Mutations in p53, p53 protein overexpression and breast cancer survival

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Abstract

p53 is an important tumour suppressor gene that encodes p53 protein, a molecule involved in cell cycle regulation and has been inconsistently linked to breast cancer survival. Using archived tumour tissue from a population-based sample of 859 women diagnosed with breast cancer between 1996 and 1997, we determined p53 mutations in exons 5–8 and p53 protein overexpression. We examined the association of p53 mutations with overexpression and selected tumour clinical parameters. We assessed whether either p53 marker was associated with survival through 2002, adjusting for other tumour markers and prognostic factors. The prevalence of protein overexpression in the tumour was 36% (307/859) and of any p53 mutation was 15% (128/859). p53 overexpression was positively associated with the presence of any p53 mutation (odds ratio [OR] = 2.2, 95% confidence interval [CI] = 1.5–3.2), particularly missense mutations (OR = 7.0, 95% CI = 3.6–13.7). Negative oestrogen and progesterone receptor (ER/PR) status was positively associated with both p53 protein overexpression (OR = 2.6, 95% CI = 1.7–4.0) and p53 mutation (OR = 3.9, 95% CI = 2.4–6.5). Any p53 mutation and missense mutations, but not p53 protein overexpression, were associated with breast cancer-specific mortality (hazard ratio [HR] = 1.7, 95% CI = 1.0–2.8; HR = 2.0, 95% CI = 1.1–3.6, respectively) and all-cause mortality (HR = 1.5, 95% CI = 1.0–2.4; HR = 2.0, 95% CI = 1.2–3.4, respectively); nonsense mutations were associated only with breast cancer-specific mortality (HR = 3.0, 95% CI = 1.1–8.1). These associations however did not remain after adjusting for ER/PR status. Thus, in this population-based cohort of women with breast cancer, although p53 protein overexpression and p53 mutations were associated with each other, neither independently impacted breast cancer-specific or all-causing mortality, after considering ER/PR status.

Keywords: breast cancer • p53 mutations • p53 overexpression • survival

Introduction

p53 is a transcription factor expressed in most cell types; its expression is induced primarily in response to genotoxic stress. The activation of p53 results in induction or repression of a number of genes involved in cell cycle regulation, DNA repair and apoptosis [1]. Thus, p53 allows DNA repair or induces apoptosis, protecting against the accumulation of genetic changes.

Mutations in p53 suppress the regulatory functions of the protein and are the most common genetic change in breast cancer, with a frequency of about 30% (range 15–71%) [2, 3]. p53 mutations are characterised by a high prevalence of missense mutations found primarily in exons 5–8, in the DNA-binding domain. The spectrum of mutations in breast cancer is similar to...
that of other cancers, with less G:C to T:A transversions and more A:T to G:C transitions.

Variations have been observed in the pattern of p53 mutations in breast cancer by geographical location and may reflect the effect of environmental factors and/or ethnicity. A higher frequency of G:C to A:T transitions and G:C to T:A transversions was observed among breast cancer cases in Western countries, suggesting exposure to tobacco smoke [2]. Deletions were more frequent in breast cancers cases from Japan [2]. There are also differences in the mutation pattern by ethnicity, with a significantly higher frequency of all types of transitions among African-American women than among Caucasians. The reason for this difference is not known, but it is hypothesized that population-specific environmental exposure or endogenous factors may play a role [4].

Immunohistochemical staining of p53 provides information on the expression of the protein and has become a widely used method of mutant p53 detection. The correlation between p53 expression and p53 protein overexpression assessed by immunohistochemistry is estimated to be less than 75% for breast cancer [5]. This discrepancy is attributed to the fact that not all mutations result in stable protein formation; also, some tumours may express increased levels of wild-type p53. However, there is also some uncertainty regarding the methodological aspects of p53 immunohistochemical detection that may be responsible for the differences in the frequency of p53 mutations and p53 protein levels [6].

Although detection of p53 overexpression by immunohistochemistry has been done to investigate its association with survival for a number of different cancers, the results are inconclusive [7–9]. In contrast, studies that use sequencing to detect p53 mutations generally have demonstrated shorter survival with the presence of mutations [10, 11], particularly for mutations within the DNA-binding domain. However, the clinical studies on p53 mutation have usually been undertaken in hospital-based series of cases, which are more likely to reflect the experience among women with more severe disease than in a sample drawn from the general population; few studies on the prognostic experience among a sample from the general population have been previously reported, and none with a large sample size. Most previous studies have also not taken into consideration the potential confounding effects of oestrogen/progesterone receptor (ER/PR) status, which have been consistently linked to breast cancer survival, along with tumour size and other characteristics of the tumour [12].

