

## REVIEW ARTICLE

## GENOMIC MEDICINE

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## Breast and Ovarian Cancer

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**D**ESPITE YEARS OF INTENSIVE STUDY AND SUBSTANTIAL PROGRESS IN understanding susceptibility to breast and ovarian cancer, these diseases remain important causes of death in women. However, several recent critical advances — sequencing of the human genome and the development of high-throughput techniques for identifying DNA-sequence variants, changes in copy numbers, and global expression profiles — have dramatically accelerated the pace of research aimed at preventing and curing these diseases. We review some of the important discoveries in the genetics of breast and ovarian cancer, ongoing studies to isolate additional susceptibility genes, and early work on molecular profiling involving microarrays.

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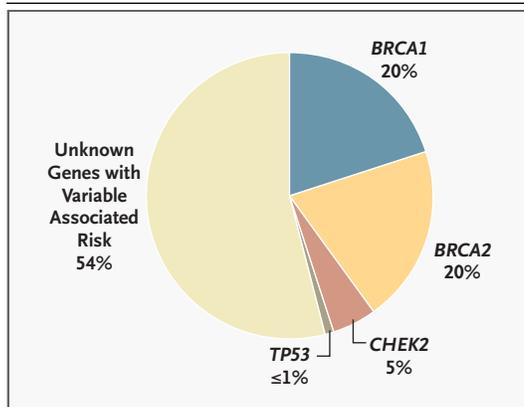
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## SUSCEPTIBILITY TO BREAST AND OVARIAN CANCER

In the United States, 10 to 20 percent of patients with breast cancer and patients with ovarian cancer have a first- or second-degree relative with one of these diseases.<sup>1</sup> Two major genes associated with susceptibility to breast and ovarian cancer — breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) — have been identified to date.<sup>2,3</sup> Mutations in either of these genes confer a lifetime risk of breast cancer of between 60 and 85 percent and a lifetime risk of ovarian cancer of between 15 and 40 percent.<sup>4,5</sup> However, mutations in these genes account for only 2 to 3 percent of all breast cancers,<sup>6,7</sup> and susceptibility alleles in other genes, such as *TP53*, *PTEN*, and *STK11/LKB1*, are even less common causes of breast and ovarian cancer (Fig. 1).

The prediction that there are common DNA-sequence variants that confer a small but appreciable enhanced risk of cancer has been validated with the recent discovery of the 1100delC mutation in the cell-cycle-checkpoint kinase gene (*CHEK2*).<sup>9</sup> This mutation was found in 1.1 percent of women without breast cancer, 1.4 percent of women with a personal but no family history of breast cancer, and 4.2 percent of index patients from 718 families in which two or more members had been given a diagnosis of breast cancer before the age of 60 years but in which there was no detectable *BRCA1* or *BRCA2* mutation. This mutation doubles the risk of breast cancer among women and increases the risk among men by a factor of 10. *CHEK2*, an important component of the cellular machinery that recognizes and repairs damaged DNA, is activated after phosphorylation by the checkpoint gene *ATM* and in turn activates *BRCA1*. The role of *ATM* mutations in the predisposition to the early onset of breast cancer remains controversial, but some missense mutations do appear to increase susceptibility to breast cancer in humans<sup>10</sup> and mice.<sup>11</sup>

There is convincing evidence that additional high-penetrance genes that increase susceptibility to breast cancer exist. In contrast, it has been suggested that, other than *BRCA1* and *BRCA2*, high-penetrance genes that confer susceptibility to ovarian cancer do not exist.<sup>12</sup> An ovarian-cancer-susceptibility locus on chromosome 3p22–25 has



