Serum antibodies against p53 in relation to cancer risk and prognosis in breast cancer: a population-based epidemiological study

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Summary  To perform an epidemiological evaluation of the predictive value of p53 autoantibodies in breast cancer, we measured antibodies against p53 in serum samples from 165 breast cancer patients in comparison with serum samples from 330 healthy controls, selected from the same population as the cases and matched for age, sex and specimen storage time. Median age of patients was 51 years (range 25–64 years). Presence of serum p53 autoantibodies was analysed by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blotting. The lower ELISA reactivities were similar for cases and controls, but presence of high-level reactivity was more common among cases than among controls [odds ratio (OR) 9.03, 95% confidence interval (CI) 2.40–50.43]. Presence of Western blot-detected p53 autoantibodies had a very similar association (OR 10.8, CI 3.0–59.4). Among the cases, we also studied whether there was any correlation between level of anti-p53 antibodies and stage of the disease or survival. There was no significant correlation between presence of antibodies and stage of the disease. There was a significant negative correlation between presence of p53 antibodies and survival (P = 0.003). A stepwise multivariate Cox regression analysis showed that T-stage, age and presence of anti-p53 antibodies were significant independent prognostic variables, with a dose-dependent negative effect on survival for all three variables. We conclude that presence of anti-p53 antibodies are of significance both for the risk of having breast cancer and the risk of dying from breast cancer.

Keywords: breast cancer; p53; antibodies; case–control study; survival

Alteration of the p53 tumour-suppressor gene is the most common genetic change found so far in malignancy (Hollstein et al, 1991), including breast cancer (Osborne et al, 1991). The p53 gene is of crucial importance for growth regulation, and it is therefore not surprising that p53 alterations are consistently associated with more aggressive tumour biological factors and poorer prognosis in breast cancer (Davidoff et al, 1991a; Ostrowski et al, 1991; Hanzal et al, 1992; Isola et al, 1992; Thor et al, 1992; Allred et al, 1993a,b; Barnes et al, 1993; Noguchi et al, 1993; Silvestrini et al, 1993; Stenmark-Askmalm et al, 1994).

Autoantibodies against p53 have been detected in sera from breast cancer patients with prevalences ranging from 1% to 48% (Crawford et al, 1982; Davidoff et al, 1991a, 1992; Schlichtholz et al, 1992; Green et al, 1994; Mudenda et al, 1994; Porzolt et al, 1994; Lubin et al, 1995; Peyrat et al, 1995; Vojtesek et al, 1995; Coomber et al, 1996; Regidor et al, 1996; Willsher et al, 1996). In the study of Vojtesek et al 1995, which utilized three different methods to detect anti-p53 antibodies [immunoprecipitation of labelled protein, Western blotting and enzyme-linked immunosorbent assay (ELISA)], p53-specific circulating antibodies, confirmed by all methods, could be detected in only 1 of 100 cases (1%). With ELISA, 25% of the sera were considered positive, which was thought to be due to cross-reactions with unspecific immunogens. In the other studies, using ELISA-based methods, the reported frequency of positive sera has been in the range 12–48%. In most of these studies, the results were validated by immunoprecipitation or Western blotting.

Antibodies can be detected against mutated as well as wild-type p53 (Labrecque et al, 1993). Mutated p53 tends to have a much longer half-life than wild-type p53, leading to accumulation of the protein (Davidoff et al, 1991b). One study reported that there was a significant correlation between presence of p53 autoantibodies and the detection of mutated p53 protein in tissue sections (Mudenda et al, 1994). That study also reported a much higher detection rate of antibodies in patients with breast cancer than in normal control volunteers. There are also observations indicating that only tumours with missense mutations of p53, resulting in p53 overexpression, elicit p53 antibodies (Davidoff et al, 1992; Winter et al, 1992; Lubin et al, 1993; Houbiers et al, 1995). Anti-p53 antibodies have also been reported to indicate a poor prognosis in patients with breast cancer (Schlichtholz et al, 1992; Mudenda et al, 1994; Peyrat et al, 1995).

The objectives of the present study were to use an elaborate epidemiological approach to evaluate whether breast cancer patients do have an increased presence of p53 autoantibodies in comparison with matched controls selected from a population-based blood bank, and to investigate whether there was any correlation between antibody responses towards p53 and the stage at diagnosis as well as the survival of patients with breast cancer.
**MATERIALS AND METHODS**

**Patients and controls**

The case material consisted of 165 consecutive patients with newly diagnosed breast cancer admitted to the department of surgery at the University hospital of Umeå, between 1985 and 1992. Umeå University Hospital is the only hospital providing specialized breast cancer care for the population of the Västerbotten county in Northern Sweden. To obtain comparability with controls (see below), we excluded patients of age, at blood sampling, over 65 years. The age of the patients was thus 25–64 years (median 51 years). The tumours were clinically and pathologically staged using the TNM classification according to UICC (1982). In the majority of cases, treatment consisted of breast conserving surgery followed by radiotherapy of the breast and, in some cases, also the ipsilateral axilla. Patients with metastases in the axillary lymph nodes and/or stage T3 also received adjuvant therapy with tamoxifen or cytostatics. Patients with relapse or progression later in the course of disease were treated according to different protocols, mostly with cytostatics and/or hormonal regimens. Some patients received radiotherapy to metastatic sites. The patients were followed from date of diagnosis until death (from all causes), or to completion of follow-up (3 Nov 1995). Thus, the follow-up time was 1–120 months (median 73 months). No patient was lost to follow-up.