In the present study, we investigated the frequency and type of p53 mutation in exons 5–8 among 859 women, participants of the Long Island Breast Cancer Study Project (LIBCSP), which is a large, population-based epidemiological study undertaken to identify determinants of developing breast cancer and, once diagnosed, prognostic indicators. We correlated the presence of p53 mutations with protein overexpression assessed by immunohistochemistry. Further, we investigated the association of these factors with selected clinical parameters of the tumours including tumour stage (in situ and invasive) and ER/PR status. Finally, we studied whether p53 overexpression and p53 mutations were associated with survival among this population-based cohort of women with breast cancer.

Materials and methods

Study population

The details of the LIBCSP case–control [13] and the follow-up cohort [14] study designs have been described previously. Briefly, the eligible cases were women residing in Nassau or Suffolk counties, who spoke English, were 20 years of age or older and were newly diagnosed with in situ or invasive breast cancer between August 1, 1996, and July 31, 1997. A total of 1508 women with breast cancer, of which 1273 had invasive breast cancer, participated in the baseline, case–control study interview. Case women were subsequently followed up for vital status through 2002; survivors or their next of kin were interviewed again in 2002. Medical records were abstracted during the case–control study and again as part of the follow-up study.

Data collection

Baseline data

The main case–control questionnaire was administered within a few months of diagnosis by a trained interviewer in the participant’s home and lasted approximately 2 hrs. Information was assessed on known and suspected breast cancer risk factors prior to or at diagnosis, including passive and active cigarette smoking, life-time alcohol use, menstrual and reproductive histories, hormone use, body size by decade of adult life, life-time participation in recreational activities, prior medical history, and family history of breast cancer (http://epi.grants.cancer.gov/LIBCSP/projects/Questionnaire.html). The usual dietary intake in the year prior to the interview was assessed using a self-administered food frequency questionnaire, as previously described [15, 16]. The majority of case interviews occurred prior to the initiation of chemotherapy or radiation therapy.

Medical records and archived tumour tissue at baseline

As part of the baseline case–control study, medical records of the 1453 women who provided a signed medical record release form were abstracted for tumour stage and ER and PR status. Also, archived pathology blocks were requested from the 33 hospitals in the Long Island study area and successfully retrieved for 962 cases. After the review by a trained pathologist, the tumour tissues from 859 subjects were available for p53 mutation detection and p53 protein overexpression analysis. The distribution of known and suspected breast cancer risk factors did not vary substantially between cases with and without tumour tissue available for these analyses (data not shown).

Follow-up data

Follow-up information on the full course of treatment for the initial breast cancer diagnosis was obtained by trained interviewers via telephone from 1098 case participants or their proxy in 2002–2004. There were 410 cases without follow-up interview data due to non-response, refusal or because they were untraceable or were deceased without an identifiable proxy. During the follow-up, medical records were abstracted for 598 women by trained abstractors to determine tumour size and nodal status and treatment regimen. There was a high concordance between treatment information abstracted from records and self-reported radiation therapy ($\kappa = 0.97$), chemotherapy, ($\kappa = 0.96$) and
hormone therapy ($\alpha = 0.92$). Thus, for this study, the analysis is based upon the self-reported treatment data.

**Study outcome**

The National Death Index (NDI) was used to ascertain all-cause and breast cancer-specific mortality among study participants. The participants were followed up from diagnosis until December 31, 2002, for a mean of 66.7 months (range 2.7 – 88.6). Among the 1508 women diagnosed with breast cancer, 198 (13.1%) deaths occurred (126 deaths among those with tissue available). On the basis of ICD codes 174.9 and C-50.9 listed as a primary or secondary code on the death certificate, 128 (64.6%) deaths were due to breast cancer. Nine women died due to other cancers in areas of common breast tumour, metastases, including the brain and lungs. Cardiovascular disease was the second most common cause of death, accounting for 21% of all deaths.

**Tumour tissue microdissection and DNA extraction**

Formalin-fixed, paraffin-embedded tumour sections were histopathologically reviewed by a trained pathologist and the cancer tissue was separated using manual microdissection. The tissues were incubated overnight at 37°C in 30 μl of the digestion buffer containing 150 μl of proteinase K (10 mg/ml) and 850 μl of 0.5% Tween 20, 1mM EDTA, pH 8.0, and 50 mM Tris, pH 8.5. On the next day, 10 μl of proteinase K solution was added and the samples were incubated for 4–6 hrs at 56°C. Proteinase K was inactivated by incubation of the samples at 100°C for 10 min. DNA samples were stored at –80°C until analysis.

