**Prognostic value of p53 molecular status in high-risk primary breast cancer**


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Received 4 July 2002; revised 14 November 2002; accepted 3 December 2002

Background: Mutations in the p53 gene are the most common genetic alterations in human primary breast carcinoma and these mutations are often associated with worse prognosis and chemoradioresistance.

Patients and methods: The analysis of the p53 gene was performed by fluorescence-assisted mismatch analysis in 13 consecutive high-risk primary breast cancer (HR-BC) patients with 10 or more involved axillary nodes to evaluate its prognostic value.

Results: Three p53 mutations (23%) and four allelic variants were detected. After a median follow-up of 52 months the HR-BC disease-free survival (DFS) was 51% and overall survival 79%. All patients harboring a p53 mutation (p53mut) relapsed within 10 months of the median DFS while 67% of those showing a wild-type p53 status (p53wt) survive disease-free at a median follow-up of 43 months. One p53mut patient is still alive while all the p53wt patients survive at 56 months median follow-up. Two out of the four p53wt relapsing breast cancer patients showed the Arg72Pro allelic variant; one of these died at 75 months.

Conclusions: p53 mutations may help identify a subset of very high risk breast cancer patients (vHR-BC) with worse prognosis.

Key words: breast cancer, FAMA, p53, prognostic factors

**Introduction**

The extent of nodal involvement has long been considered to be the most reliable indicator of prognosis in breast cancer patients and has been used as the decisive tool in therapeutic decision-making.

High-risk, node-positive breast cancers (HR-BC) with ≥10 involved axillary lymph nodes show a 5-year disease-free survival (DFS) of 30–40% and 5-year overall survival (OS) ranging between 50% and 71%, despite different adjuvant treatments [1–4]. The wide distribution of survival intervals (DFS and OS) may be due to the different extent of micrometastatic involvement at the time of diagnosis as well as to the biological aggressiveness and sensitivity of cancer cells [5, 6]. The identification of a different subset of very high risk (vHR-BC) patients using molecular determinants could help to better address therapeutic strategies (i.e. conventional, high-dose, experimental).

p53 gene inactivation represents a critical step in the development of human malignancies due to its pivotal role in multiple cellular pathways such as cell cycle control at the G1/S checkpoint, DNA repair, programmed cell death and neoangiogenesis [7]. p53 mutations characterize 20–40% of breast cancers [8, 9]. Most reported studies, in which different molecular diagnostic approaches were used, have established that p53 molecular status is an independent marker of poor prognosis in breast cancer patients, while p53 status assessed by immunohistochemistry has shown conflicting results [10–22].

Semiautomatic scanning approaches based on chemical cleavage of mismatch, such as fluorescence assisted mismatch analysis (FAMA), show optimal diagnostic accuracy in detecting mutations of large and complex genes such as BRCA1 [23, 24]. In the present study, complete analytical scanning of the p53 gene coding sequence (exons 2–11) was performed by FAMA in tumor DNA samples from 13 HR-BC patients in order to prospectively assess its prognostic value.

**Patients and methods**

Patient characteristics

Between 1991 and 1999, samples of histologically proven primary invasive breast cancers were collected during surgery at the Division of Oncologic Surgery, S. Salvatore Hospital, L’Aquila, Italy. Pathological staging fulfilled American Joint Committee on Cancer classification criteria [25]. Thirteen consecutive HR-BC samples were selected for p53 analysis.
Quadrantectomy plus adjuvant radiotherapy or modified mastectomy combined with axillary dissection were the primary treatments. All patients received systemic adjuvant chemotherapy at the end of which patients with positive estrogen receptor (ER) status were also treated with hormone therapy (Tamoxifen). Recurrent cancers were treated with systemic chemotherapy. Clinical staging consisted of serum chemistry including tumor markers (CEA and CA 15-3), radiologic imaging using X-ray, ultrasounds and computed tomographic (CT) scans of the abdomen and chest. Bone scans were performed every year. All patients were seen every 3–6 months for 5 years according to standard follow-up procedures; after 5 years, they were seen on a yearly basis. Follow-up was carried out at the Division of Medical Oncology, S. Salvatore Hospital, L’Aquila.

Analysis of p53 status

Breast cancer tissue fragments and nearby normal breast tissue were immediately snap-frozen and kept at −80°C. Genomic DNA was obtained by proteinase K digestion and phenol/chloroform extraction [26]. Complete analytical scanning of the p53 coding frame (exons 2–11) was planned by FAMA, a semiautomated scanning procedure based on chemical cleavage of mismatch [24], which has recently been demonstrated to guarantee optimal diagnostic accuracy in scanning large PCR amplicons, up to 1.4 kb in length [23], also at the somatic level [27]. Altogether, the p53 scanning strategy was based on fluorescent amplification of four different DNA regions: two amplicons covering exons 5–9 (p53.1, 1264 bp, spanning exons 5–7 and adjacent introns, and p53.2, 831 bp, spanning exons 8–9 and flanking introns) were designed as already described [27]; one covering exons 2–4 (p53.3, 1078 bp), and one covering exons 10–11 (p53.4, 1303 bp).

