the literature, 1979–1999*

| Methodology | Positive | Total | Comment | Reference |
|---|---|---|---|---|
| Control studies[a] | | | | |
| IP[b] | 0 | 164 | Healthy women | (8) |
| IP | 1 | 88 | Children with nonmalignant disease | (9) |
| Wb | 0 | 51 | Healthy control | (38) |
| Wb | 0 | 67 | Acute and chronic liver diseases | (110) |
| ELISA[c] | 2 | 206 | Healthy control | (24) |
| Wb | 1 | 73 | Healthy women | (111) |
| ELISA | 1 | 76 | Healthy control | (65) |
| ELISA | 0 | 49 | Nonmalignant lung disease | (112) |
| ELISA | 0 | 17 | Healthy control | (113) |
| ELISA | 0 | 15 | Healthy control | (69) |
| Wb | 0 | 48 | Healthy control | (70) |
| ELISA + Wb | 3 | 330 | Healthy control | (66) |
| ELISA | 0 | 40 | Healthy control | (84) |
| ELISA | 2 | 36 | Nonmalignant liver disease | (84) |
| ELISA | 4 | 63 | Healthy control | (92) |
| ELISA | 0 | 41 | Healthy control ($n = 29$)<br>Nonmalignant lung disease ($n = 12$) | (114) |
| ELISA + Wb + IP | 0 | 30 | Healthy control | (91) |
| ELISA + Wb + IP | 1 | 19 | Nonmalignant esophageal disease | (91) |
| Wb | 0 | 24 | Healthy control ($n = 10$)<br>Nonmalignant lung disease ($n = 14$) | (76) |
| ELISA | 0 | 195 | Healthy control | (115) |
| ELISA | 0 | 20 | Healthy control | (93) |
| ELISA | 0 | 9 | Gastric polyps | (71) |
| ELISA | 0 | 40 | Nonmalignant liver disease | (116) |
| ELISA | 0 | 10 | Healthy control | (85) |
| ELISA | 1 | 69 | Healthy women | (117) |
| ELISA | 0 | 140 | Chronic hepatitis C | (118) |
| ELISA | 0 | 41 | Healthy control | (119) |
| ELISA | 2 | 50 | Healthy control | (120) |
| Wb | 0 | 10 | Healthy control | (121) |
| ELISA + Wb | 0 | 11 | Healthy control ($n = 6$) + HBV-infected patients ($n = 5$) | (122) |
| ELISA + Wb | 0 | 60 | Healthy control | (123) |
| ELISA + Wb + IP | 0 | 50 | Healthy control | (124) |
| ELISA + IP | 0 | 51 | Healthy women | (26) |
| ELISA | 0 | 24 | Nonmalignant digestive disease | (82) |
| ELISA | 0 | 9 | Healthy control | (125) |
| ELISA | 0 | 41 | Healthy control | (81) |
| ELISA | 0 | 17 | Chronic inflammatory diseases of head and neck | (81) |
| Wb | 0 | 50 | Healthy control | (79) |
| ELISA | 17[d] | 70 | Nonmalignant oral diseases | (126) |
| Breast | | | | |
| IP | 14 | 155 | Association with high grade | (8) |
| IP | 14 | 122 | Association with high grade | (127) |
| Wb | 7 | 60 | Association with specific p53 mutation binding to hsp70 | (39) |
| IP + Wb | 15 | 100 | Association with high grade and with tumor negative for steroid hormone receptor | (45) |
| ELISA | 3 | 105 | | (128) |
| ELISA | 12 | 93 | | (47) |
| ELISA | 10 | 290 | Association with tumor negative for steroid hormone receptor | (24) |
| Wb | 45 | 176 | Association with p53 accumulation in the tumor | (111) |
| ELISA | 48 | 182 | Association with high histological grade and p53 accumulation in the tumor | (65) |
| IF | 21 | 50 | Association with good survival | (68) |
| ELISA | 42 | 353 | Association with short survival | (64) |
| ELISA + Wb | 15 | 165 | Association with short survival | (66) |
| ELISA | 2 | 12 | | (120) |
| ELISA | 9 | 61 | Association with tumor size | (129) |
| ELISA | 39 | 82 | No clinical correlation detected | (67) |
| Lung | | | | |
| Wb | 6 | 46 | Correlation with missense mutation and p53 accumulation | (38) |
| ELISA | 10 | 42 | | (47) |
| ELISA | 6 | 73 | | (24) |
| ELISA | 10 | 42 | | (48) |
| ELISA | 16 | 136 | | (112) |
| ELISA + IP + Wb | 9 | 107 | Association with p53 accumulation in the tumor | (130) |
| ELISA | 18 | 67 | NSCLC; association with better survival after radiotherapy | (131) |
| ELISA | 13 | 62 | NSCLC; association with p53 accumulation in the tumor | (114) |
| ELISA + Wb | 38 | 188 | NSCLC; association with advanced stage and p53 accumulation in the tumor; no association with survival | (132) |
| Wb | 9 | 111 | NSCLC; association with poor survival | (76) |
| Wb | 1 | 14 | SCLC | (76) |
| ELISA | 19 | 84 | NSCLC; association with poor survival, especially for SCC | (75) |
| ELISA | 17 | 140 | NSCLC; association with p53 accumulation in the tumor and short survival for SCC only | (77) |
| Wb | 27 | 170 | SCLC; no association with clinical parameters | (79) |
| ELISA | 54 | 231 | SCLC | [e] |
| ELISA | 20 | 97 | SCLC; association with poor prognosis for limited-stage SCLC patients | (78) |
| Colon cancer | | | | |
| IP | 2 | 16 | | (127) |
| ELISA | 13 | 82 | | (24) |
| ELISA | 63 | 249 | Association with poor differentiation and short survival | (73) |
| ELISA | 59 | 184 | Association with p53 accumulation and short survival | (72) |
| ELISA | 42 | 235 | | (115) |
| ELISA | 10 | 41 | Association with p53 accumulation in the tumor | (85) |