**p53 mutations classification**

The mutations in p53 were classified according to type (point mutations, including transitions and transversions, and insertions or deletions) or their effect (missense, nonsense, silent and frameshift/in-frame mutations). Missense mutations were further split into mutations within the DNA-binding domains (DBD) and mutations within non-DBD [11]. DBD mutations included mutations in the L2 and L3 loops (codons 164–194 and 237–250, respectively) and in the LSH motif (codons 119–135 and 272–285). These codons are in contact with DNA in the minor and major grooves. Non-DBD mutations were mutations outside the previously defined DBD mutations.

**p53 mutation detection**

For 859 women, mutations were detected in exons 5–8 of p53 gene of only 128 samples. The samples were first PCR-amplified using the following primers and PCR conditions: exon 5, forward primer ATC TGT TCA GTG CCC TG, reverse primer AAC CAG CCC TGT CTC TC, amplification at 95°C for 15 min, 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec; exon 6, forward primer AGG TCC CCC AGG AGG CCT AT, reverse primer CAC CCT TAA CCC CTC CTC CC, amplification at 95°C for 15 min, 35 cycles at 94°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec; exon 7, forward primer CCA AGG CGC ACT GGC ATC, reverse primer CAG AGG CGG CAC AGC AGG, amplification at 95°C for 15 min, 35 cycles at 94°C for 30 sec, 66°C for 30 sec and 72°C for 30 sec; and exon 8, forward primer TTC TCT ACT GGC TCT TGC TT, reverse primer TGT CCT GCT TGG TT, amplification at 95°C for 15 min, 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. PCR amplification was not successful in all samples and/or exons. The failure rate varied from 3.7% to 11.5% for individual exons, depending on the size of amplicons (a larger amplicon resulted in a higher failure rate). Overall, the percentage of PCR failures for all samples and exons was 7.2%. For 10% of samples, DNA extraction, PCR amplification and mutation detection were repeated.

As a screening method for the detection of p53 mutations, the Surveyor Mutation Detection Kit (Transgenic, Omaha, NE, USA) was used. The method is based on the hybridisation between wild-type and mutated DNA strands, followed by the treatment with celery DNA endonuclease, which cleaves DNA specifically at sites of base-substitution mismatch and DNA distortion. The samples are then separated by gel electrophoresis, and the presence of more than one band indicates mutation in the DNA [17]. The PCR products were first hybridised by incubation at 95°C for 10 min and subsequent cooling to 85°C at a rate of 0.2°C/sec, followed by cooling to 25°C at a rate of 0.1°C. The hybridised samples were then incubated with the endonuclease according to the manufacturer’s recommendation at 42°C for 20 min. The reaction was stopped, DNA loading dye was added and the samples were either stored at –20°C or analysed immediately on 4% NuSieve 3:1 agarose (Cambrex, East Rutherford, NJ, USA) in 1× TBE buffer. DNA was stained by SYBR Green I (Roche, Indianapolis, IN, USA), which had been added to the agarose solution before casting the gel. Electrophoresis was run at 10–12 V/cm for 1 hr and the gel was inspected using a FluorImager 595 (Molecular Dynamics, Sunnyvale, CA, USA). Positive and negative DNA samples (DNA extracted from cell lines of known mutation status) were used in every experiment to confirm that PCR amplification and endonuclease treatment were successful. The samples containing multiple bands were then selected for further confirmation and identification of mutations by PCR amplification and sequencing using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA).
p53 protein had an intensity score of moderate or strong, had at least 10% or more of cells showing evidence of expression and was considered positive by both study pathologists (HH and LM). The rationale for the cut-off point was based on the background level of the normal adjacent breast tissue on the tumour sections; tumour tissue that showed staining below this threshold was considered negative for p53 protein expression by immunohistochemical detection. Appropriate positive and negative (staining lacking primary antibodies) controls were used in each batch of staining.

### Statistical analysis

The chi-square tests were used to assess the association between mutation type (any, missense, nonsense, silent and frameshift) and protein overexpression (data not shown). We further assessed the association by estimating odds ratios (ER) and 95% confidence intervals (CI), with adjustments made for age at diagnosis and race using an unconditional logistic regression [18]. Protein overexpression was defined as having strong staining and positive cells or having moderate staining and at least 10% positive cells. An unconditional logistic regression was used to estimate the association between any p53 mutation and, in separate models, specific type of mutation (missense, nonsense, silent and frameshift) and p53 protein overexpression after adjusting for age, race, ER/PR status and tumour stage. We also used the regression to assess the association between ER/PR status, tumour stage and p53 protein overexpression and overall p53 mutations, adjusting for age and race.