**Figure 1. The Genetics of Breast Cancer.**

*BRCA1* and *BRCA2* mutations occur in approximately 20 percent of families with evidence of inherited susceptibility to breast cancer. Germ-line mutations in *TP53* cause the Li-Fraumeni syndrome and account for no more than 1 percent of cases of familial breast cancer, but women who survive the childhood cancers associated with the Li-Fraumeni syndrome have as much as a 90 percent risk of breast cancer.<sup>8</sup> Mutations in the cell-cycle-checkpoint kinase gene (*CHEK2*) account for about 5 percent of all cases of familial breast cancer (defined by the diagnosis of breast cancer in two or more family members before the age of 60 years), but the risk for individual mutation carriers is probably less than 20 percent.<sup>9</sup> All other cases of breast cancer are presumed to be due to an undefined number of additional susceptibility genes with various degrees of penetrance, exposure to hormonal and environmental factors, and stochastic genetic events.

putatively been identified, but this finding has yet to be confirmed by an independent group.<sup>13</sup>

Many additional genetic variants in low-penetrance susceptibility alleles may moderately increase the risk of breast cancer, ovarian cancer, or both. These genetic variants are much more common in the population than are high-penetrance gene mutations and, thus, in aggregate may make a substantially greater contribution to breast and ovarian cancer in the population than mutations in high-risk genes.<sup>14</sup> However, genetic heterogeneity and the rarity of high-penetrance genes make both high- and low-penetrance genes difficult to identify.

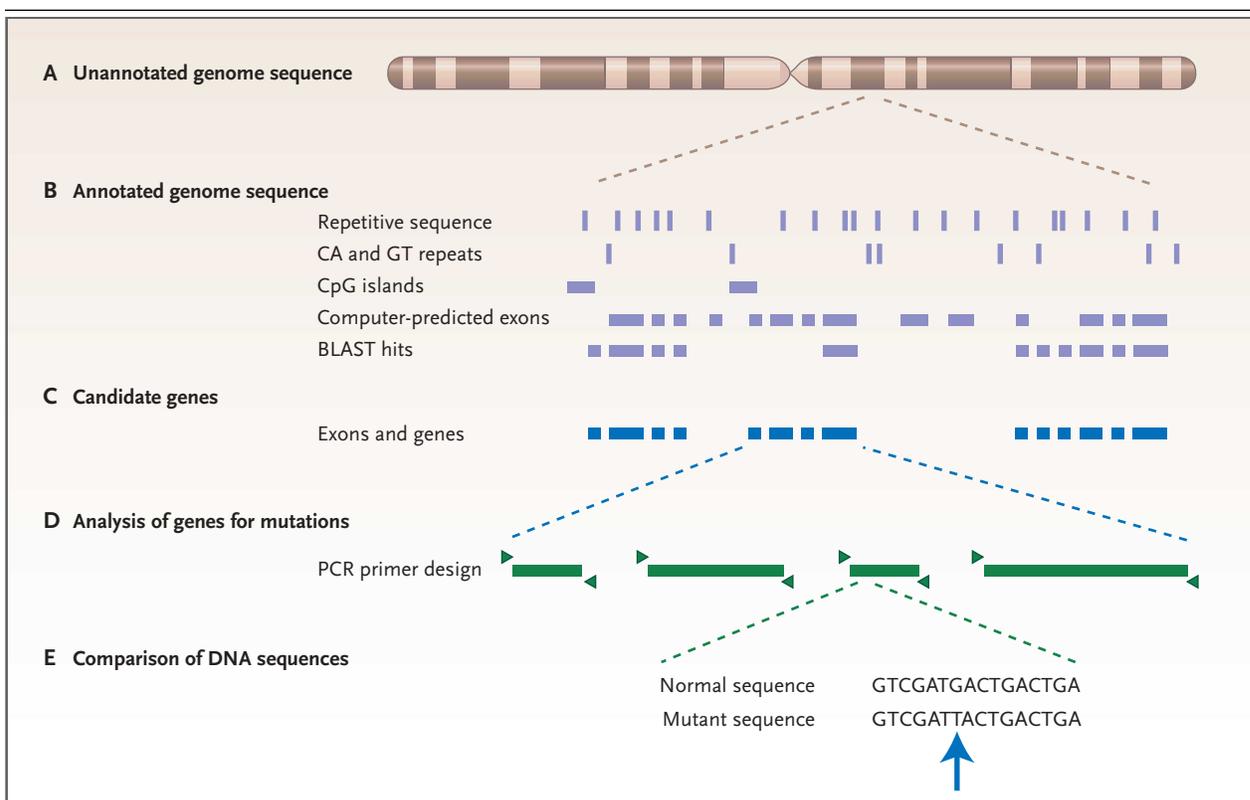
#### IDENTIFICATION OF GENES THAT INCREASE SUSCEPTIBILITY TO BREAST AND OVARIAN CANCER