Table 1 *Continued*

| Methodology | Positive | Total | Comment | Reference |
|---|---|---|---|---|
| ELISA | 10 | 42 | | (120) |
| Wb | 32 | 47 | | (121) |
| IP + Wb + ELISA | 9 | 65 | | (124) |
| ELISA | 14 | 54 | Association with p53 accumulation in the tumor | (82) |
| ELISA | 53 | 229 | No clinical correlation detected | (74) |
| Lymphoma | | | | |
| IP | 14 | 119 | High prevalence in Burkitt[1] lymphoma and T-cell lymphoma | (9) |
| ELISA | 3 | 115 | | (24) |
| ELISA | 2 | 14 | From patients with Sjögren syndrome | (133) |
| Gastric cancer | | | | |
| ELISA | 23 | 120 | Association with p53 accumulation in the tumor and short survival | (69) |
| Wb | 61 | 501 | Association with large tumor, poor differentiation and short survival | (70) |
| ELISA | 8 | 25 | Association with poor differentiation, high stage, and poor survival | (71) |
| ELISA | 13 | 81 | No clinical correlation detected | (134) |
| Oral cancer | | | | |
| Wb | 7 | 14 | | (135) |
| ELISA | 24 | 70 | Leukoplatia | (92) |
| ELISA | 15 | 50 | Association with poor differentiation and p53 accumulation in the tumor | (92) |
| ELISA | 32 | 74 | No clinical correlation detected | (136) |
| ELISA | 18 | 82 | | (137) |
| ELISA | 23 | 117 | Association with p53 accumulation in the tumor | (119) |
| ELISA | 15 | 80 | Association with p53 accumulation in the tumor and short survival | (80) |
| ELISA | 9 | 39 | | (125) |
| ELISA | 39 | 143 | Association with therapy failure | (81) |
| Wb | 7 | 30 | Associated with tumors with p53 complexed to Hsp70 | (40) |
| ELISA | 37 | 97 | | (126) |
| ELISA | 11 | 30 | Patient with recurrence | (126) |
| ELISA | 47 | 177 | Association with tumor size | (138) |
| ELISA | 32 | 73 | Association with p53 accumulation in the tumor | (139) |
| Esophageal cancer | | | | |
| ELISA + Wb + IP | 11 | 33 | Esophageal cancer, association with p53 accumulation in the tumor | (91) |
| ELISA + Wb + IP | 3 | 36 | Barrett's metaplasia | (91) |
| ELISA | 6 | 20 | | (93) |
| IP + Wb + ELISA | 16 | 65 | Association with p53 accumulation in the tumor and p53 mutation in the core domain | (44) |
| ELISA | 16 | 63 | Association with p53 mutation in the tumor; no relationship with clinical parameter | (140) |
| ELISA | 33 | 57 | Not done | (141) |
| Liver cancer (HCC) | | | | |
| Wb | 20 | 80 | European patients<br>Advanced disease | (110) |
| ELISA | 9 | 130 | Association with p53 accumulation in the tumor and better survival | (84) |
| ELISA | 5 | 39 | | (142) |
| ELISA | 28 | 86 | Association with poor overall survival | (116) |
| ELISA | 3 | 7 | | (118) |
| ELISA + Wb | 14 | 38 | Association with p53 accumulation in the tumor | (122) |
| Ovarian cancer | | | | |
| ELISA | 10 | 72 | | (128) |
| ELISA | 11 | 86 | | (24) |
| ELISA | 12 | 33 | Association with poor survival | (113) |
| ELISA | 3 | 17 | Benign ovarian tumors | (113) |
| ELISA | 15 | 40 | | (86) |
| ELISA | 18 | 86 | No clinical correlation detected | (143) |
| ELISA | 8 | 30 | | (28) |
| ELISA | 11 | 38 | Association with poor histological differentiation and p53 accumulation in the tumor | (117) |
| ELISA | 10 | 30 | | (144) |
| ELISA | 41 | 174 | Association with poor histological differentiation and short survival | (145) |
| ELISA + IP | 4 | 46 | | (26) |
| Pancreatic cancer | | | | |
| ELISA | 2 | 46 | | (128) |
| ELISA | 14 | 73 | | (47) |
| ELISA | 2 | 46 | | (24) |
| ELISA + Wb | 5 | 78 | | (146) |
| ELISA | 8 | 29 | | (147) |
| ELISA + Wb | 5 | 96 | Chronic pancreatitis | (123) |
| ELISA + Wb | 23 | 145 | Association with high grading (grade III), short survival, and p53 accumulation in the tumor | (123) |
| ELISA | 6 | 33 | 1/19 acute pancreatitis<br>4/33 chronic pancreatitis | (148) |
| Prostate cancer | | | | |
| ELISA | 2 | 83 | | (128) |
| ELISA | 2 | 65 | | (24) |
| Thyroid cancer | | | | |
| ELISA | 4 | 108 | | (47) |
| Bladder cancer | | | | |
| ELISA | 8 | 29 | | (47) |
| Leukemia | | | | |
| ELISA | 4 | 88 | | (47) |
| ELISA | 1 | 107 | | (24) |
| Wb | 1 | 50 | HTLV-1 asymptomatic carrier | (149) |
| Wb | 2 | 50 | ATL | (149) |
| Wb | 3 | 50 | HAM/TSP | (149) |
| ELISA | 3 | 83 | Association with p53 mutation | (150) |