Each of the following fluorescent primers was selected in intronic DNA sequences (≥100 nucleotides away from exons) and contained a ‘GG’ dinucleotide at the 5’ end as a spacer between the fluorophore and the DNA sequence: p53.1 forward/5'-FAM-gttcagaaaggtcctaa-3'; p53.1 reverse/5'-HEX-ggtatagaaatgattga-3'; p53.2 forward/5'-FAM-gttcatacttccggg-3'; p53.2 reverse/5'-HEX-ggaagatctctcagtcg-3'; p53.3 forward/5'-FAM-gggatgccctccttgatg-3'; p53.3 reverse/5'-HEX-gggtgtttggtgggatgc-3'; p53.4 forward/5'-FAM-ggctttttgtacgctataa-3'; p53.4 reverse/5'-HEX-ggacagagaaggtcctac-3'.

Polymerase chain reactions (35 cycles: 30 min at 90°C, 30 min at 57°C, 1:20 min at 72°C) were performed using 200 ng of genomic tumor DNA; 10 pmol of primers labeled at the 5’ end with 6-FAM or HEX fluorophore; 200 µM dNTPs (Amersham Pharmacia, Buckinghamshire, UK); 1× PCR buffer [Tris 50 mM, pH 9.2, (NH₄)₂SO₄ 16 mM, MgCl₂ 2.25 mM] (Sigma, St.Louis, MO, USA) and AmpliTaq DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) 1.25 U/µl in a total volume of 25 µl.

The FAMA protocol for p53 mutation scanning was as previously described [27]. Briefly, after heteroduplex formation, 0.2 pmol of fluorescent PCR product were subjected to chemical cleavage reaction using either hydroxylamine or osmium tetroxide, which interact with cytosines and thymines, respectively, at the level of mismatched nucleotides along heteroduplex DNA molecules.

The reaction products were loaded onto a 4% polyacrylamide denaturing gel and electrophoresis was performed on an ABI PRISM 377 DNA Sequencer (Applied Biosystems). The electrophoretic fluorescent profiles were analyzed using the GeneScan 3.1 software. All the mismatches observed by FAMA analysis were confirmed by semiautomated sequencing analysis using Big Dye Terminator Kit (Applied Biosystems) according to the manufacturer’s instructions.

Statistical analysis

Disease recurrence was defined as either local recurrence or metastatic disease. Time to recurrence was defined as the period from surgery to first recurrence, and survival time, defined as the interval from surgery to death, were calculated by the Kaplan–Meier method [28] and compared according to the p53 status (mutated versus wild-type).

Analysis of survival data was planned after the detection of metastatic disease in 50% of patients and performed each year thereafter.

Results

Complete analytical scanning of p53, by FAMA, was performed in 13 HR-BC samples (eight ER+/five ER–). Table 1 shows the clinical and pathological features of the HR-BC patients and their respective p53 molecular status: altogether, three mutations (23%) and four allelic variants were detected. Of the three p53 mutations, two were missense (Ala159Pro; Pro278Arg) and one was a nonsense (Gln192STOP) mutation. Two of these p53 mutations were observed in ER-negative patients. The allelic variant (CCG12139CCC, Arg72Pro) was detected in four samples: M11, M153, M36 and M187. The latter two also harbored a p53 pathologic mutation. Figure 1 shows the exon 5/p53 mutation (Ala159Pro), detected by FAMA, in M187. The chemical cleavage of mismatched nucleotides in fluorescently labeled heteroduplex DNA molecules, obtained from the random hybridization of normal and mutated p53 alleles, gives rise to a specific fluorescent pattern of mutation signals at the gel image level as well as in electropherograms. The mutation was also confirmed by direct sequencing.

After a median follow-up of 52 (range 26–111) months, seven patients relapsed giving a 51% DFS [95% confidence interval (CI) 23% to 80%] (Table 2). At a median DFS of 38 months the survival difference between p53mut and p53wt was statistically significant (P = 0.02). The three HR-BC patients harboring a p53 mutation (p53mut) relapsed within a median of 10 (range 4–23) months (Table 1); four patients showing a wild-type p53 status (p53wt) relapsed giving a 67% DFS (CI 55% to 98%). Two of the four relapsing p53wt HR-BC patients showed the p53 allelic variant Arg72Pro: they relapsed at 29 and 44 months. Two patients died, giving an overall survival of 79% (CI 52% to 100%). The median time-to-death was 28 months. Two p53mut patients died and one was still alive (33%; CI 0% to 88%) after median follow-up of 32 (range 26–52) months; all 10 p53wt patients survived (100%) after 56 months median follow-up (range 28–111 months). This survival difference was statistically significant (P = 0.01). One p53mut patient showing the p53 allelic variant Arg72Pro relapsed at 44 months and died at 75 months.