We used the Cox proportional hazard models to estimate the association between p53 protein overexpression and p53 mutation by type (any, missense, nonsense, silent and frameshift) [19] and breast cancer-specific and all-cause mortality. Multivariate hazard ratios (HRs) and 95% CIs were calculated from the Cox proportional hazard models. We first ran separate models for p53 protein overexpression and p53 mutation type, adjusting for age at diagnosis. We examined potential confounding of the p53 protein overexpression and p53 mutation associations with survival using the following variables: ER/PR status of the tumour, race, tumour stage (in situ vs. invasive), nodal status of tumour, tumour size, treatment type (chemotherapy and radiation), cigarette smoking (non-smoker, current and former smoker), alcohol use (ever and never), oral contraceptive use (ever and never), age at first childbirth, the number of pregnancies and body mass index (BMI) at diagnosis. Only ER/PR status confounded the associations between p53 mutation type and breast cancer-specific and all-cause mortality based on the 10% criteria; therefore, ER/PR status remained in the final multivariate survival models. All statistical methods were carried out using SAS (version 9.1, SAS Institute, Cary, NC, USA).

### Results

Table 1 shows the number and types of p53 mutations, the mutation effect and a comparison of these variables with the International Agency for Research on Cancer (IARC) p53 database [20]. Of the 859 tumour samples with available mutation data, a total of 151 mutations were identified; 128 tumours (15%) contained at least one mutation. In the latest IARC p53 database (R12 database, November 2007), the prevalence of mutations in exons 5–8 for breast cancer was 25%.

| Mutation type        | LIBCSP (n) | LIBCSP (%) | IARC (%) |
|----------------------|------------|------------|----------|
| Point mutations      | 125        | 82.8       | 85.0     |
| Transitions          |            |            |          |
| G:C–A:T at CpG       | 35         | 23.2       | 24.4     |
| G:C–A:T at non-CpG   | 46         | 30.5       | 19.8     |
| A:T–G:C              | 15         | 9.9        | 13.7     |
| Transversions        |            |            |          |
| G:C–T:A              | 17         | 11.3       | 10.2     |
| G:C–C:G              | 6          | 3.9        | 7.8      |
| A:T–T:A              | 5          | 3.3        | 4.5      |
| A:T–C:G              | 1          | 0.7        | 4.6      |
| Insertions or deletions | 26     | 17.2       | 13.7     |
| Other                | 0          | 0          | 1.3      |
| Total number of mutations | 151   | 100.0      | 100.0    |
| Number of tumours with at least one mutation | 128 | 14.9  |
| Number of tumours with no mutations | 731 | 85.1 |
| Total number of tumours | 859 | 100.0 | 13,585|

Table 1 Distribution of p53 mutations, their type and effect among 859 case women diagnosed with a first primary breast cancer in 1996–1997 and who participated in the Long Island Breast Cancer Study Project (LIBCSP), as compared with the IARC p53 mutations database

Mutations were further classified according to their location in the DBD, as described in Materials and Methods. The total number of tumour samples with at least one missense mutation in the DBD was 44 (34% of all tumours with at least one mutation); the number of missense mutations in non-DBD was 30 (23%).

Data on p53 immunostaining were available for 859 tumours, and of these, 307 (36%) were positive for p53 overexpression. Table 2 reports the association between the results of p53 overexpression detected by immunohistochemistry and frequency of all p53 mutations analysed by sequencing. There was a strong association between the two methods; the OR for p53 mutations and p53 protein overexpression was 2.2 (95% CI = 1.5–3.2). The results were consistent even on using different cut-off points for
the determination of p53-overexpressing samples (data not shown). Examination of the individual mutation effects revealed that p53 overexpression was very strongly associated with missense mutations (OR = 7.0, 95% CI = 3.6–13.7), but not with the others (nonsense, silent and frameshift/in frame).

Table 3 shows the association between p53 protein overexpression, p53 mutation, ER/PR status and tumour stage. Women with ER/PR-negative tumours had a 3.9-fold higher probability of p53 mutation in the tumour (OR = 3.9, 95% CI = 2.3–6.4) and a 2.6-fold higher probability of p53 protein overexpression (OR = 2.6, 95% CI = 1.7–4.0). Tumour stage was associated with p53 protein overexpression (OR = 2.8, 95% CI = 1.0–7.8), but not with p53 mutations.

Table 4 reports the distribution of clinicopathological characteristics of case women by vital status as of December 31, 2002. p53 overexpression and silent, nonsense, as well as frameshift p53 mutations were not associated with mortality. In contrast, the presence of missense p53 mutations was significantly associated with both breast cancer-specific and all-case mortality. Tumour stage, ER/PR status and tumour size were other parameters associated with mortality.