Table 1 *Continued*

| Methodology | Positive | Total | Comment | Reference |
|---|---|---|---|---|
| Testicular cancer | | | | |
| ELISA | 0 | 144 | | (24) |
| Hepatoma | | | | |
| ELISA | 1 | 150 | | (24) |
| Melanoma | | | | |
| ELISA | 0 | 58 | | (24) |
| Multiple myeloma | | | | |
| ELISA | 5 | 165 | | (24) |
| ELISA | 18 | 119 | | (151) |
| ELISA | 0 | 80 | | (150) |
| Glioma | | | | |
| ELISA | 2 | 12 | | (36) |
| ELISA | 0 | 70 | | (35) |
| ELISA | 4 | 107 | | [b] |
| Endometrial cancer | | | | |
| ELISA | 2 | 10 | | (144) |

[a] We have included all individuals both healthy and with nonmalignant disease.
[b] IP, immunoprecipitation; WB, Western blot; HTLV-1, human T-cell lymphotrophic virus, type 1; ATL, adult T-cell leukemia; HAM/TSP, HTLV-associated myelopathy/tropical spastic paraparesis; IF, immunofluorescence; HCC, hepatocellular carcinoma.
[c] ELISA, 20% of the assays were homemade, whereas 80% were commercial (2 main distributors).
[d] This single study represented 50% of positive cases in healthy controls.
[e] P. Murray, T. Soussi, and M. Tavassoli. Serum p53 antibodies: predictors of survival in small-cell lung cancer?, manuscript in preparation.
[f] Unpublished observations.

were obtained only after a special immunization or selection procedure (53, 54).

Taken together (*a*) the presence of immunodominant epitopes outside the hot spot region of the p53 mutation; (*b*) the correlation between p53 accumulation (and *p53* gene mutation) in tumor cells and p53-Abs responses; (*c*) the similarity of humoral responses in patients independent of the cancer type; and (*d*) the similarity of antigenic site profiles in patients and hyperimmunized animals—all suggest that p53 accumulation is the major component of the humoral response in patients with cancer. This accumulation could lead to a self-immunization process culminating in the appearance of p53-Abs. As stated above, the level of p53 proteins in a normal organism is very low, which suggests very weak (if any) tolerance to endogenous p53. Isotyping of p53-Abs has shown that they correspond mainly to IgG1 and IgG2 subclasses, although some patients exhibit a predominant IgA response (25). Some patients also had IgM, although none had p53 IgM as the only isotype. No IgG3 or IgG4 was detected. Again, this result strengthens the hypothesis of an active humoral response to p53.