**IDENTIFICATION OF HIGH-PENETRANCE GENES**  
Genetic linkage was used to identify the *BRCA1* and *BRCA2* loci on chromosomes 17q and 13q, respec-

tively.<sup>15,16</sup> In both cases, no information was initially available on the location, structure, or function of the genes, and they were identified through positional cloning. Loss-of-heterozygosity mapping was of little assistance in either search; however, a homozygous deletion on chromosome 13 in a pancreatic adenocarcinoma helped identify the location of *BRCA2*.<sup>17</sup> Finally, critical data in the search for *BRCA2* came from studies of breast cancer in Iceland, whose population derives from a small group of settlers from Norway and Ireland.<sup>18,19</sup> Such populations share more genetic information than large, admixed populations and have been used successfully many times in gene mapping.<sup>20</sup> After the *BRCA1* locus was identified, it took almost four years to isolate the gene and involved several labor-intensive strategies.<sup>2</sup> By contrast, the *BRCA2* locus was one of the first genomic intervals to be systematically sequenced as part of the Human Genome Project. These data, together with other information about the genes in the region, reduced the time it took to isolate *BRCA2* to two years.<sup>3</sup> Thus, information from the human genome sequence greatly enhances the utility of linkage analysis for gene identification (Fig. 2).

#### BRCA3 AND BEYOND

Several candidate regions for *BRCA3* have been proposed, including chromosome 13q21<sup>21</sup> and chromosome 8p12–22,<sup>22</sup> but both have been strongly refuted by analysis of data from independent families.<sup>23,24</sup> The search for *BRCA3* has been difficult for several reasons. First, ovarian cancer and male breast cancer were recognized as components of syndromes of breast-cancer susceptibility before either *BRCA1* or *BRCA2* was isolated, allowing targeted identification of affected families. Since no such phenotype has been associated with the putative *BRCA3* gene or genes, families in current studies are selected only on the basis of a young age at the diagnosis of breast cancer and the absence of ovarian and male breast cancer. Ideally, these families should have multiple members with early-onset breast cancer and strong evidence against the involvement of either *BRCA1* or *BRCA2*. However, the breast cancers in most such families are in fact due to germ-line mutations in *BRCA1* or *BRCA2*,<sup>25</sup> and those that are not may represent the effects of multiple susceptibility alleles (genetic heterogeneity), reducing the power of linkage analysis. What is needed to further this effort are larger families to increase the statistical power of such studies, as well



**Figure 2. Effect of Sequencing the Human Genome on Gene-Discovery Strategies.**

The annotated DNA sequence of the human genome can be used to locate genes, repeat sequences, and other features and has revolutionized the identification of cancer genes. A sequence without annotation is of limited utility (Panel A). As shown in Panel B, an annotated sequence shows genetic markers such as CA and GT repeats along with other data, such as CpG islands, known genes, genes predicted to exist on the basis of computational models, and Basic Local Alignment Search Tool (BLAST) matches. Using publicly available data (<http://www.ensembl.org>, <http://www.ncbi.nlm.nih.gov>, and <http://www.genome.ucsc.edu>), it is possible to jump from a genetic region of interest to the identification of candidate genes in a matter of seconds and download the relevant data (Panel C). With these data in hand, experiments, such as those involving the polymerase chain reaction (PCR), can be designed to analyze the genes for mutations (Panel D). The final step in the identification of genes is to compare the sequence from patients with the disease of interest with the normal reference sequence to discover the mutations (Panel E).

as novel means of clustering families into subgroups most likely to represent single-gene disorders.