Discussion

In this subset of HR-BC patients, the 23% prevalence rate of p53 mutations, detected by FAMA, is in the range of that reported by other groups using different molecular diagnostic approaches [8, 9]. Preliminary findings of p53 mutations detected by FAMA in breast cancer patients in our group showed an equivalent rate indicating that the p53 mutation rate does not vary significantly according to stage of disease [27] and nodal status [22]. p53 analysis, by FAMA, confirms that this technique is able to accurately detect and localize mutations even in somatic tumor cells,
Table 1. Clinical and pathologic features of HR-BC patients

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AA, amino acids; DFS, disease-free survival; duct, ductal carcinoma; ER, estrogen receptor; lob, lobular carcinoma; mut, mutated; n, positive lymph nodes; ntot, total examined lymph nodes; OS, overall survival; T, tumor size (TNM staging system); wt, wild-type.

Figure 1. Profile of the p53 exon 5 GCC13155CCC (Ala159Pro) mutation by FAMA. (A) Gel image and electrophoretic profiles of a mutated sample and a wild-type sample showing two extra bands (arrows) on complementary DNA strand (green, antisense; blue, sense) involving C nucleotides (C lanes, samples treated with hydroxylamine; T lanes, samples treated with osmium tetroxide) and corresponding fluorescent peaks. *T cleavages corresponding to a DNA T-stretch along the p53 sequence. (B) Sequence of the heteroduplex with evidence of the mismatch. (C) Evidence of the mutation (arrow) using direct sequencing in the mutated sample.
thus representing a clinically relevant molecular diagnostic approach.

After a median follow-up of 52 months, the survival rates (51% DFS and 79% OS) relative to this small but specific cohort of HR-BC patients correspond to those reported in patients with 10 or more involved axillary nodes, treated with conventional and high-dose chemotherapy [3]. The role of high-dose chemotherapy in vHR-BC patients is currently under evaluation in randomized phase III trials and remains questionable.

The meta-analysis of published studies concerning the prognostic value of the molecular detection of p53 mutations shows a relative hazard estimate of death of 2.0 (CI 1.7% to 2.5%) in all breast cancer patients and 2.6 (CI 1.7% to 3.9%) in node-positive breast cancer patients [29]. The prognostic evaluation according to p53 molecular status (Table 2) in the cohort of HR-BC patients shows a statistically significant, worse prognosis in the p53mut patients in terms of DFS and OS at 52 months median follow-up, thus identifying a very high risk breast cancer subset: each of the three patients relapsed within 2 years; two out of three patients died within a median of 32 months. Conversely, in the p53wt subset 67% of patients are disease-free and all are alive at 52 months. High-risk breast cancers represent a wide range of different and unmeasurable micro-metastatic diseases [3]. Thus, in this subset of patients (vHR-BC) the detection of p53 mutations may exert its prognostic value as a molecular factor of biological aggressiveness as well as a clinical indicator of early metastatic disease [29–31]. Furthermore, the present observation of long-surviving p53 wt HR-BC patients as well as the comparable survival rates already reported in node-positive and negative p53 wt breast cancer patients [22] requires the need to redefine the prognostic relevance of nodal involvement in the p53wt subset.

Four patients show the exon 4/Arg72Pro allelic variant and therefore we suggest considering the functional implications of specific p53 mutations, or allelic variants, in terms of prognosis and treatment-response prediction. The Arg72Pro polymorphism affects a proline-rich region of the p53 protein that is essential for triggering p53-dependent apoptosis [2]. Furthermore, in the presence of p53 conformational mutations, the allelic variant frequently reported at this level has been demonstrated to induce a gain of function of the p53 gene, through the inactivation of the p73 gene, that loses its role as a transcriptional regulator and apoptosis trigger [3]. In our cohort of HR-BC patients, two patients carrying a p53 pathologic mutation, associated to the allelic variant, relapsed and one died. Two patients showing the same allelic variant relapsed within the p53wt subset with a median DFS of 36 months (29 and 44 months); one of these died at 75 months. These results preliminarily suggest the possibility of identifying patients with an intermediate prognosis within the p53 wt HR-BC subset.

Present data represent findings specifying the prognostic value according to p53 molecular status in HR-BC patients using a highly accurate molecular diagnostic approach. Further prospective studies will need to better define the prognostic and predictive role of the p53 molecular status in different subsets of breast cancer.

References


