Primary research

P53 autoantibodies in 1006 patients followed up for breast cancer

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Statement of findings

Serial plasma samples from 1006 patients with breast cancer revealed: (i) no correlation of p53 autoantibody status with disease status at the time of sample collection, or with menopausal status at time of primary diagnosis of breast cancer; (ii) 155 out of 1006 (15%) of patients were positive for p53 autoantibodies, and these patients tended to have a persistent autoantibody status throughout follow up, irrespective of disease behaviour; and (iii) where a negative autoantibody status was found at primary diagnosis of breast cancer, this negative status persisted throughout follow up, irrespective of later disease behaviour. We conclude that screening for p53 autoantibody status is not informative on residual tumour activity nor on therapeutic responsiveness.

Keywords: breast, cancer, monitoring, p53 autoantibodies

Synopsis

Introduction: Dysfunction of the tumour-suppressor protein, p53, may be due to either mutational or epigenetic factors, each of which may lead to accumulation of cytoplasmic p53. Abnormal accumulation of p53 in breast cancer tissue is predictive of poor prognosis [1,2]. Humoral studies [3,4] have shown that cancer patients may develop immunity to abnormally expressed p53, as revealed by p53 autoantibodies in the blood. Again, prognostic correlates have been noted, with presence of circulating p53 autoantibodies at diagnosis of breast cancer being associated with reduced overall survival [5,6] and with poor prognostic factors such as high histological grade and the absence of hormone receptors [5,7,8].

Little is known of the potential value of p53 autoantibody in follow up of cancer. In lung cancer there is evidence that autoantibodies to p53 may provide a useful tool to monitor response to therapy [9,10], whereas serial measurements of autoantibodies to p53 in 40 patients with advanced ovarian cancer were not found to be clinically useful [11]. In breast cancer some 30% of node-negative patients will relapse within 5 years, but there is no current means to predict those who are at risk.

We performed the present study to ask if the presence of autoantibodies to p53 has any association with breast cancer progression.

Materials and methods: A library of plasma samples were collected from all patients attending one general oncology clinic for postoperative follow up of breast cancer. The clinical status of each patient at the time of sampling was summarized. An average of eight plasma samples were cryopreserved for each patient over a period of 15 years.

The enzyme-linked immusorbent assay (ELISA) for p53 autoantibodies was developed in-house, based on the ELISA procedure of Lubin *et al* [3]. Our in-house method is detailed in the full text of this article. In one assay series we compared a commercial ELISA kit for p53 autoantibodies with our in-house ELISA. A total of 20 patients' samples were tested,

ELISA = enzyme-linked immunosorbent assay; OD = optical density; PBS = phosphate-buffered saline.

representing a range of positive and negative readings. Two samples scored as strongly positive with the in-house assay, but only one of these two scored positive with the commercial assay. Having established sensitivity, specificity and reproducibility of the in-house assay, we judged that this was superior to the commercial assay both in terms of sensitivity and of cost (£1 per test compared with £23 per test). The in-house assay was thus used throughout the present study.

Results: Serial plasma samples from 1006 patients with breast cancer revealed the following: (i) no correlation of p53 autoantibody status with disease status at the time of sample collection (Table 1), or with menopausal status at time of primary diagnosis of breast cancer (Table 2); (ii) 155 out of 1006 (15%) of patients were positive for p53 autoantibodies, and these patients tended to have a persistent autoantibody status throughout follow up, irrespective of disease behaviour; and (iii) where a negative autoantibody status was found at primary diagnosis of breast cancer, this negative status persisted throughout follow up, irrespective of later disease behaviour (Table 3).