Table 2 *p53-Ab frequency in various types of cancer: statistical evaluation*

| Status | Frequency of p53-Abs | $P$[a] |
|---|---|---|
| Healthy | 35/2404[b] | |
| Esophageal cancer | 85/274 | $<10^{-4}$ |
| Oral cancer | 309/1062 | $<10^{-4}$ |
| Bladder cancer | 8/29 | $<10^{-4}$ |
| Colon cancer | 307/1244 | $<10^{-4}$ |
| HCC[c] | 82/387 | $<10^{-4}$ |
| Ovarian cancer | 140/635 | $<10^{-4}$ |
| Lung cancer | 219/1282 | $<10^{-4}$ |
| Breast cancer | 296/2006 | $<10^{-4}$ |
| Gastric cancer | 105/727 | $<10^{-4}$ |
| Pancreatic cancer | 60/650 | $<10^{-4}$ |
| Multiple myeloma | 23/364 | $<10^{-4}$ |
| Lymphoma | 19/248 | $<10^{-4}$ |
| Leukemia | 14/428 | 0.005 |
| Glioma | 6/144 | 0.03 |
| Prostate cancer | 4/148 | NS |
| Testicular cancer | 0/144 | NA |
| Melanoma | 0/58 | NA |
| Hepatoma | 0/150 | NA |
| Total cancers | 1600/9489 | $<10^{-4}$ |

[a] Correlation between cancer patients and healthy individuals were tested by the $\chi^2$ test. The levels of significance were set at $P < 0.05$.
[b] As shown in Table 1, one study contributed to 17 (50%) positives in healthy controls. All of the statistical analyses including this study were performed emphasizing the strength of these trends. If this study is omitted (18 positive controls), prostate carcinoma reaches significance with a $P$ of 0.015.
[c] HCC, hepatocellular carcinoma; NS, not significant; NA, not applicable.

It is not clear whether p53 mutation is really required for the production of p53-Abs or whether the sole accumulation of p53 protein can lead to this humoral response. This question is difficult to answer because there is no normal situation of wild-type p53 accumulation in humans that could be used to test this hypothesis. On the other hand, such a situation occurs in animals. p53-Abs have been discovered in the sera of animals that bear tumors induced by SV40 (1, 5). In such tumors, wild-type p53 is stabilized through its interaction with SV40 large T antigen. These observations indicate that p53 accumulation alone is the main component of this autoimmunization.

The mechanism by which p53 is presented to the immune system is unknown. It is possible that it is released after cell necrosis, but thus far, p53 has not been found reproducibly in human sera (see below; 55, 56). Either the p53 protein is very rapidly eliminated from sera, or else other mechanisms of p53 presentation are involved.

X-ray crystallography of human p53 was an important step in the understanding of the structure of this protein. The central region (amino acids 102–292) was crystallized in the form of a protein-DNA complex (57). This core region has been shown to include the following motifs: (*a*) two antiparallel $\beta$ sheets composed of four and five $\beta$-strands, respectively. These two sheets form a kind of compact sandwich that holds the other elements; (*b*) a LSH containing three $\beta$-strands, an $\alpha$-helix, and the L1 loop; (*c*) an L2 loop containing a small helix; and (*d*) an L3 loop composed mainly of turns. It is quite remarkable to note the striking correspondence between these various structural elements and the four evolutionarily conserved blocks (II to V). The LSH motif and the L3 helix are involved in direct DNA interaction (LSH with the major groove and L3 with the minor groove). The L2 loop is presumed to provide stabilization by associating with the L3 loop. These two loops are held together by a zinc atom tetracoordinated to Cys176 and His179 on the L2 loop and to Cys278 and Cys242 on the L3 loop.

Furthermore, the conformational changes in p53 were dissected by a new battery of monoclonal antibodies directed against the central region of the protein (53). None of these antibodies was able to recognize native, wild-type p53. On the other hand, regardless of the location of their epitopes, they were all able to recognize p53 mutants that had undergone conformational changes (53). This result indicates

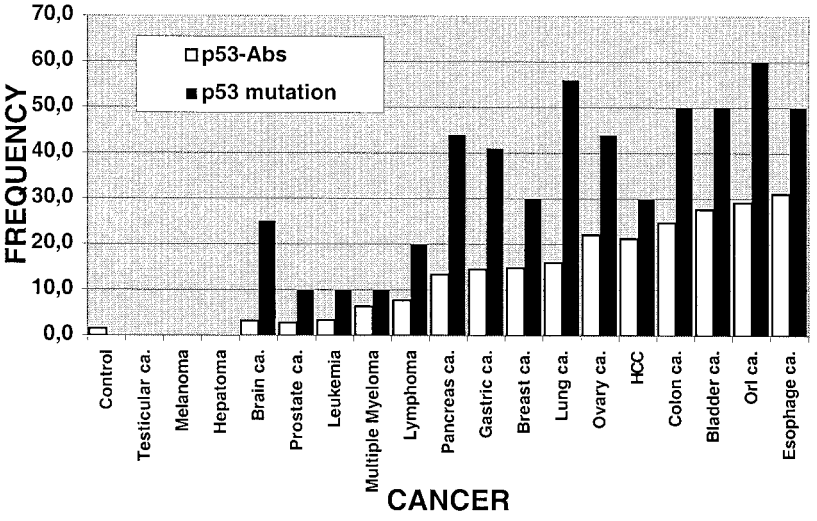


Fig. 1. Relationship between the frequency of p53 mutation and p53 antibodies in various types of cancer. The frequency of p53 mutations is taken from the literature, and the frequency of p53-Abs is taken from Table 1.

that the central region of wild-type p53 protein has a very compact structure that is held in place by the two antiparallel B sheets. Such conformation and the observation that this region is highly hydrophobic explain its poor immunogenicity.