Pretreatment serum samples were, after informed consent, taken consecutively from all patients. The samples were frozen and stored at –80°C.

For each case (n = 165), two controls were selected from the blood bank connected with the Västerbotten Project, which is a project offering health controls for the entire population of Västerbotten county in the age range 30–60 years. Currently, samples from about 50 000 subjects have been collected and stored at –80°C. The controls (n = 330) were matched for gender and age at blood sampling (±3 years). To assure storage times for cases and controls were as comparable as possible, we attempted to match also for time of sampling. For 55% of the triplets, this difference in sampling time between case and each of the corresponding two controls was less than 3 years, but for the rest of the material the difference was larger, up to 10 years at most.

**Serology**

Levels of antibodies against p53 were analysed by ELISA using p53 protein, purified as described previously (Ryder et al, 1996). Analyses were performed with the laboratory blinded to case–control status of the specimens. Cases and controls belonging to the same triplet were analysed together, minimizing the risk of bias due to possible interassay variations of the analysis procedure.

The p53 preparation used for ELISA assays was analysed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis on a 10–15% gradient gel, followed by silver staining, to verify its purity and that it had not been degraded in transit. A major p53 band was detected (see Figure 1). p53 was coated onto ELISA plates (Costar, Cambridge, MA, USA) at 0.4 μg ml⁻¹ (the lowest coating concentration that gave maximal absorbance with reactive sera) in 0.1 M tris-HCl, pH 8.8, and incubated at 4°C overnight. After blocking with 10% horse serum in phosphate-buffered saline (PBS) for 1 h at 37°C, human sera [diluted 1:100 in 10% horse serum in PBS (HS-PBS)] was incubated on the plates for 2 h at 37°C. After washing with PBS/0.05% Tween 20 (PBS-T), a mouse monoclonal antibody to human IgG (gamma chain) (Eurodiagnostika, Malmö, Sweden), diluted 1:800 in HS-PBS, was incubated on the plates for 90 min at 37°C. After washing with PBS-T, a horseradish peroxidase conjugated antibody to mouse IgG (Southern Biotechnology) was incubated on the plates for 1 h at 37°C. After washing with PBS-T, the peroxidase substrate ABTS (Boehringer) was incubated on the plates for 1 h, whereafter the levels of absorbance were recorded at 405 nm.

For 96 sera (case–control triplets with two controls per case), the in-house optimized ELISA method was compared with a commercial p53 autoantibody detection kit (Dianova, Hamburg, Germany). The absorbances of reactive sera were similar by both methods.

**Western blot**

All serum samples with ELISA absorbances of 0.180 or more and some randomly chosen sera with lower reactivity, in total 48 serum samples, were also analysed for p53 reactivity by Western blot. Forty microgram per gel of p53 was run on 10–15% polyacrylamide gradient gels in a semi-automated gel electrophoresis system (Phast system, Pharmacia, Uppsala, Sweden). The proteins of the gels were transferred to nitrocellulose sheets, which were cut into 15 strips per gel. The strips were blocked in 5% non-fat dry milk in PBS for 1 h, incubated with the human sera [diluted 1:100 in 2% non-fat dry milk in PBS (Whatman filtered)] for 2 h at room temperature, washed with 5% milk for 40 min with three changes of wash fluid, incubated with rabbit anti-human IgG-horseradish peroxidase conjugate (Dako, Copenhagen, Denmark) [diluted 1:1000 in 2% non-fat dry milk in PBS (Whatman filtered)] for 30 min at room temperature and then washed with PBS containing 0.5% Tween 20 for 2 h with ten changes of washing fluid. The strips were then developed using enhanced chemiluminescence as described by the manufacturer (Amersham-Pharmacia, Uppsala, Sweden).

**Statistical methods**

Statistical analyses were carried out after division of antibody levels into three groups. The mean of the case mean and control mean was used as lower cut-off, and the mean of the controls plus two standard deviations as upper cut-off.

Multivariate conditional logistic regression for stratified data was used to calculate odds ratios with age at blood sampling, categorized into the three groups 25–49, 50–59 and 60–64 years, and
antibody levels, categorized as described above, in the model. Exact confidence intervals for odds ratios were calculated using the permutational distribution of the sufficient statistics.

Associations between antibody levels and T-, N- and M-stage of the disease were tested with the Jonckheere–Terpstra test. Kaplan–Meier estimate of survival and the log-rank test for trend was used to compare survival for different groups of antibody levels.