Discussion: As a working hypothesis, we proposed that levels of autoantibodies to p53 would reflect tumour behaviour. However, we found that the presence or absence of p53 autoantibodies was not predictive of presence or absence of recurrent disease. There was an equivalent incidence of active disease at the time of sampling in both the autoantibody-negative and autoantibody-positive groups, these being 25.2 and 28.7%, respectively. Thus, humoral immune activity against p53 appeared to be relatively restricted to a subgroup of patients in whom, once an autoantibody response had been generated, antibody was likely to persist regardless of tumour behaviour. Conversely, where no detectable p53 autoantibody was present at the time of primary diagnosis, these patients remained similarly negative for antibody, irrespective of subsequent disease activity (Table 3).

In contrast to shed markers that correlate with tumour mass, such as CA15.3 for cancer of the breast, any tumour-related immune response will be subject to complex regulation. Autoantibody responses to p53 will require appropriate primary immunization; initial low-dose antigen exposure may induce immune tolerance and lack of response. Higher antigen doses may activate either antibody-mediated immunity, or cellular immunity.

In breast cancer patients, our results suggest that, once an active humoral response against p53 is established, then this remains active. This persistent humoral reaction may be driven by persistent antigenic stimulation by p53 protein derived from overexpression of p53 at distant metastatic sites; alternatively, irradiated normal tissue may be a source of continued antigenic stimulation, because a long-term side effect of radiation therapy is an increased expression of p53 in normal breast tissue that persists for several years [12]. Since the great majority of our total patient cohort had received radiotherapy, humoral immunity to p53 associated with primary disease might persist, even in those patients who enter remission, due to tumour-independent antigenic stimulation.

Table 1

Incidence of autoantibodies to p53 compared with disease status at last clinic attendance

Disease status	% Anti-p53 positive
Primary remission	14.7% (82/557)
Secondary remission	17.4% (27/155)
Secondary recurrent	19.4% (20/103)
Continuous active	16.5% (24/145)
Total	15.9% (153/96)*

Pearson χ^2: $P = 0.606$. *Information on disease status at last sample time was available for 960 out of 1006 patients

Table 2

Incidence of autoantibodies to p53 compared with menopausal status at diagnosis

Menopausal status	Anti-p53 positive (%)
Premenopausal	15.0% (104/693)
Postmenopausal	16.8% (51/313)
Total	15.4% (155/1006)

Pearson χ^2: $P = 0.788$.

Table 3

Anti-p53 negative patients do not become positive with recurrent disease

Current disease status	Anti-p53 status at diagnosis	Current anti-p53 status
Nonactive disease	38/38 negative	38/38 negative
Active disease	21/22 negative	22/22 negative

Sixty patients who were negative for p53 autoantibodies had also had a plasma sample taken within 30 days of their primary diagnosis of breast cancer. To determine whether antibody status at diagnosis might have been predictive of later disease behaviour (ie independent of the current negative status), we compared two patient subgroups: patients with current nonactive disease and patients with current active disease. With one exception, all patients were antibody negative within 30 days of initial diagnosis. This showed that recurrent disease is highly unlikely to induce a humoral anti-p53 response in those patients who were initially antibody negative.

Loss of p53 function is known to correlate with loss of efficacy of cancer therapy *in vivo* [13,14]. This raised the possibility that autoantibodies to p53 that develop during follow up might indicate those patients whose tumor has become resistant to therapy. However, the present results show that, if no immunity has been generated at the time of primary diagnosis, then later immunity is unlikely to occur. This corresponds to the finding that expression of p53 antigen in biopies of locally advanced breast cancer did not correlate with drug resistance [15,16].

Overall, the present observations show that screening for p53 autoantibody status is not informative on residual tumour activity, or on therapeutic responsiveness. We conclude that the potential value of p53 autoantibody screening in patients with breast cancer is limited to the prognostic information obtained at diagnosis.