A recent study (58) indicates that critical residues of epitopes recognized by several anti-p53 monoclonal antibodies correspond to key residues of p53 involved in interaction of the mdm2 protein with the $NH_2$ terminus of the p53 protein. This can explain why such a region is at the surface of the molecule. It should also be mentioned that the p53 protein, whatever its phylogenetic origin, is an incredibly potent antigen. Immunization of mice with p53 requires only a small amount of protein, and adjuvant is not necessary to obtain a high titer of antibodies.[5]

Recently, several p53 homologues have been identified (59–62). Although, the conservation of the sequence is only partial, it is conceivable that p53-Abs could either cross-react with such proteins or were produced toward one of the homologues. Extensive assays of sera with or without p53-Abs indicate that most sera are specific for p53. Nevertheless, specific antibodies toward p73 have been detected.[6]

### p53 Antibodies and Clinical Parameters

Numerous studies have attempted to evaluate the clinical value of p53-Abs (Table 1). As for p53 mutation and p53 immunohistochemical analysis, these studies have reported contradictory results. Before examining them, one should bear in mind one question that has not been assessed thus far, *i.e.*, the role of such antibodies in the neoplastic process. This question has never been thoroughly discussed or experimentally tested. During our lifetime, it is quite possible that we experience several unknown precancers that arise after a genetic variation in an oncogene or a tumor suppressor gene. Such preneoplastic cancers could be quickly eliminated either because the mechanisms that control cellular proliferation have been able to overcome these tumoral cells or because they have been eliminated from the organism through various surveillance mechanisms including the immune system. It is plausible that early p53 accumulation, such as that seen in lung or oral cancer, can lead to the production of a humoral and cellular response that participates in the elimination of the tumor. Such a hypothesis is supported by the work of Roth *et al.* (63). Immunization with canarypox virus recombinants that expressed human or murine p53 protected BALB/c mice from a challenge with a highly tumorigenic mouse fibroblast tumor cell line that express high levels of mutant p53. This tumor protection was equally effective regardless of whether wild-type or mutant p53 was used for the immunization, which indicated that the immunological response was not dependent on any particular p53 mutation and that immunization with this live virus vaccine works effectively against mutant p53 protein expressed in a tumor cell (63). Although there is no formal proof that a natural response toward p53 can protect from precancerous tumor, it is not possible to exclude such a hypothesis. However, it is quite possible that such an immune response is totally neutral toward the organism and is simply the consequence of self-immunization toward a self-protein. In that case, any clinical correlation with p53-Abs would be due to the presence of p53 inactivation via mutation.

In breast cancer, several studies indicate that p53-Abs are found in patients with tumors that have high grades and/or that are negative for steroid hormone receptors (8, 24, 45, 64, 65), two clinical parameters already known to be associated with p53 mutations and bad prognosis. Two studies, on 353 and 165 patients, found an association between p53-Abs and short survival (64, 66), whereas one study (82 patients) did not find any association (67), and another study (50 patients) found an association with good survival (68). In gastric cancer, three of four studies found an association between p53-Abs and poorly differentiated tumors and short survival (69–71). In colon cancer, two of three studies also found an association between p53-Abs and short survival (72–74). In lung cancer, as for p53 mutations, controversies exist concerning the clinical value of p53-Abs (Table 1). In NSCLC,

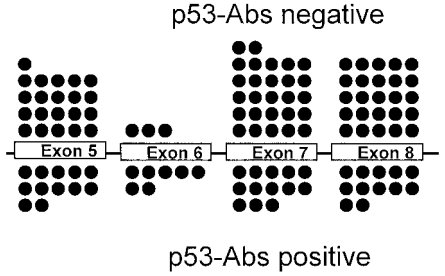


Fig. 2. Distribution of p53 mutations and p53-Abs in patients in whom both serological and molecular analyses were performed.

[5] Y. Legros and T. Soussi, unpublished observation; D. Lane, personal communication.

[6] O. Tominaga, K. Unzal, and T. Soussi. p73 antibodies are found in the sera of patients with various types of cancer, manuscript in preparation.

