Relating Genotype and Phenotype in Breast Cancer: An Analysis of the Prognostic Significance of Amplification at Eight Different Genes or Loci and of p53 Mutations

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ABSTRACT

Breast cancer heterogeneity can be related directly to its variability at the genetic level. Thus, tumor genotyping could be a valuable approach to define breast tumor subtypes. It has been shown that it is possible to delineate subgroups of breast tumors according to specific sets of DNA amplifications. The aim of the present work was to study the prognostic significance of these DNA amplifications. We studied DNA amplification at eight genes or loci (AIB1, CCND1, EMS1, ERBB2, FGFR1, MDM2, MYC, and RMC20C001) as well as p53 mutations in a series of 640 breast cancer patients who had not received presurgical therapy and analyzed the correlations with survival. DNA amplification was assessed by Southern blotting and was scored positive when exceeding three to five copies. Mutations in the p53 gene were searched by four-color fluorescent single-strand conformational polymorphism, using an automated sequencer. Of the nine genetic alterations tested, four (CCND1, EMS1, FGFR1, and p53 mutations) showed a significant association with reduced disease-free (DFS) and/or overall survival (OVS) in the unselected set of patients by univariate test. Correlations for p53 were found only when selecting mutations in exons 5 or 7. Analysis of node-negative and -positive subgroups of patients showed that MDM2 amplification and p53 mutations bore prognostic significance in node-negative patients, whereas amplification of CCND1, EMS1, and FGFR1 correlated with poor outcome in node-positive patients. Multivariate analysis on an unselected set of patients retained significance for the amplification of EMS1, FGFR1, and MDM2 with DFS, of CCND1 with OVS, and of RMC20C001 with both DFS and OVS. Interestingly, stratified analysis according to nodal status confirmed results obtained in the univariate tests: significance of MDM2 amplification and p53 mutations in node-negative and that of CCND1, EMS1, and FGFR1 in node-positive patients. We also observed an association between the number of genetic alterations observed in a tumor and poor prognosis. Patients with two or more amplified loci had a worsened outcome. Strongly correlating coamplifications such as CCND1 and FGFR1, as well as ERBB2 and MYC, were associated with a significant reduction of patient survival, thus indicating cooperative effects. Our data support the idea that genetic alterations in breast cancer are not only helpful for phenotyping purposes, but can also represent powerful prognostic indicators in the clinical practice.

INTRODUCTION

Breast cancer is a highly heterogeneous disease, and there is a growing body of evidence that this heterogeneity finds its source in genetic variability. This calls for tumor classifications based on the identification of novel biological and/or molecular markers. Although these have been recognized as potentially interesting, they are not yet recommended for routine use in clinical practice (1). Indeed, the clinical management of breast cancer remains based on classical bioclinical markers, namely tumor size/node/metastasis and steroid receptor levels as well as a number of biological or molecular markers. Classical molecular genetics as well as molecular cytogenetics approaches have demonstrated that in breast cancer, almost every chromosome presents at least one site involved in cancer-related genetic alterations (chromosomal losses, DNA amplifications, mutations, or altered DNA methylation patterns (2). As a consequence, the number of genes identified as being qualitatively or quantitatively altered in breast cancer has been steadily rising over the years. This supports the idea that genetic profiling of tumors may be used to delineate phenotypic subsets in breast cancer (3).

In a recent study, we analyzed a cohort of 1875 breast tumor DNAs for the amplification status of 26 genes or markers mapping at 15 chromosomal localizations (4). These genes were selected because they bore functions related to cancer or because they were localized in a chromosomal region known to be frequently amplified. Groups were defined according to correlations observed with classical bioclinical markers as well as associations found among distinct amplification events. Our data showed that it was possible to delineate subgroups of breast tumors according to sets of DNA amplifications. In the present work, we sought to obtain a more complete view of the phenotypic significance of DNA amplifications and therefore investigated their prognostic significance. We therefore studied a cohort of 640 breast cancer patients for whom complete follow-up data could be collected and who corresponded to a subset of the population studied by Courjalt et al. (4). All patients had undergone surgery between 1987 and 1992. DNA amplification events included in this study concerned AIB1 at 20q11, CCND1 and EMS1 at 11q13, ERBB2 at 17q12-q21, FGFR1 at 8p12, MDM2 at 12q13, MYC at 8q24, and RMC20C001 at 20q13. Because the amplification of MDM2 is functionally equivalent to an inactivation of the p53 gene, we searched for p53 mutations by SSCP4 and compared their prognostic significance with that of DNA amplifications.