Table 5 shows findings from the age-adjusted and multivariate-adjusted regression models for the associations between p53 protein overexpression and p53 mutations and breast cancer-specific and all-cause mortality. In the age-adjusted model, protein overexpression was not associated with breast cancer mortality, whereas...
Table 4  The distribution of clinicopathological characteristics of LIBCSP case women by vital status as of December 31, 2002

|                     | Breast cancer-specific mortality |                     | All-cause mortality |                     |
|---------------------|----------------------------------|---------------------|---------------------|---------------------|
|                     | Alive                           | Deaths              | P-value             | Alive               | Deaths              | P-value             |
|                     | n   | %    | n   | %    | P-value | n   | %    | n   | %    | P-value |
| Age                 |     |      |     |      |         |     |      |     |      |         |
| <40                 | 40  | 5.7  | 7   | 8.4  | 0.46    | 40  | 6.0  | 7   | 5.8  | <0.01  |
| 41–50               | 131 | 18.6 | 20  | 24.1 | 0.01    | 130 | 19.5 | 21  | 17.4 |
| 51–60               | 180 | 25.6 | 17  | 20.5 | 0.01    | 177 | 26.6 | 20  | 16.5 |
| 61–70               | 169 | 24.0 | 16  | 19.3 | 0.01    | 165 | 24.8 | 20  | 16.5 |
| >70                 | 184 | 26.1 | 23  | 27.7 |         | 154 | 23.1 | 53  | 43.8 |
| Tumour stage        |     |      |     |      |         |     |      |     |      |         |
| In situ             | 90  | 12.8 | 1   | 1.2  | <0.01   | 86  | 94.5 | 580 | 83.3 | 0.01   |
| Invasive            | 614 | 87.2 | 82  | 98.8 |         | 5   | 5.5  | 116 | 16.7 |
| p53 overexpression  |     |      |     |      |         |     |      |     |      |         |
| –                   | 452 | 64.2 | 50  | 60.2 | 0.48    | 425 | 84.7 | 241 | 84.6 | 0.97   |
| +                   | 252 | 35.8 | 33  | 39.8 |         | 77  | 15.3 | 44  | 15.4 |
| p53 mutation        |     |      |     |      |         |     |      |     |      |         |
| Missense            |     |      |     |      |         |     |      |     |      |         |
| –                   | 650 | 92.3 | 71  | 85.5 | 0.03    | 618 | 85.7 | 48  | 72.7 | 0.01   |
| +                   | 54  | 7.7  | 12  | 14.5 |         | 103 | 14.3 | 18  | 27.3 |
| Silent              |     |      |     |      |         |     |      |     |      |         |
| –                   | 681 | 96.7 | 81  | 97.6 | 0.67    | 644 | 84.5 | 22  | 88.0 | 0.63   |
| +                   | 23  | 3.3  | 2   | 2.4  |         | 118 | 15.5 | 3   | 12.0 |
| Nonsense            |     |      |     |      |         |     |      |     |      |         |
| –                   | 679 | 96.4 | 79  | 95.2 | 0.56    | 641 | 84.6 | 25  | 86.2 | 0.81   |
| +                   | 25  | 3.6  | 4   | 4.8  |         | 117 | 15.4 | 4   | 13.8 |
| Frameshift          |     |      |     |      |         |     |      |     |      |         |
| –                   | 679 | 89.3 | 25  | 92.6 | 0.59    | 642 | 84.5 | 24  | 88.9 | 0.53   |
| +                   | 81  | 10.7 | 2   | 7.4  |         | 118 | 15.5 | 3   | 11.1 |
| ER/PR status        |     |      |     |      |         |     |      |     |      |         |
| ER+/PR+             | 94  | 18.0 | 31  | 41.3 | <0.01   | 88  | 17.9 | 37  | 35.6 | <0.01  |
| ER+/PR–             | 16  | 3.1  | 4   | 5.3  | 0.33    | 16  | 3.3  | 4   | 3.8  |
| ER–/PR+             | 85  | 16.3 | 14  | 18.7 |         | 76  | 15.4 | 23  | 22.1 |
| ER–/PR–             | 326 | 62.6 | 26  | 34.7 |         | 312 | 63.4 | 40  | 38.5 |

Continued
Table 4 Continued.

| Tumour size (cm) | Alive | Deaths | P-value | Alive | Deaths | P-value |
|------------------|-------|--------|---------|-------|--------|---------|
|                  | n     | %      | n       | %     | n      | %       |
| <1.0             | 488   | 69.3   | 71      | 85.5  | 0.01   |         |
| 1.0–1.5          | 86    | 12.2   | 1       | 1.2   | 84     | 12.6    |
| 1.5–2.0          | 46    | 6.5    | 4       | 4.8   | 45     | 6.8     |
| >2.0             | 84    | 11.9   | 7       | 8.4   | 83     | 12.5    |

Table 5 Univariate and multivariate hazard ratios (HR) and 95% confidence intervals (CI) for breast cancer-specific and all-cause mortality in relation to p53 protein overexpression and p53 mutations (missense, nonsense, silent and frameshift/in-frame) among LIBCSP case women diagnosed with a first primary breast cancer in 1996–1997 and followed up for vital status until 2002.