One approach is to classify families with breast cancer according to the molecular profile of the associated tumors. These analyses could be based either on expression profiling or on array-based comparative genomic hybridization, both of which provide unique molecular signatures (Fig. 3). Nonetheless, hundreds of small pedigrees may be needed to identify the BRCA3 locus.

**USE OF THE HUMAN GENOME SEQUENCE TO IDENTIFY LOW-PENETRANCE GENES**

As noted, the susceptibility genes identified to date are not responsible for most breast and ovarian

cancers, leaving a considerable potential contribution from less penetrant genes. One of the implicit problems in isolating low-penetrance genes is that such genes will rarely produce striking familial patterns involving multiple cases that can be used in traditional linkage studies. An additional concern is that very large studies, with statistical power to evaluate multiple interactions between genes, may be needed before genetic profiles involving this class of genes can be used for risk prediction.<sup>14</sup> As the computational methods for finding coding sequences embedded in a sequence of genomic DNA become increasingly powerful, the value of the human genome sequence as a tool for identifying unknown genes also increases. These algorithms for finding

genes have largely replaced laborious experimental techniques to identify potential coding sequences of unknown genes for mutation analysis within linkage regions. These methods are another illustration of the fact that it is the annotation of the genomic sequence (i.e., the identification of genes and their function) that brings the sequence to life. Annotation parses the sequence into genes and noncoding regions. By including genomic features such as CpG islands, which mark the promoter regions of many genes, annotations produce a complete rendering of each sequence. Annotated sequences are publicly available in several data bases (<http://www.ensembl.org>, <http://www.ncbi.nlm.nih.gov>, and <http://www.genome.ucsc.edu>) with associated genome browsers.

The depth and value of annotation have also grown through the addition of millions of single-nucleotide polymorphisms, which are invaluable in the search for susceptibility genes.<sup>26</sup> One such example is the recent demonstration that a silent single-nucleotide polymorphism in *LIG4*, a gene encoding a DNA ligase important in the repair of breaks in double-stranded DNA, is associated with survival among patients with breast cancer.<sup>27</sup> This effect was demonstrated in a British population-based study that included 2430 cases of breast cancer. DNA from these patients was genotyped for polymorphisms in 22 DNA-repair, hormone-metabolism, carcinogen-metabolism, and other genes, and the effect of each single-nucleotide polymorphism on the outcome was assessed by Cox regression analysis. The silent polymorphism D501D (t>c) in *LIG4* had the largest effect. The estimated hazard ratio for death among patients homozygous for the polymorphism, as compared with those homozygous for the wild-type sequence, was 4.0 (95 percent confidence interval, 2.1 to 7.7;  $P=0.002$ ), and this effect remained significant after stratification according to tumor stage, grade, and type (hazard ratio, 4.2; 95 percent confidence interval, 1.8 to 9.4;  $P=0.01$ ). The inclusion of these single-nucleotide polymorphisms in the annotation of the human genome sequence greatly facilitated this analysis, which would otherwise have had to have been preceded by an extensive sequence-based effort to identify single-nucleotide polymorphisms.