Full article

Introduction

The tumour-suppressor protein p53 is a nuclear transcription factor that is autoregulatory in terms of expression, this being low in normal cells. Loss of p53 function is often associated with high accumulation of p53 and its retention in the cytoplasm. Abnormal accumulation of p53 in breast cancer tissue is predictive for poor prognosis [1,2]. Humoral studies [3,4] have shown that cancer patients may develop immunity to abnormally expressed p53, as revealed by p53 autoantibodies in the blood. Again, prognostic correlates have been noted, with presence of circulating p53 autoantibodies at diagnosis of breast cancer being associated with reduced overall survival [5,6] and with poor prognostic factors such as high histological grade and the absence of hormone receptors [5,7,8]. P53 dysfunction may be due to either mutational or epigenetic factors, each of which may lead to accumulation of cytoplasmic p53.

Little is known of the potential value of p53 autoantibody in follow-up of cancer. In lung cancer there is evidence that autoantibodies to p53 may provide a useful tool to monitor response to therapy [9,10], whereas serial measurements of autoantibodies to p53 in 40 patients with advanced ovarian cancer were not found to be clinically useful [11]. In breast cancer, some 30% of node-negative patients will relapse within 5 years, but there is no current means to predict those who are at risk. We have asked if the presence of autoantibodies to p53 have any association with disease progression by testing plasma samples taken from 1006 patients with breast cancer with a median follow-up period of 4 years.

Materials and methods

Patient plasma samples

A library of plasma samples were collected from all patients attending one general oncology clinic for postoperative follow up of breast cancer; samples were taken without any patient selection and irrespective of clinical stage, menopausal status, histological type or degree of differentiation. The operative management of each patient was according to the tenets of their surgeons at the time of presentation; the breast was conserved wherever possible. The majority of patients were treated with adjuvant radiotherapy. Adjuvant cytotoxic chemotherapy, with or without tamoxifen, was the subject of clinical trial at the time.

The clinical status of each patient at time of sampling had been summarized on a database using Helix Express (version 1.0.1; Helix Technologies, Northbrook, IL, USA). The plasma library had been established over a 15-year period, with an average of eight plasma samples per patient being cryopreserved. It is known that cryopreserved p53 autoantibodies are stable in either sera or plasma, and that several cycles of freezing/thawing do not cause loss of titre (Soussi T, personal communication). Although it is theoretically possible that shed p53 protein might partially block autoantibody in some patients, it has already been shown [17] that autoantibody to p53 is normally in excess, and is thus detectable by ELISA.

P53 autoantibody detection by enzyme-linked immunosorbent assay

The ELISA assay for p53 autoantibodies was developed in-house, based on the ELISA procedure of Lubin *et al* [3] in which the amount of specific p53 autoantibody recorded by ELISA was confirmed by Western blot and immunoprecipitation analyses. Briefly, 96-well assay plates (Falcon 3912; Becton Dickinson and Co, Oxnard, CA, USA) were precoated with baculoviral-derived, purified, wild-type, full-length, human recombinant p53 (0.1 ng/ml phosphate-buffered saline [PBS]) using 30 µl per well. After overnight incubation at 4°C, the plates were washed three times with PBS–0.1% Tween at room temperature. Wells were then blocked for 2 h with 250 µl PBS–0.5% Tween. After five washes with PBS–0.1% Tween, 30 µl of each plasma sample was incubated on ice for 2 h. The plate was again washed five times as above before addition of horse radish peroxidase-linked goat antiserum to human immunoglobulin (Sigma A8667; Sigma Aldrich Co Ltd, Poole, Dorset, UK) and incubation on ice for 1 h. After a further five washes, bound horse radish peroxidase was detected by conventional methods using *O*-phenylenediamine dihydrochloride (30 min), stopping with 3 mol/l H_2SO_4, and reading the optical density (OD) at 492 nm.

An OD reading of less than 0.4 was taken as a negative result. This cutoff was determined by titration of positive and negative samples (1/10, 1/50, 1/100 and 1/500) and gave good discrimination between positive and negative samples at 1/500 dilution. All assay runs included the same internal standards of a sample known to be positive at 1/500 dilution and a sample known to be negative at

1/10 dilution, in order to confirm assay reproducibility throughout the series.