Breast cancer-specific mortality

|          | Age-adjusted HR (95% CI)* | Multivariate-adjusted HR (95% CI)† |
|----------|---------------------------|-----------------------------------|
| p53 overexpression | 1.00 | 1.00 |
| Any p53 mutation | 1.00 | 1.00 |
| p53 mutations | Missense | 1.69 (1.01–2.81) | 1.04 (0.59–1.85) |
|                  | 1.97 (1.07–3.64) | 1.17 (0.56–2.44) |
| Nonsense | 1.00 | 1.00 |
| Silent | 2.98 (1.09–8.12) | 1.51 (0.53–4.32) |
| Frameshift/in-frame | 1.00 | 1.00 |
| +        | 0.78 (0.19–3.17) | 0.57 (0.14–2.34) |

All-cause mortality

|          | Age-adjusted HR (95% CI)* | Multivariate-adjusted HR (95% CI)† |
|----------|---------------------------|-----------------------------------|
| p53 overexpression | 1.00 | 1.00 |
| Any p53 mutation | 1.00 | 1.00 |
| p53 mutations | Missense | 1.53 (0.99–2.35) | 1.04 (0.64–1.69) |
|                  | 2.04 (1.24–3.36) | 1.28 (0.72–2.28) |
| Nonsense | 1.00 | 1.00 |
| Silent | 2.11 (0.78–5.72) | 1.23 (0.45–3.42) |
| Frameshift/in-frame | 1.00 | 1.00 |
| +        | 0.71 (0.23–2.25) | 0.86 (0.27–2.72) |

*Adjusted for age.
†Adjusted for age, ER/PR status, type of mutation (missense, nonsense, silent and frameshift/in-frame) and tumour stage.

the presence of any p53 mutation increased the HR for breast cancer-specific mortality by 1.7-fold (HR = 1.7, 95% CI = 1.0–2.8); p53 missense mutations increased the risk of breast cancer-specific mortality by two-fold (HR = 2.0, 95% CI = 1.1–3.6) and of nonsense mutations by three-fold (HR = 3.0, 95% CI = 1.1–8.1). Other p53 mutations had no effect. For all-cause mortality, any p53 mutation was associated with a 1.5-fold increased risk of mortality (HR = 1.5, 95% CI = 1.0–2.4) and missense mutations, with a doubling of the risk (HR = 2.0, 95% CI = 1.2–3.4). Further analysis of the effect of p53 mutations on survival revealed that missense mutations in the DBD were responsible for the effect, whereas the effect of missense non-DBD mutations was not significant (data not shown). These strong associations, however, did not remain after adjusting for ER/PR status (HR = 1.2, 95% CI = 0.6–2.4 for breast cancer-specific mortality and HR = 1.3, 95% CI = 0.7–2.3 for all-cause mortality).

When stratifying by ER/PR status (data not shown), nonsense mutations were positively, but not statistically significantly,
associated with breast cancer-specific and all-cause mortality (HR = 2.0, 95% CI = 0.7–13.3 and HR = 2.2, 95% CI = 0.5–9.2) among women with ER+/PR+ tumours only. There was no association between nonsense mutation and mortality among women with OR+/PR− tumours (HR = 1.0, 95% CI = 0.2–3.4 and HR = 0.6, 95% CI = 0.2–2.7, respectively). There were no associations with the other mutation types after stratifying by ER/PR status, but these results are limited by the small numbers of subjects in some strata.

Discussion

In the present study, we observed a significant association between the presence of overexpression of p53 protein assessed by immunohistochemistry and p53 mutations, particularly missense mutations. However, in the multivariate analysis, adjusting for age, ER/PR status, tumour stage and type of mutation, we failed to demonstrate a role for either mutations in p53 or p53 protein overexpression in breast-cancer specific or all-cause mortality.