A multivariate Cox regression model was used to study prognosis in relation to antibody levels together with other prognostic factors. As explanatory variables, antibody level, categorized into three groups as explained above, T-stage (T1, T2 and T3), N-stage (N– and N+), M-stage (M– and M+) and age at diagnosis, categorized into the three groups 25–49, 50–59 and 60–64 years, were used. Antibody level and age at diagnosis were included in the model as categorical variables with the lowest group as reference category. Backward stepwise analysis was used to select the final model.

**RESULTS**

**Anti-p53 antibodies in relation to breast cancer risk**

The distribution of antibody levels against p53 in cases and controls is shown in Table 1 and Figure 2. For cases and controls, the median absorbance values were 0.115 and 0.109, respectively, whereas the mean values were 0.198 and 0.106 respectively. The reason for the discrepancy between median and mean was the presence of a few patients with very high values. This skewness is
evident in the highest quartile, in which 30% of cases and 21% of controls were found. This discrepancy between cases and controls was, furthermore, confined to the highest values.

Lower and upper cut-off points for antibody levels were 0.152 and 0.291 respectively. In the logistic regression analysis, age at blood sampling did not have a significant effect, and removing it from the model yielded estimated odds ratios of 1.88 (95% confidence interval 0.96–3.69) and 9.03 (95% confidence interval 2.40–50.43) for the middle and upper groups of p53 antibodies compared with the lowest group respectively (Table 1). Dividing the material at the lower cut-off point yielded an odds ratio of 2.60 (95% confidence interval 1.44–4.80) for those above compared with those under the cut-off value.

All sera with ELISA absorbances of 0.180 or more were also analysed by the confirmatory method, Western blotting (Figure 3). Most reactivities detected with the p53 band were very strong. Among the sera with ELISA absorbances above the high cut-off level (0.291), the reactivity was confirmed by Western blotting for all subjects except one. In the OD range 0.250–0.291, a p53 Western blot reactivity was detected in three out of six serum samples. In the OD range 0.180–0.249, a p53 Western blot reactivity was detected only in 1 out of 20 serum samples. The breast cancer risk associated with Western blot confirmed presence of p53 autoantibodies was similar to that of the ELISA high cut-off-detected p53 autoantibodies (3 out of 330 controls; 15 out of 165 cases; OR, 10.9; CI, 3.0–59.4).

Estimated odds ratios did not change significantly after excluding the 96 case–control triplets in which the age difference between case and control was more than 3 years, or the date of blood sampling differed by more than 3 years.

**Anti-p53 antibodies in relation to disease status**

There was no significant correlation between serum level of p53 antibodies and T-, N- or M-stage of the disease (Table 2). Western blot reactivity was also not significantly associated with the T-, N- or M-stage of the disease (not shown).

**Anti-p53 antibodies in relation to prognosis**

The univariate survival analysis was based on 165 cases, of which 54 were observed to die during follow-up. Survival was correlated with anti-p53 antibody level; the higher the antibody level, the shorter was the survival. Mean survival was 97, 73 and 71 months for the low, middle and upper group of antibody levels respectively. In Figure 4, the Kaplan–Meier estimate of the survival distributions is shown for each group of antibody levels. The log-rank test for trend showed a significant difference ($P = 0.003$) in survival between the groups.

The multivariate survival analysis was based on 99 cases with complete data on all variables. Of these cases, 35 were observed to
Antibodies against p53 in breast cancer

DISCUSSION

The main finding of the present study was that a high serum level of antibodies directed against p53 was found more often in breast cancer cases than in age- and sex-matched normal subjects from the general population. In patients with breast cancer, presence of p53 autoantibodies also indicated a shortened survival. In this study, a population-based reference material was utilized, which is advantageous from an epidemiological point of view. To our knowledge, a population-based selection of controls has not been used in previous studies.

The finding of serum p53 autoantibodies in breast cancer is consistent with other studies. The first was Crawford et al (1982), who reported 9% p53-positive sera in 155 breast cancer patients by immunoprecipitation whereas none was positive among 164 healthy controls. Mudenda et al (1994) detected significantly higher antibody levels in breast cancer patients than in normal control volunteers with ELISA methodology. In the present study, population-based controls were used, matched for sex, age and time of specimen storage. The difference in anti-p53 antibody levels, measured with an ELISA-based method, was smaller between cases and controls in this study. Nevertheless, the difference was clearly evident for subjects with the highest absorbance levels of anti-p53 serum antibodies (absorbance values above 0.292), which correlated closely with the presence of p53 autoantibodies detectable also by Western blotting. Presence of ELISA reactivity that was not confirmed by Western blotting could be non-specific, directed against p53 conformational epitopes destroyed by denaturing in Western blotting or present in too low amounts to be detectable by the less sensitive Western blot technique. The fact that this low-level p53 ELISA reactivity (<0.297 absorbance) was significantly different from the reference group only in the prog-

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