Table 3 *Frequency of p53-Abs in high-risk individuals: a survey of the literature 1979–1999*

| Methodology | Selected Individuals/ Possible Carcinogen | Clinical Status at the Time of p53-Ab Detection | Description | p53 Alteration | Type of Study (Reference) |
|---|---|---|---|---|---|
| ELISA, IP, WB | Heavy smokers/tobacco residues | No detectable malignant or premalignant lesion | Two individuals with p53-Abs | | Prospective with follow-up (48, 88) |
| | | | Patient 90: no follow-up; died 8 months later from lung cancer | ND[a] | |
| | | | Patient 37: effective follow-up; developed a lung cancer 2 years later; tumor was detected before clinical manifestation of the cancer; patient still alive after therapy | IHC+[b] | |
| ELISA, IP, WB | Factory workers in industry/vinyl chloride | | Patient 24 with ASL: p53-Abs 10 yr before diagnosis | ND | Retrospective study (42) |
| | | | Patient 23 with ASL: p53-Abs 3 mo before diagnosis | ND | |
| | | ND | Patient 19 with Raynaud's syndrome: p53-Abs 4 yr before diagnosis | ND | |
| | | | Worker 22 without diagnosed cancer: p53-Abs 4 yr before assay | ND | |
| | | | Worker 21 without diagnosed cancer: p53-Abs 6 yr before assay | ND | |
| ELISA, IP, WB | Heavy smokers with COPD/tobacco residue | | Patient 12 with lung cancer; p53-Abs 7 mo before diagnosis | ND | Retrospective study (90) |
| | | | Patient 10 with lung cancer; p53-Abs 6 mo before diagnosis | Mutant p53 | |
| | | ND | Patient 8 with breast cancer; p53-Abs 7 mo before diagnosis | IHC+ | |
| | | | Patient 11 with prostate cancer; p53-Abs 11 mo before diagnosis | IHC+ | |
| ELISA | Betel nut chewer/tobacco and copper in betel nut | Leukoplakia | Exposed individuals: 4/63[c] | | Prospective study (92) |
| | | | Premalignant (leukoplakia): 15/50 | ND | |
| | | | Cancer: 24/70 | | |
| | | | No follow-up of these patients | | |
| ELISA, IP, WB | Barrett's metaplasia | Barrett's metaplasia | Patient 88 with Barrett's esophagus; p53-Abs 1 mo before detection of ADC | ND | Retrospective study (91) |
| | | | Patient 2 with ADC; p53-Abs 1 mo before diagnosis | Mutant p53 | |

[a] ND, not done; ASL, angiosarcoma of the liver; IHC, immunohistochemistry.
[b] IHC +, p53 accumulation in the tumor.
[c] These four normal subjects were heavy consumers of tobacco and betel quid.

p53-Abs seem to be associated with poor survival, especially in squamous cell carcinoma (75–77), whereas in SCLC, the studies are very divergent (78, 79). In oral cancer, two studies have also demonstrated an association between p53-Abs and short survival (80, 81).

Taken together, in all of the these studies, there is a trend toward an association between p53-Abs and tumors with poor differentiation, a feature already observed with p53 mutations. The value of p53-Abs in terms of survival is promising, but additional studies are necessary before this can be clearly established.

## p53 and Follow-Up of Patients during Therapy

Because p53 accumulation is the main trigger of this humoral response, it was of interest to examine the behavior of these p53-Abs during therapy to see whether there was a relationship between tumor disappearance and a decrease in p53-Abs. Several studies have addressed this question in various types of cancer (24, 28, 82–87). Such studies can only be performed using a quantitative assay, but this has not been taken into account in many reports. Using immunoprecipitation and two different ELISA formats, Zalcman *et al.* (83) showed that there is a good correlation between the specific evolution of the p53-Abs titer and the response to therapy in patients with lung cancer. A similar situation was described in colorectal (82) and ovarian cancer (24). In other studies, clinical data were not available. In several patients, the disappearance of p53-Abs was very rapid, nearly as rapid as the half-life of human IgG (83, 88). Several arguments demonstrate the specificity of p53-Abs variation during therapy: (*a*) there is no variation in total serum immunoglobulins; (*b*) there is no variation in the amount of antibodies directed toward other antigens; and (*c*) a decrease of p53-Abs can occur in patients who have been treated by surgery without any chemo- or radiotherapy. All of these observations indicate that constant stimulation of the immune system is necessary to maintain a high level of p53-Abs. Removing the tumor would prevent such stimulation.

In breast cancer, it is possible to detect the reappearance of p53-Abs 2 years after initial therapy. This increase in p53-Abs has been detected 3 months before the detection of a relapse (5). Thus, in such tumor types, p53-Abs could be a useful tool for controlling the response to therapy and for monitoring certain early relapses before they are clinically detectable. As indicated above, several studies have demonstrated that sera that score negative at the time of diagnosis never turn positive during follow-up (82, 83, 86).