When compared with the IARC database, we observed a lower proportion of missense mutations (53.0% vs. 73.3%) and a higher frequency of silent and frameshift/in-frame mutations (19.2% vs. 4.7% and 17.2% vs. 10.5% for our study and for the IARC database, respectively). For mutational screening, we selected a method based on the use of Surveyor nuclease (Transgenomic, Omaha, NE, USA) previously shown to have a high sensitivity, comparable with the ABI 3100 capillary sequencer [17]. The method has been successfully used in various applications, including detection of heteroplasmic mitochondrial DNA mutations [21], detection of mutations in the hCDC4 gene in patients with acute myeloid leukaemia [22] and p53 mutations in exons 5–8 in patients with haematological malignancies [23]. Owing to the decreasing costs of sequencing, future studies will not be required to utilise this pre-screening method because it is no longer cost-effective.

Immunohistochemical staining for p53 protein provides information on the concentration and localisation of the protein. In our study, we detected p53 overexpression in 36% of tumour samples. This frequency is comparable with other studies that reported positive p53 immunostaining in 30–40% of samples [24–29]. Immunohistochemical staining is widely used; however, the method is semiquantitative and subjective and the results may depend on the threshold set during scoring. Moreover, a recent study [30] reported that the results could be strongly affected by the concentration of antibody used for staining, even reversing observed relationships.

However, in our study, we observed a significant association between p53 mutations and p53 protein overexpression even if various cut-points for determination of p53 positive staining were used. A further analysis revealed that missense mutations were responsible for this association, whereas nonsense, silent and frameshift/in-frame mutations had no effect on immunohistochemistry results. A significant correlation between p53 overexpression and p53 mutations was observed by others [24, 31, 32], although some authors report no correlation between these two parameters [33, 34].

ER/PR status is an important molecular marker of breast tumours with both prognostic and predictive functions [12, 35]. ER/PR-positive tumours are usually better differentiated and have better prognosis and survival rate [36]. We observed a significant association between ER/PR-negative status and p53 protein overexpression, which is consistent with the results of at least one [37, 38], but not all other, studies. We assume that any discrepancy across studies may be caused by the reported variability of the p53 immunohistochemistry assay [30].

We observed a borderline difference in p53 protein overexpression between in situ and invasive breast cancer, thus confirming the results of others [24]. Some studies, however, did not find any difference [25, 26, 39]. The study of Wernberg et al. suggests that p53 expression reflects grade rather than invasiveness of the disease [39].

The association of p53 mutation status with other clinical and tumour characteristics including ER/PR status has been observed in a number of studies [40–44]. Similar to these results, our study found women with ER/PR-negative tumours had almost a fourfold higher risk of having p53 mutations than women carrying ER/PR-positive tumours.

Mutations in p53 are correlated with higher histological grade of a tumour [41, 42]. It has been shown that mutations occur in ductal carcinoma in situ before the development of invasive breast cancer, and that their frequency increases with higher grade of the disease [45]. Although p53 mutations are more frequent in advanced breast carcinoma [33, 46, 47], we observed a slightly higher prevalence of p53 mutations among those with invasive versus in situ disease, but the association was not statistically significant, which could be due to the substantially lower number of cases with in situ disease in our sample.

Whether the association between p53 mutations and other clinical parameters of poor prognosis is causal (e.g. p53 mutations lead to the development of tumours with a poor prognosis), has a shared aetiology (an upstream event/exposure/genetic profile), but not p53 mutations, causes a woman to develop a tumour with multiple indicators of poor prognosis) or reflects a shared behavioural trait (little or no screening for breast cancer results in the diagnosis of a tumour with multiple indicators of a poor prognosis) is not clear. Thus, it is also unclear whether statistical analyses undertaken to examine the effect of p53 mutations on mortality should be adjusted for the possible confounding effects of hormone receptor status or other clinical indicators of poor prognosis. Because it is possible that clinical indicators (other than p53 mutations) are on the causal pathway between the exposure (p53 mutations) and the outcome (mortality), adjustment for causal intermediates would be inappropriate and could yield biased results [48]. Currently, there is no clinical evidence to rule out that these clinical parameters are not on the causal pathway linking p53 mutations and mortality. Thus, the most prudent course is to report our findings, both unadjusted and adjusted, for
these other clinical parameters. However, our strong positive findings for the association between p53 mutations and mortality are only evident when we do not adjust for other clinical parameters, such as hormone receptor status.