#### WHOLE-GENOME APPROACHES TO THE ANALYSIS OF BREAST AND OVARIAN CANCER

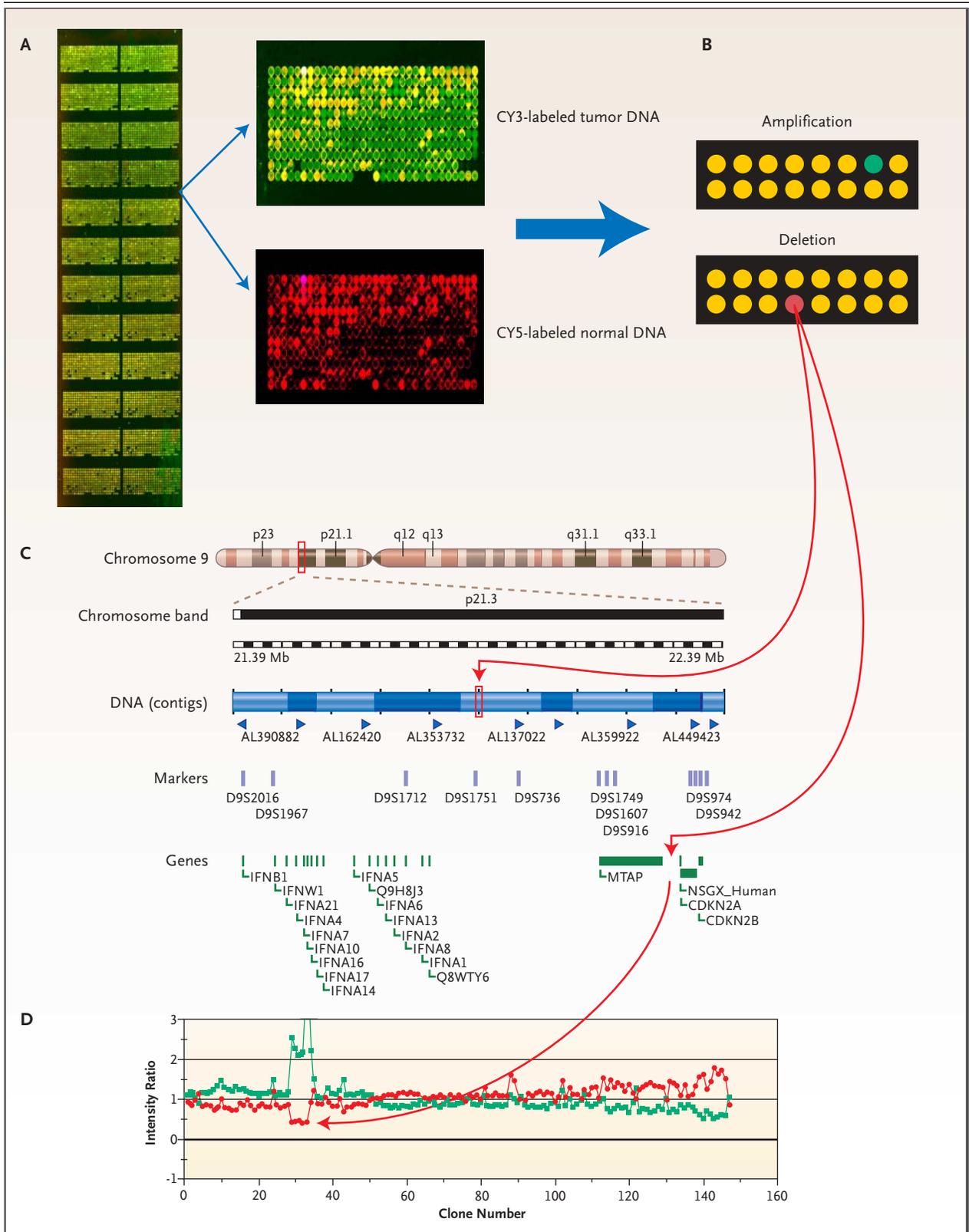
Many genomic approaches to the identification of cancer genes are based on microarray techniques.

For gene-expression profiling, each element usually represents one gene and is created with the use of a complementary DNA (cDNA) or oligonucleotide for the gene in question. Similar arrays have been produced with large genomic clones for array-based comparative genomic hybridization to identify changes in the number of copies of DNA. This approach replaced lower-resolution comparative genomic hybridization of cells in metaphase and provides a direct link to genes in the altered region (Fig. 3).