## p53 Antibodies and Populations at High Risk of Cancer

As demonstrated in the previous section, p53 accumulation is the major component in the appearance of these p53-Abs. These antibodies are usually IgG indicating a secondary response after a prolonged immunization before the diagnosis of the disease (25). All of the studies described up until now used sera taken at the time of diagnosis before any treatment. Thus, it is reasonable to presume that such p53-Abs could be used as an early indicator of p53 mutations in tumors in which such alterations occur early during tumoral progression (Table 3). One good model for testing this hypothesis is that of

Table 4 *Quantification of p53 in human sera*

| Assay | Patients | Amount of p53 | Comment | References |
|---|---|---|---|---|
| ELISA[a]/PAb240 | 71 controls | 0.37 ± 0.44 ng/ml | No statistical difference between controls and cancers | (97) |
| | 40 colon cancers | 0.31 ± 0.46 ng/ml | | |
| | 12 polyps | 0.84 ± 0.44 ng/ml | | |
| ELISA[a]/PAb240 | 58 controls | 0.31 ± 0.41 ng/ml | No statistical difference between controls and cancers | (95) |
| | 23 lung cancers | 0.55 ± 1.16 ng/ml | | |
| ELISA | 100 controls | <0.04 ng/ml | No p53 protein | (55) |
| | 13 nonmalignant lung diseases | <0.04 ng/ml | No p53 protein | |
| | 114 patients with lung cancers | <0.04 ng/ml[b] | No p53 protein | |
| ELISA[a]/PAb240 | 47 controls | 0.55 ng/ml | Statistical difference between controls and cancer, $P < 0.02$ | (96) |
| | 61 "normal" with history of colon cancer | 0.09 ng/ml | | |
| | 54 adenomas | 0.44 ng/ml | | |
| | 22 carcinomas | 0.55 ng/ml | | |
| ELISA[a]/PAb1801 | 10 controls | 0.29 ng/ml | No statistical difference between controls and cancer | (94) |
| | 36 asbestos controls | 0.61 ± 0.33 ng/ml | | |
| | 27 lung cancers | 0.33 ± 0.07 ng/ml | | |
| ELISA[a]/PAb1801 | No controls | | | (93) |
| | 15 esophageal cancer | 0.6–8.6 ng/ml | | |
| ELISA | 800 cancer patients | Negative | | (56) |
| ELISA | 67 lung cancer patients | Negative | | (38) |
| HPLC[c] | 144 controls | 0.22–0.6 mg/ml | Should be taken with caution | (98) |
| | 184 colon cancers | 1.8–5.6 mg/ml | | |

[a] All of these ELISA were performed with the same commercial kit using a specific monoclonal antibody as a capture antibody.
[b] Two sera were shown to be false positive after checking for heterophilic antibodies.
[c] HPLC, high-performance liquid chromatography.

lung cancer and heavy smokers. It is well established that p53 accumulation is an early event in lung cancer and that such cancer is strongly associated with tobacco smoking. Indeed, in 1994, p53-Abs were found in two heavy smoker who were negative for any detectable lung cancer (48). One of the patients could not be followed but died 8 months later from a rapidly growing lung tumor. The second patient, PT37, was placed under surveillance, with regular assay for p53-Abs and thoracic X-rays. Two years later, lung cancer was detected in this patient before any clinical manifestations of the disease (88). The patient showed good response to therapy that paralleled the total disappearance of p53-Abs (83, 88). Since 1996, this patient has been tumor-free, and a recent check-up indicated that neither the tumor nor p53-Abs had reappeared. To our knowledge, this is the only prospective study that addressed the importance of p53-Abs in individuals at high risk for cancer, and that used such assays for clinical management of the patient. Since that work, several studies have demonstrated that p53-Abs can be found in the sera of high-risk individuals.

Angiosarcoma of the liver is an extremely rare cancer in humans; it is found in individuals, including workers in several types of industries, who have been exposed to several carcinogens such as vinyl chloride. p53-Abs were detected in the sera of individuals several years before the diagnosis of the tumors (Table 3; Ref. 42). This work is of importance because it is known that p53 mutations are frequent in individuals exposed to various carcinogens, and such mutations usually occur early in the transforming process (89). Therefore, this assay could be useful for early identification of cancer in individuals occupationally exposed to carcinogens. Similarly, p53-Abs have been detected in the sera of patients with chronic obstructive pulmonary disease and in heavy smokers (Table 3; Ref. 90).

There exist certain clinical situations in which nonmalignant lesions can predate their progression toward cancer. This is the case in Barrett's esophagus. The histopathological sequence for (Barrett's) metaplasia, which develops—as a consequence of chronic reflux—to dysplasia and then to carcinoma is well established for these tumors. In Barrett's esophagus, a variety of molecular changes have been characterized and correlated with tumor initiation and progression. Mutations and accumulation of p53 are found mainly in the transition from low- to high-grade dysplasia and are associated with an increased risk of cancer. The finding of p53-Abs in patients with Barrett's esophagus may be promising if confirmed in a larger population because it may predate clinical diagnosis of esophageal ADC (91). A similar situation occurs in individuals with premalignant oral lesions (leukoplakia) due to tobacco or betel nut chewing. Such individuals are at high risk of developing oral cancer (5–10%). p53-Abs have been found at high frequency in patients with premalignant and malignant lesions, which suggests that such antibodies could be used for early detection of cancer (Table 3; Ref.92). Unfortunately, no follow-up has been performed on these patients. Due to the high frequency of this type of cancer in countries such as India or Pakistan, this kind of diagnosis could be of importance. The recent discovery that p53-Abs can be found in saliva indicates that easy screening could be organized to verify the value of these antibodies (27).