PATIENTS AND METHODS

Patients. Clinical follow-up data were collected retrospectively on a cohort of 640 breast cancer patients who had undergone surgery at the Cancer Center Val d’Aurelle-Paul Lamarque, Montpellier, France, between 1987 and 1992. All patients included in this study had primary cancer, with unilateral breast tumors showing no macroscopic metastatic disease, and had not received any treatment prior to surgery. Follow-up was of 5 years minimum. Patients who died from causes other than breast cancer were considered as censored observations at the time of their deaths. Seven patients (1.1%) were lost to follow-up.

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4 The abbreviations used are: SSCP, single-strand conformational polymorphism; ER, estrogen receptor; PR, progesterone receptor; DFS, disease-free survival; OVS, overall survival; SBR, Scarff, Blum, and Richardson.
Breast cancer-specific OVS was defined as the time from surgery to death if the patient died from breast cancer or to last contact. Five-year survival rates were estimated, and survival curves were plotted according to Kaplan and Meier (5). Differences between groups were calculated by the log-rank test (6). In multivariate analysis, relative risk of recurrence or death from breast cancer, 95% confidence intervals, and P values for censored survival data were calculated by use of Cox’s proportional hazards regression model (7). All P calculations were two-sided and P was considered significant at <0.05. Different Cox models were built in which only clinical parameters bearing prognostic significance were included. Cox model I (cohort 3) included nodal status, clinical tumor size, SBR grade, and ER status; Cox model II (node-negative patients selected from cohort 2) included clinical tumor size and ER status; Cox model III (node-positive patients selected from cohort 2) included PR status. Clinical parameters were dichotomized as follows: nodal status (≥1 versus no positive lymph node), clinical tumor size (T1–T2 versus T3–T4), SBR tumor grade (1–2 versus 3), ER [low (<10 fmol/mg protein) versus high (≥10 fmol/mg protein)], PR [low (<10 fmol/mg protein) versus high (≥10 fmol/mg protein)]. A stepwise regression analysis was performed that included nodal status, clinical tumor size, SBR grade, ER status, PR status, and the molecular parameters that were significant at the 0.1 level in the univariate analysis. For each molecular parameter, patients with missing information were considered as a separate category and included in the analysis as well. In each case, we verified whether the category “missing information” was associated with a significant prognostic value.

RESULTS

Patients and Clinicopathological Parameters

Three types of analyses were conducted: univariate analysis on the whole population of 640 patients, labeled cohort 1; and multivariate analysis on cohorts 2 and 3 (554 and 459 patients). Cohort 2 corresponded to patients for whom complete information on tumor size, nodal status, ER status, and PR status was available; cohort 3 corresponded to the subset of patients for whom information on SBR tumor grade could also be collected.

Table 1 presents the clinicopathological characterization. The distribution of bioclinical markers was in good agreement with previously reported data, indicating that our cohort was representative of breast cancer patients. DFS and breast cancer-specific OVS rates were estimated and compared by univariate analysis on these characteristics. Statistically significant associations were observed for clinical tumor size, nodal involvement, tumor grading, and ER status with both DFS and OVS. PR status correlated only with OVS. No significant differences were observed for age and histological type.