A comparative genomic hybridization can be used to identify the loss of one or both copies of a given gene as well as regions of amplification. Arrays made with cDNAs can be used for expression profiling and comparative genomic hybridization simultaneously.<sup>28</sup> This approach allows a direct comparison between the number of copies of a gene and the level of expression of that gene, but the results of comparative genomic hybridization may be variable, presumably because the cDNA sequence and the genomic sequence are not collinear. However, the alternative approach of using large cloned segments of genomic DNA in the bacterial artificial chromosomes consistently provides excellent data.<sup>29</sup> The genomic clones can be spaced evenly across the genome, and the array set can be enriched with selected clones that contain candidate cancer genes to enhance resolution. The use of DNA microarrays has been suggested for other applications; however, epigenetic changes such as changes in DNA methylation, which are likely to be a critical

#### Figure 3 (facing page). Array-Based Comparative Genomic Hybridization.

In Panel A, bacterial artificial chromosome clones or complementary DNAs are placed on glass slides at high density; tumor and normal DNA are labeled with CY3 and CY5, respectively; and the combined probe is hybridized to the array. The array is analyzed with use of a laser scanner that reads each color channel individually and then calculates an intensity ratio for each spot. In Panel B, spots with intensity ratios greater than 1.25 (green spots) represent increases in copy number (amplification), and those with intensity ratios of less than 0.75 (red spots) represent decreases in copy number (deletion). Each spot is a DNA segment that can be linked directly to the human genome sequence (Panel C), thus defining changes in the number of copies of a specific gene. In Panel D, the plotting of intensity ratios for the chromosome 9 bacterial artificial chromosome clones on the array in linear order identifies a homozygous loss of *CDKN2A* in a melanoma cell line.



component in the development of cancer, have been notoriously difficult to assay regardless of the format.

The power of a comparative genomic hybridization was recently demonstrated by Albertson and colleagues, who used this approach to map the recurrent breast-cancer amplicon at chromosome 20q12.3.<sup>30</sup> This approach clearly demonstrated that what had previously been described as a single amplicon was, in fact, two distinct amplicons, one containing the putative oncogene ZNF217<sup>31</sup> and the other containing CYP24, which encodes vitamin D24-hydroxylase.<sup>32</sup> The overexpression of this enzyme alters the control of growth mediated by vitamin D. There were two distinct peaks of high copy numbers within this 2-Mb region, with a gene at the peak of each amplicon. The ability of comparative genomic hybridization to show peaks in increases in copy numbers across regions of recurrent abnormality at high resolution is very useful for locating oncogenes in many human cancers.

Much less advanced, but critically important, are techniques involving proteomics, which examine the entire complement of proteins expressed in a specific tissue or cell. The information supplied complements that provided by a comparative genomic hybridization, expression profiling, and screening for mutations in cancer research,<sup>33</sup> since the genetic code does not indicate which proteins are expressed, in what quantity, and in what form. For example, post-translational modifications, such as phosphorylation or glycosylation, may determine the function or stability of a protein and are not detected by transcriptional analyses. Many differences between normal tissue and malignant tumors are due to post-translational modifications, and a complete analysis of the cancer phenotype will require a whole-proteome approach.

Diffuse large  $\beta$ -cell lymphoma was the first human cancer to undergo gene-expression profiling, and a microarray containing 17,800 cDNAs was used.<sup>34</sup> Breast and ovarian cancer have now been subjected to molecular profiling as well. In the first such study, Perou and colleagues used a cDNA microarray containing 8000 genes to assay 65 breast-biopsy specimens, primarily invasive breast cancers.<sup>35</sup> Perhaps not surprisingly, estrogen-receptor status was a key predictor of the outcome and treatment response, with estrogen-receptor and coregulated genes being the primary elements needed for these tumors to cluster. In addition, a profile of tumors that overexpress ERBB2 was easily identified.