The intial screen of all patients was at a plasma dilution of 1/10 in PBS–0.1% Tween. Samples from all patients with a positive OD reading at the 1/10 dilution were then re-assayed at 1/500 (see Sample processing, below). The positive and negative internal standards ensured that all results were directly comparable. Background controls received no p53 protein. Specificity controls used soluble p53 protein added to the positive plasma sample before assay; this resulted in loss of signal.

In one assay series we compared a commercial ELISA kit for p53 autoantibodies (produced by Dianova GmbH; licenced to CalBiochem-Novabiochem Corp; distributed by Oncogene Research Products, Cambridge, MA, USA; cat no. QIA 16) with our in-house ELISA. A total of 20 patients' samples were tested, representing a range of positive and negative readings. Two samples were scored strongly positive by the in-house assay, but only one of these two scored positive by the Dianova assay. Having established sensitivity, specificity and reproducibility of the in-house assay, we judged that this was superior to the commercial assay both in terms of sensitivity and of cost (£1 per test compared with £23 per test). The in-house assay was thus used throughout the study presented here.

Sample processing

The last sample obtained from each of the 1006 patients was screened for anti-p53 antibodies at a 1/10 dilution. Those patients who proved to be positive for p53 autoantibodies at this low dilution were then tested for autoantibody status using all samples from each patient; here a 1/500 dilution of plasma was used because this was found to give good specificity and sensitivity for known positive and negative controls. Of those patients who were antibody negative in the initial screen, 60 had had a plasma sample obtained around the time of their primary diagnosis of breast cancer; these primary samples were assayed (1/10 dilution) to look for any positive to negative switches in autoantibody levels during clinical follow up.

Clinical fields

The presence or absence of autoantibodies to p53 was compared with the clinical status of each patient, which was classified: (i) primary remission; (ii) secondary remission following a previous relapse; (iii) active relapsed disease; or (iv) continuous active disease since first diagnosis. For those patients who were anti-p53 positive, their clinical history was compared with levels of measured p53 autoantibodies throughout their follow-up period.

Results

Of the 1006 breast cancer patients screened, 155 (15%) had autoantibodies to p53 in the most recently obtained plasma sample. Of the total patient cohort, the disease status corresponding to the time of collection of this sample was known in 960 patients. Autoantibody status was compared with disease status (Table 1). There was no correlation with those patient groups who had known active disease (either continuous active, or recurrent) or with those in primary or secondary clinical remission; the incidence of p53 autoantibody positivity was approximately 16% in all groups. The possibility that patient age may influence humoral immunity was tested by comparing premenopausal with postmenopausal patients; there was no correlation with the patients' menopausal status at diagnosis with autoantibodies to p53 (Table 2).

For those patients whose most recent sample contained p53 autoantibodies, all previous samples were screened. This longitudinal review showed that autoantibody tended to be persistent. Although preoperative plasma samples were not available, some patients had been included in this study from the time of their first appointment at the oncology clinic; these samples were those that were most likely to contain any residual p53 autoantibody associated with the primary tumour. For those who had antibody present at early follow up, levels tended to persist throughout follow up. This was in contrast to those patients who had no autoantibody detectable within 30 days of surgery (see below). Overall, any fluctuations in autoantibody levels within the positive patient cohort gave no consistent pattern when compared with the clinical history of each patient. Although there was no correlation between p53 autoantibody status and disease behaviour, in one patient there was a strong correlation with prednisolone therapy and a fall in p53 autoantibodies, presumably as a result of steroid-induced immunosuppression. Shortly after steroid treatment, the patient developed cerebral metastases which were marked by a rapid rise in CA15.3.