DNA Amplification and p53 Gene Mutations: Incidences and Correlations

DNA amplification at eight genes or loci, AIB1 (20q11), CCND1 (11q13), EMS1 (11q13), ERBB2 (17q12-q21), FGFR1 (8p12), MDM2 (12q13), MYC (8q24), and RMC20C001 (20q13), was analyzed by Southern blotting, and clinicopathological correlations were assessed (Table 2). These genes or loci were chosen on the basis of three criteria, two of which had to be met: amplification in at least 5% of the analyzed tumors; existence of positive correlations with clinicopathological parameters; or proven oncogenic properties. CCND1 amplification correlated with ER and PR positivity, nodal involvement, and invasive lobular carcinoma; EMS1 amplification correlated with ER and PR; ERBB2 with loss of ER and PR and carcinoma of the ductal

\footnote{Several of the patients belong to more than one group.}
type; and MYC with loss of ER and PR. Trends of association were observed for AIB1 and FGFR1 with the presence of ER. Correlations between amplifications occurring at nonsyntenic loci were also found.

As described previously, strong correlations were observed between amplifications of CCND1 and/or EMS1 (11q13) and FGFR1 (8p12), MYC (8q24) and ERBB2 (17q21), as well as MDM2 (12q13) and AIB1 (20q11) (4, 8). In addition, we searched for p53 mutations at the genomic DNA level using fluorescent PCR-SSCP. We screened exons 5, 6, 7, and 8, and overall, 14.1% of tumors presented variant migrating bands when analyzed by SSCP in either exon (Table 2). The occurrence of p53 mutations (identified solely on the basis of SSCP) correlated with loss of ER and PR, as well as high-grade tumors. Noticeably, no positive correlation was observed between p53 mutations and amplification of any of the tested loci. On the contrary, an exclusion was observed between p53 mutations and MDM2 amplification.

**Prognostic Significance of Genetic Alterations**

**Univariate Analysis.** We next sought to evaluate how genetic alterations related to patient’s outcome. Univariate results are shown in Table 2. Amplification of CCND1, EMS1, and FGFR1 correlated with reduced DFS and OVS, whereas p53 mutations (for exons 5 and 7; no correlation was observed when all exons were considered) correlated with reduced OVS only. Amplification of MDM2 presented a trend of association with DFS, and that of the RMC20C001 locus presented a trend with both reduced DFS and OVS. MYC, ERBB2, and AIB1 could not be related to a worsened course of the disease.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosomal localization</th>
<th>Frequency</th>
<th>No. of patients</th>
<th>5-year rate</th>
<th>DFS</th>
<th>Breast cancer-specific OVS</th>
<th>5-year rate</th>
<th>P</th>
<th>DFS</th>
<th>Breast cancer-specific OVS</th>
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<td></td>
<td>89.6</td>
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<tr>
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<td>353</td>
<td>77.0</td>
<td>NS</td>
<td>85.2</td>
<td>NS</td>
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<td>Exons 5, 6, 7, 8 wt</td>
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<td></td>
<td></td>
<td></td>
<td>81.0</td>
<td>88.9</td>
<td></td>
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<tr>
<td>p53</td>
<td>Mutated</td>
<td>17p13</td>
<td>8.9</td>
<td>359</td>
<td>69.4</td>
<td>NS</td>
<td>80.1</td>
<td>0.03</td>
<td>80.1</td>
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</table>

*NS, not significant; wt, wild type.*
Because nodal status bears strong prognostic significance and is a key parameter in clinical management, we searched for correlations in the node-negative (N−) and node-positive (N+) subgroups of patients. In N− patients, MDM2 amplification and p53 mutations correlated with reduced DFS and OVS, whereas amplification of FGFR1, CCND1, and EMS1 correlated with DFS and OVS in the N+ group. In this latter group, we also noted a weak correlation of ERBB2 amplification with OVS (data not shown).

**Multivariate Analyses.** Multivariate analyses were undertaken on cohorts 2 and 3 to evaluate the risk of relapse and death related to each of the nine molecular markers tested here. Each marker was adjusted based on nodal status, clinical tumor size, ER and PR status, and tumor grade. Different Cox models were used according to the population of patients tested, including the clinical parameters that remained significant. Cox model I was used for cohort 3, whereas Cox models II and III were used for the N− and N+ subgroups of patients selected from cohort 2. The relative hazard rates of recurrence and death from breast cancer are shown in Fig. 1 for the significant parameters only. Amplification of EMS1, FGFR1, MDM2, and RMC20C001 were retained in Cox model I for DFS, whereas only CCND1 and RMC20C001 were significantly associated with reduced OVS (Fig. 1). Interestingly the amplification of RMC20C001, which showed borderline significance in the univariate test, correlated with both reduced DFS and OVS in the multivariate analysis. When stratified according to nodal status, MDM2 and p53 mutations (selecting mutations in exons 5 and 7) showed an association with reduced DFS and OVS in N− patients. Similarly, CCND1, EMS1, and FGFR1 correlated with reduced DFS and OVS in the N+ group (Fig. 1).