A number of clinical studies have analysed the effect of p53 mutations on breast cancer survival [10, 11, 26, 41–44, 49] and reported that the presence of p53 mutations was associated with poorer survival, although most are based on small case series of breast cancer patients (which yield unstable results). From these studies, only two were population-based [26, 49], and two were adjusted to hormone receptor status [42, 43]. In our large population-based sample, however, once adjustments were made for ER/PR status, there was no effect of mutations on survival. To our knowledge, only two studies larger than ours have been published on the prognostic value of p53 mutations in breast cancer [11, 42]. In the first, a hospital-based case series of Australian women reported by Powell et al. [42], 1037 breast tumours were screened for mutations in exons 4–8 by a PCR-SSCP method and 178 samples with mutations were confirmed by sequencing. Although an association between p53 mutations and ER/PR negative tumours was observed, in contrast to our study, in the final multivariate analysis, based on 675 cases, both p53 mutations and OR status were significant predictors of poor survival. The second large study, by Olivier et al., was recently published and is based on a pooled analysis of 1794 breast cancer patients with 308 mutations in p53 [11]. The samples originated from 10 hospitals in seven European countries and mutation detection was done in five different laboratories using three different screening methods, with the exception of one cohort where direct sequencing of cDNA was used. The methods used inevitably differ in sensitivity, which may impact on the number of successfully detected mutations. They also found that in multivariate analyses based on 1470 patients, p53 mutations interacted with PR-negative status to significantly increase the risk of breast cancer-specific death.

Unlike these previously published large studies, we found that p53 mutations did not have an independent prognostic value in breast cancer, nor was there an effect on mortality among women with hormone receptor-negative tumours. We cannot find any easy explanation for these discrepancies. Although the large sample size of both studies might explain why their results differed from previously published substantially smaller studies, our sample size was still relatively large (and actually larger than the final sample analysed in the Australian study). The differences between the studies might be attributed to different screening methods for p53 mutations utilised across these internationally based studies. Or perhaps, the differences in the sampling strategies used to assemble the study populations could contribute to the differences in the results. For example, our study is a population-based sample, drawn from over 33 hospitals in a single geographic area in the northeastern United States. In contrast, the two previous reported studies are based on hospital-based case series, drawn from multiple institutions across Europe or several institutions in Australia. The results derived from the hospital-based series may be influenced by the large number of referral patients with more advanced stages of disease who have sought care at these higher quality institutions; the much higher mortality rate of 20% reported in the Australian case series [42], as compared with the 10% mortality observed among our population-based sample, is consistent with this possibility. Thus, the previously published reports are more likely to reflect the experience of very ill breast cancer patients who are referred to large hospitals, whereas our findings may be more applicable to women in the general population. Also, we have to take into account the possible effect of the stage of the disease on the frequency of p53 mutations. The tumour samples analysed in our study were obtained from women with probably less advanced stage than those of women from hospital-based studies. p53 mutation frequency increases with advanced stages of breast cancer. Therefore, our estimate of p53 mutation frequency may be lower than that in both the previously mentioned studies. We cannot rule out the possibility of misclassification of p53 mutation status in our study and its impact on breast cancer survival analysis. The tumour tissues obtained after the initial surgery may have been without p53 mutations, whereas the tumours that re-emerged later and caused the death of patients likely carried p53 mutations. For the survival analysis, DNA extracted from tumours from initial surgery was used. Thus, tumours classified as p53 mutation-negative may have been incorrectly associated with poor survival. In more advanced tumour samples obtained in hospital-based studies, this misclassification is less likely. Alternatively, the differences in study findings could be due to differences in the underlying genetic profile of these diverse populations. For example, a recent study found that the association between tumour p53 mutation status and breast cancer was modified by polymorphisms in MDM2 [50]. Tumour p53 status was not associated with survival among carriers of the variant MDM2 SNP309 allele (G/T or G/G), suggesting that other factors may impact the effects of p53 on survival.

Studies investigating the relationship between p53 overexpression and breast cancer survival have also produced conflicting results [10, 35, 51]. Some studies, including ours, reported no relationship with breast cancer survival [38, 52, 53], whereas other authors found a significant decrease in survival associated with p53 expression [54–56].

In our study, we analysed p53 mutations only in exons 5–8, which is sufficient due to the fact that these exons contain >90% of the mutations reported in breast cancer [11]. Although our sample size was smaller than that in the two previously mentioned studies which used p53 mutation analysis, our study has several advantages. First, unlike the study by Olivier et al. [11], all the samples were processed and analysed in one laboratory with one screening method, resulting in maximal reliability of the data. Second, p53 protein overexpression was assessed and correlated with p53 mutation data in the same study in a larger sample size than was previously studied. Lastly, our tumour tissue was drawn from a population-based sample of women diagnosed with a first primary breast cancer who reside in two counties in New York, rather than from a case series drawn from single hospitals from multiple countries in Europe or Australia.
In conclusion, in our population-based sample of women with breast cancer, p53 protein overexpression was associated with the frequency of p53 mutations in tumour tissue, but neither marker was associated with survival, once we adjusted for the effects of hormone receptor status. Although p53 mutations, particularly missense mutations in DBD, may have some impact on survival, in our study, they did not appear to be a major independent prognostic factor.

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