Thus, the primary clusters recognized were tumors that expressed estrogen receptor and had a luminal-cell pattern of gene expression, tumors that did not express estrogen receptor and had a myoepithelial-cell pattern of expression, tumors that overexpressed ERBB2, and a fourth group of tumors that clustered with normal breast tissue. More recently, Hedenfalk and colleagues suggested that transcriptional profiling can also accurately differentiate breast cancers with underlying germ-line mutations in BRCA1 or BRCA2 from those without such mutations, an advance that could facilitate the identification of high-risk families on the basis of molecular phenotyping, as well as identify characteristic molecular differences that may be useful clinical targets for directed therapy.<sup>36</sup>

Ovarian cancers have been subjected to transcriptional profiling with similar results. In one series, 27 serous papillary ovarian cancers and 3 samples of normal ovarian tissue underwent gene-expression profiling with oligonucleotide-based arrays representing more than 6000 human genes.<sup>37</sup> Normal ovarian tissue was clearly distinguishable from malignant tissues, and three types of tumors were identified. The first subtype clustered with normal tissue and was well differentiated on conventional histologic analyses. The second group was characterized by the expression of genes from admixed stromal cells and infiltrating lymphocytes. Although this profile could represent a random admixture of cell types, it could also represent an immune response to the tumor, as suggested recently by Zhang and colleagues.<sup>38</sup> The third group of tumors had a high level of expression of cell-cycle-associated genes, most likely reflecting a high proliferative rate. More than half the tumors in this cluster were poorly differentiated on histologic analysis. Which of the differentially expressed genes in this and other series represent the root causes of malignant transformation rather than markers of progression is not known but must be determined in order to distinguish diagnostic markers from therapeutic targets.

Microarrays have also been used to show how an expression profile can change as cancer cells develop resistance to doxorubicin-based therapy.<sup>39</sup> In these experiments, a set of genes that were transiently overexpressed after initial exposure to doxorubicin included a subgroup of genes that became constitutively overexpressed as resistance to doxorubicin developed. These experiments, although just the beginning of what will be a fundamental change in molecular oncology owing to the deci-

phering of the human genome sequence, demonstrate the power of this approach.

In perhaps the most extensive and informative study to date, the expression profiles of 117 primary breast cancers were compared with known prognostic markers and the clinical outcome at least five years after diagnosis.<sup>40</sup> Expression profiling with the use of 25,000 genes separated the tumors into two groups, one in which distant metastases developed in 34 percent at five years and one in which metastases developed in 70 percent at five years. From the original 25,000 genes in the array, 70 were identified as having the greatest accuracy in predicting recurrent disease. When the tumors were sorted on the basis of this smaller set of genes, fewer than 10 percent of the tumors in the poor-prognosis group were misclassified. A comparative multivariate analysis using clinical prognostic factors that included tumor grade, tumor size, the presence or absence of angiolymphatic invasion, patients' age, and tumor estrogen-receptor status demonstrated that as compared with the good-prognosis gene-expression signature, the poor-prognosis microarray profile was an independent predictor of recurrence, with an odds ratio of 18 (95 percent confidence interval, 3 to 94). This approach has now been tested in 295 consecutive patients with stage I or II breast cancer.<sup>41</sup> Of these, 180 had the poor-prognosis profile and 115 had the good-prognosis profile. Ten years after the diagnosis of breast cancer, the probability of remaining free of metastases was 51 percent among women with a poor-prognosis profile and 85 percent among those with a good-prognosis profile. These data provide compelling evidence that the genetic program of a cancer cell at diagnosis defines its biologic behavior many years later, refuting a competing hypothesis that the genetic changes driving the development of metastatic disease are acquired in residual cells after adjuvant treatment.