The independence of the different variables tested here was assessed using a stepwise analysis. As expected, it showed that CCND1 and EMS1 were collinear and could thus not be analyzed concomitantly. Moreover, inclusion of FGFR1 in the stepwise analysis excluded the 11q13 genes and inclusion of the 11q13 genes excluded FGFR1. As shown in Table 3, tumor size, nodal status, tumor grade, and amplification of RMC20C001, MDM2, and EMS1 were the major independent prognostic indicators for DFS. When the selected molecular parameters were ranked according to P, the following relative order was observed: RMC20C001 > MDM2 > EMS1. Parameters selected for OVS were very similar except that PR was selected and EMS1 was replaced by CCND1; their relative rank was RMC20C001 > MDM2 > CCND1. In N− patients, tumor size, ER, MDM2 amplification, and p53 mutations were selected as independent prognostic indicators for both DFS and OVS. In N+ patients, EMS1 amplification was the only molecular parameter selected together with SBR grade for DFS and with PR for OVS (data not shown).

**Concerted Amplification and Clinical Outcome**

The occurrence of preferential coamplifications, illustrated by correlations between amplifications involving non syntenic loci, suggested a possible cooperative effect. This is why we analyzed the disease outcome according to the number of amplified loci. As shown in Fig. 2A (DFS) and Fig. 2B (OVS), patients with tumors presenting no amplification at any of the tested loci did not show a significant difference in survival time compared with those with only one amplified locus. Interestingly, a significant difference was seen when patients showing two or more amplified loci were compared. This was true for DFS as well as OVS. We next tested a cooperative effect for strongly correlating coamplifications. We analyzed the survival rates of patients showing either concomitant amplifications of CCND1 and FGFR1 or coamplification of ERBB2 and MYC to determine whether these fared worse than those with only one of these genes amplified. Coamplifications of CCND1 and FGFR1 as well as of ERBB2 and MYC were associated with significantly lower survival rates. The increased risk associated with the CCND1-FGFR1 coamplification could be evidenced both at the DFS and OVS levels and was strongest in the N− subgroup of patients (data not shown). Interestingly, the survival time of patients with either CCND1 or FGFR1 amplified was intermediate to that of patients presenting the coamplification (Fig. 2C). This contrasted with the ERBB2 and MYC situation. Noticeably, patients bearing either ERBB2 or MYC amplified did not present an increased risk compared with patients showing neither of the two. Only when both genes were coamplified was reduced DFS or OVS demonstrated (Fig. 2D).

**Table 3** Stepwise multivariate analysis in cohort 3 (459 patients)

<table>
<thead>
<tr>
<th></th>
<th>DFS</th>
<th>Breast cancer-specific OVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>1.8 (1.1–3.0)</td>
<td>0.03</td>
</tr>
<tr>
<td>Nodal status</td>
<td>2.1 (1.4–3.1)</td>
<td>0.0005</td>
</tr>
<tr>
<td>SBR grade</td>
<td>2.4 (1.6–3.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PR status</td>
<td>2.3 (1.4–3.9)</td>
<td>0.002</td>
</tr>
<tr>
<td>RMC20C001</td>
<td>4.8 (2.1–11.2)</td>
<td>0.0002</td>
</tr>
<tr>
<td>EMS1</td>
<td>3.4 (1.7–6.7)</td>
<td>0.006</td>
</tr>
<tr>
<td>CCND1</td>
<td>4.8 (2.1–11.2)</td>
<td>0.0002</td>
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<tr>
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<td>3.4 (1.7–6.7)</td>
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<table>
<thead>
<tr>
<th>Relative risk (95% CI)</th>
<th>P</th>
<th>Relative risk (95% CI)</th>
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* a CI confidence interval.
  b Amplified.
amplification of CCND1 correlated with reduced DFS and OVS in the N group. Our results for FGFR1 (8), our data do not associate AIB1 (20q11), CCND1 (11q13), EMS1 (11q13), ERBB2 (17q12-q21), FGFR1 (8p12), MDM2 (12q13), MYC (8q24), and RMC20C001 (20q13), and of mutations of p53. These genetic anomalies were selected because of their frequent occurrence in sporadic breast tumors and because of the existence of clinicopathological correlations (4, 8). The rational of our study was to test whether genetic anomalies that affect different genes or loci bore prognostic significance in different patient subsets. Furthermore, we were interested in verifying whether specific combinations of genetic alterations were associated with a worsened course of the disease.