## p53 Protein in Sera

This question continues to be a subject of debate with highly divergent results (Table 4; Refs. 55, 56, 93–98). It should be pointed out that most assays use a commercial ELISA kit that was developed for the detection of p53 protein in cell or tumor extracts but that has not been fully verified on serum samples. Sera have always been tested undiluted, which can lead to high background and false-positives. Indeed, in carefully controlled experiments, Levesque *et al.* (55) demonstrated that some false-positive sera were caused either by the presence of human antibodies with broad antispecies specificities that can cross-react with some antibodies used in the assay or by other nonspecific reactants that interfere with the assay.

Due to the lack of reliability of the various assays used, serum p53 protein should not be considered as valid as long as the protein has not been formally identified in sera using a reliable assay.

## Antibodies toward Other Oncogenes and Tumor Suppressor Genes

Only a few published studies have addressed this question. Antibodies to ras (99), c-myb (100), L-myc (101), c-myc (102), mdm2 (103) or HER2-neu (104, 105) have been detected in sera of patients with various types of cancers.

The presence of antibodies to HER-2/neu were detected in 12

(11%) of 107 breast cancer patients *versus* none (0%) of 200 normal controls (104). The presence of antibodies to HER-2/neu was also correlated with the accumulation of HER-2/neu protein in the patient's primary tumor. Such accumulation of HER-2/neu protein is well known to be associated with a poor prognosis. No information concerning the association of such antibodies with clinical data has been published thus far.

The paucity of these data compared with p53 suggests that p53 immunogenicity is a rather specific situation related to the striking immunogenicity of the p53 protein.

This field is advancing, especially with the use of new methodology, such as SEREX, that enables identification of specific antibodies associated with gene overexpression in tumors (106, 107). Such methodology can shed light on new tumor antigens, which could lead to the discovery of new cancer genes, repeating the history of the p53 with the identification of the protein prior to cloning the gene.

### p53 Antibodies: Future Directions

Although several authors have questioned the specificity of p53-Abs, a review of the literature (Tables 1 and 2) clearly demonstrates the specificity of this serological analysis because such antibodies are truly rare in the normal population. It is possible to estimate that the specificity of this assay attains 95%. Such specificity is supported by our knowledge of p53 that accumulates specifically in the nucleus of tumor cells after gene mutation. Among the various TAAs that have been analyzed over the years, the production of p53-Abs is surely the best studied and most clearly explains humoral response. One of the disadvantages of this assay is its lack of sensitivity inasmuch as only 20–40% of patients with p53 mutations will develop p53-Abs. This lack of sensitivity totally precludes the use of the assay to evaluate p53 alteration in human tumor. Nevertheless, if we estimate that there are 8 million patients with various types of cancer throughout the world, and 50% of them have a mutation in their *p53* gene, then we can deduce that about 1 million of these patients have p53-Abs.

There exist several situations in which p53-Abs could have clinical utility. The first is in the monitoring of sera during therapy. Only prospective studies on various types of cancer in which relapses occur several months or years after treatment will enable us to validate this assay. The use of standardized assays that have been validated for quantitative analysis should help in such studies.

The second situation concerns p53-Abs in high-risk individuals. One of the challenges of the next millennium is the early detection of tumors using highly sensitive assays with gene probes specific for tumor genetic alterations (108). Such approaches are still under development and remain costly. I do believe that there is still room for serological assays of tumors markers such as those described in the present review. In developing countries, there is an increased burden of tumors due to carcinogen exposure. This is the result of an increase in cigarette consumption, higher pollution caused by political laxism, uncontrolled industrial development, and the absence of regulation in waste evacuation. It is possible that p53 mutations in cancer related to such exposure are high (109). The use of a low-cost assay for the detection of p53-Abs could be of public health benefit in such countries.

Over the past 10 years, a tremendous amount of work has been performed on *p53*. Such an effort, both in academic and private laboratories has never been made for any other single gene thus far. This work has led to the development of numerous clinical studies including gene therapy protocols, with the aim of achieving more efficient cure of the disease.

Finally, let me dedicate this review to Patient PT37 (88). I think that this patient is the first to have benefited from significant improvement, and, indeed, whose very life has been saved, thanks to the knowledge we have gained about p53, particularly from the TAA studies published 20 years ago (7).

## ACKNOWLEDGMENTS

I am grateful to L. J. Old for reading the manuscript.

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