Of the 851 patients who were negative for p53 autoantibodies at a 1/10 plasma dilution, 60 had had a plasma sample taken within 30 days of diagnosis of breast cancer. Of these, 22 had current active disease and 38 had current inactive disease, at the time when the autoantibody status of all 60 patients was negative. The first sample (taken less than 30 days after diagnosis) was screened and showed that 59 out of the 60 proved to have also been negative for p53 autoantibodies around the time of diagnosis (Table 3). This suggests that, if a patient is negative for p53 autoantibodies at diagnosis of active primary disease, then that patient is highly unlikely to develop humoral immunity to p53, even in the presence of recurrent disease.

Discussion

This large, single-centre study was designed to explore a possible relationship between p53 autoantibody status and breast cancer activity. One thousand and six patients

with breast cancer attending a single referral center were included in the study, irrespective of age or disease characteristics at the time of primary diagnosis. The median patient follow up was 4 years. An average of eight serial plasma samples per patient had been cryopreserved, and corresponding clinical information at the time of each sample's collection had been entered into the database. This fully documented library of over 8000 samples was used to look for correlates between tumour behaviour and autoantibodies to p53 during the clinical follow-up period.

Anti-p53 levels were independent of changes in tumour status

As a working hypothesis we proposed that levels of autoantibodies to p53 would reflect tumour behaviour. Thus, for those patients who were positive for p53 autoantibodies at diagnosis, we reasoned that surgical removal of primary tumour might result in reduced p53 autoantibody levels. Should these levels then show a secondary increase associated with relapsed disease, then increasing levels of p53 autoantibodies might act as a biochemical marker of tumour progression. For those patients who were negative for p53 autoantibodies, then, development of recurrent disease may be associated with changes in p53 expression within the metastatic tumour, leading to a switch to an autoantibody-positive status.

We found that the presence or absence of p53 autoantibodies was not predictive for presence or absence of recurrent disease. There was an equivalent incidence of active disease at the time of sampling in the autoantibody-negative and autoantibody-positive groups, these being 25.2% and 28.7%, respectively.

We found that humoral immune activity against p53 appeared to be relatively restricted to a subgroup of patients in whom, once an autoantibody response had been generated, antibody was likely to persist regardless of tumour behaviour. Thus, antibody-positive patients without clinical recurrence remained antibody positive throughout the follow-up period. Conversely, where no detectable p53 autoantibody was present at the time of primary diagnosis, these patients remained similarly negative for antibody irrespective of subsequent disease activity (Table 3).

Immune regulation and potential responsiveness to breast cancer

In contrast to shed markers that correlate with tumour mass, such as CA15.3 for cancer of the breast, any tumour-related immune response will be subject to complex regulation. Autoantibody responses to p53 will require appropriate primary immunization. Initial low-dose antigen exposure may induce immune tolerance and lack of response. Higher antigen doses may activate either antibody-mediated immunity, or cellular immunity.

In breast cancer patients, the present results suggest that, once an active humoral response against p53 is established, this response remains active. This persistent humoral reaction may be driven by persistent antigenic stimulation by p53 protein derived from overexpression of p53 at distant metastatic sites. Alternatively, irradiated normal tissue may be a source of continued antigenic stimulation, because a long-term side effect of radiation therapy is an increased expression of p53 in normal breast tissue, which persists for several years [12]. Since the great majority of our total patient cohort had received radiotherapy, humoral immunity to p53 associated with primary disease might persist, even in those patients who enter remission, due to tumour-independent antigenic stimulation.

Loss of p53 function is known to correlate with loss of efficacy of cancer therapy *in vivo* [13,14]. This raised the possibility that autoantibodies to p53 that develop during follow up might indicate those patients whose tumor has become resistant to therapy. However, the present results show that if no immunity has been generated at the time of primary diagnosis, then later immunity is unlikely to occur. This corresponds to the finding that expression of p53 antigen in biopsies of locally advanced breast cancer did not correlate with drug resistance [15,16]. Overall, our observations show that screening for p53 autoantibody status is not informative on residual tumour activity, nor on therapeutic responsiveness. We conclude that the potential value of p53 autoantibody screening in patients with breast cancer is limited to the prognostic information obtained at diagnosis.

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