Of the nine parameters studied, six (FGFR1, CCND1, EMS1, RMC20C001, MDM2, and p53 mutations) correlated with bad prognosis in either the univariate or multivariate analysis on the whole population of patients. Using a stepwise procedure, we could show that RMC20C001, MDM2, and EMS1 were independent prognostic indicators for DFS; interestingly for OVS, EMS1 was replaced by CCND1. Because nodal invasion is a determining factor in clinical management of breast cancer, the prognostic significance of these genetic anomalies was also studied in node-negative and node-positive subgroups of patients. It was noticeable that amplification of MDM2 and mutations of p53 correlated with reduced DFS and OVS in the N− group. Amplification of CCND1, EMS1, and FGFR1 correlated with reduced DFS and OVS in the N+ group.

This is the first time to our knowledge that amplifications of AIB1, FGFR1, and MDM2 have been studied in terms of prognostic significance in breast cancer. Despite its correlation with ER+ breast tumors (8), our data do not associate AIB1 amplification with a worsened course of the disease. Our results for FGFR1 amplification are consistent with recent data by Blancaerts et al. (9), who described an association between increased binding activity of basic fibroblast growth factor receptors and reduced DFS and OVS in breast cancer patients. Similarly, the overexpression of p95/MDM2 in breast cancer has been correlated with poor prognosis in an unselected set of patients (10). Our data thus are in keeping with these observations with the exception that in that study (10), 46% of the breast tumors analyzed were scored positive, which is almost 10-fold higher than our rate of MDM2 gene amplification. MDM2 gene amplification and p53 mutations were the only abnormalities bearing prognostic significance in N− patients, suggesting that both anomalies could have similar phenotypic consequences in breast cancer. This can be related to the role of the MDM2 protein, which is known to act as a negative regulator of the p53 protein by routing it toward active degradation (11). Our results are in concordance with most studies performed at the molecular level on p53 mutations in breast cancer, which have shown a correlation with poor outcome (12–21). Interestingly, in our set of patients, the prognostic significance of p53 was restricted to mutations in exons 5 and 7 (22). The difference in prognostic significance according to which codon or exon is mutated remains disputed; however, our data are in concordance with an analysis suggesting that mutations affecting two regions encompassing codons 163–195 (corresponding to conserved region III and part of exon 5) and codons 236–251 (conserved region IV and exon 7) are associated to a worse prognosis (18, 23).

Other parameters analyzed here have all been studied at variable extents in terms of their prognostic significance, and our data are globally in agreement with published results. Some small differences could be seen however; Tanner et al. (24) reported that amplification of RMC20C001 correlated with reduced DFS in node-negative patients, whereas our data showed evidence of a correlation with bad prognosis in the total population. It is of note that RMC20C001 is an anonymous probe subcloned from a cosmid and that two recently
identified genes map in its vicinity; the transcription factor ZNF217 (25) and the serine threonine kinase STK15 (26). Both genes have been found amplified and overexpressed in 5–10% of breast tumors. We noted that ERBB2 amplification showed only a weak correlation with reduced OVS in univariate analysis in N* patients (data not shown). This does not strongly support the prognostic value of ERBB2 in breast cancer, and this issue has been debated, leading to the proposition that this could be related to the fact that ERBB2 amplification is not an independent factor (27). Some reports have indicated that CCND1 amplification (or that of nearby mapping genes INT2/ HST) has prognostic value in unselected populations (28, 29). In our study, CCND1 and EMS1 were closely associated in terms of prognostic significance, and both correlated with worsened outcome in the N* group of patients. This is in keeping with the literature, which show a prognostic significance of CCND1 in N* patients (28, 30, 31). However, our results are more contrasted for EMS1, because Hui et al. (31) reported a correlation with a worsened course of the disease in N* patients while finding a correlation for CCND1 in the N* group. This dissociation between CCND1 and EMS1 is in contrast with our data, which show that 87% of the tumors amplified for EMS1 show CCND1 amplification as well. This is explained by the fact that both genes map ~1.5 Mb from each other on chromosome 11q13 (32). In line with this, we observed in the stepwise analysis that both variables were colinear. Moreover, we observed that inclusion of FGFR1 in the stepwise analysis excluded both CCND1 and EMS1 and that the reverse was also true, thus indicating that these three markers were linked. This can be related to the frequent coamplification of CCND1-EMS1 with FGFR1 (40% of the tumors amplified for FGFR1 are coamplified for CCND1; Ref. 4). This coamplification can lead to the formation of a hybrid amplification unit (33).

Cooperative Effect of Concomitant Amplifications. It has long been known that the more advanced a cancer is, the more rearranged the genome is (34). We were, therefore, interested in verifying whether there was an association between the number of genetic alterations observed in a tumor and a worsened outcome of the disease. Our analysis of patient survival according to the number of amplified loci revealed that patients whose tumors presented two or more amplified loci had a significantly reduced survival compared with patients showing only one or none at all. We investigated whether this was due solely to the number of amplified loci or whether the type of genes involved made a difference. To address this question, we selected pairs of frequently coamplified markers and studied their relation to disease outcome. The amplifications of CCND1 and FGFR1 as well as ERBB2 and MYC presented a strong correlation in breast tumors, and this could indicate the existence of a selective advantage associated with their coamplification. This hypothesis is supported by our findings showing that concomitant amplification of either CCND1 and FGFR1 or ERBB2 and MYC is associated with a significant reduction of the patient’s survival. It is of note that this cooperative effect was not observed when alternative pairs, such as CCND1 + x (x being any gene other than FGFR1 or EMS1) or ERBB2 + x (x excluding MYC) were tested. This could suggest a cooperative effect. It may also be of interest that neither ERBB2 nor MYC were associated with a worsened course of the disease when tested on their own in our data set.

Genotyping Breast Tumors to Delineate Phenotypic Subsets. Over the past few years, a considerable effort has been made to characterize genetic abnormalities in cancer, the general idea being that tumor genotyping would be valuable in defining cancer phenotypes. In a previous study, we showed that it was possible to delineate subsets of breast tumors according to specific combinations of DNA amplifications (4). The present work allowed us to extend the phenotypic description to prognostic significance. We show here that some of the markers tested presented prognostic significance in specific subsets of patients. This was particularly evident for MDM2 amplification and p53 mutations, which showed a strong prognostic value in the N* subset of patients, or for the amplification of CCND1, EMS1, and FGFR1 in N* patients. During the course of this study, we also made some observations that suggest the existence of correlations clustering in other patients subsets, such as MYC in patients under 50 years or MDM2 in ER* patients (data not shown). Our data constitute an attempt to delineate tumor subsets according to their genotypic specificity. Knowing the complexity of the genetic rearrangements in breast cancer, the nine events studied here probably correspond to a small portion of the genes involved in tumorigenesis. Genotyping of breast tumors will involve the analysis of an ever larger number of parameters and sorting of the significance of complex combinations. Because different combinations of genes or genetic anomalies may bear a meaning in different populations of patients, the analysis of specific phenotypic subsets will be necessary, thus leading to an increase of the number of comparisons. This will require the analysis of very large cohorts of patients (several thousand) and consequently the use of high-throughput analytical methods (35) in association with statistical tools especially devised for multiple-comparison analyses.

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Relating Genotype and Phenotype in Breast Cancer: An Analysis of the Prognostic Significance of Amplification at Eight Different Genes or Loci and of p53 Mutations

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