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CLINICAL MANAGEMENT  
OF INHERITED SUSCEPTIBILITY  
TO BREAST AND OVARIAN CANCER

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Several computational models have been developed to predict an individual woman's risk of breast cancer, including one in which family history is the predominant risk factor. This model, developed by Claus and colleagues and published as a series of tables clinicians can use,<sup>42</sup> is based on the number and degree of relatedness of family members with

breast cancer and their age at diagnosis. However, this model does not provide estimates of the likelihood that an individual woman will have a germ-line mutation in *BRCA1* or *BRCA2*. Several studies have identified factors that are associated with an increased likelihood that a *BRCA1* or *BRCA2* mutation will be identified, including early-onset breast cancer, the occurrence of breast and ovarian cancer in the same woman, a history of male family members with breast cancer, and Ashkenazi Jewish ancestry. These characteristics have also been included in predictive models designed for use by clinicians.<sup>43-45</sup>

Testing for germ-line mutations in *BRCA1* and *BRCA2* is an important tool for predicting the risk of breast cancer and developing management strategies. Once such mutations are identified, we recommend that the woman choose between annual screening mammography and prophylactic mastectomy, which significantly reduced the risk of breast cancer in a small, retrospective study of mutation carriers.<sup>46</sup> We recommend that women who choose surveillance also investigate the possibility of participating in a clinical trial evaluating the utility of magnetic resonance imaging for screening high-risk women. Several studies have shown that in women with germ-line *BRCA1* and *BRCA2* mutations, breast cancers are likely to occur as interval cancers<sup>47</sup> and that standard mammograms are more likely to be negative than in women at low or moderate risk.<sup>48-50</sup>

With respect to the risk of ovarian cancer among carriers of *BRCA1* and *BRCA2* mutations, we strongly recommend that such women undergo prophylactic oophorectomy as soon as they have completed childbearing, since no surveillance regimen to date has been shown to decrease the percentage of women who receive a diagnosis of advanced disease. Among mutation carriers, this procedure has been shown to reduce the risk of breast and ovarian cancer by more than 60 percent and 95 percent, respectively.<sup>51,52</sup> Again, although no prospective data are available, we recommend that these women receive hormone-replacement therapy until the age of 50 years, approximately the time of natural menopause. Although extending exposure to estrogens beyond the age of 50 years has been associated with a small increase in the risk of breast cancer, these younger women would be producing endogenous estrogens in the absence of prophylactic oophorectomy. The addition of hormone-replacement therapy makes this choice acceptable to women who

would otherwise refuse it because of concern about premature menopause, and the risk–benefit ratio is strongly in favor of oophorectomy, with or without hormone-replacement therapy.

The use of tamoxifen to prevent breast cancer in carriers of *BRCA1* and *BRCA2* mutations remains controversial. One retrospective study suggested that adjuvant tamoxifen therapy in carriers of *BRCA1* and *BRCA2* mutations with estrogen-receptor–positive breast cancer reduced the risk of contralateral breast cancers by the same amount as that in unselected patients with breast cancer.<sup>53</sup> However, data showing that most *BRCA1*-associated breast cancers are negative for estrogen receptors<sup>54</sup> and recent data from the Breast Cancer Prevention Trial (BCPT) of the National Surgical Adjuvant Breast and Bowel Project have led to widespread speculation that tamoxifen will not prevent breast cancer in women with germ-line *BRCA1* mutations.<sup>55</sup> It is important to consider that all available data suggest that endogenous exposure to hormones has a central role in defining the risk of cancer among carriers of *BRCA1* mutations, that breast cancer developed in only eight carriers of *BRCA1* mutations in the BCPT, and that the lack of a preventive effect of tamoxifen was statistically insignificant (odds ratio, 1.67; 95 percent confidence interval, 0.41 to 8.00). The length of treatment in the BCPT is also consistent with an early treatment effect, rather than true prevention, which, given the preponderance of estrogen-receptor–negative tumors among carriers of *BRCA1* mutations, would produce the data seen in this study. Thus, we recommend that

carriers of *BRCA1* mutations consider taking tamoxifen once they discontinue hormone-replacement therapy at about the age of 50 years.

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#### SUMMARY

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The past decade has been a period of unparalleled discovery in the field of the genetics and genomics of breast and ovarian cancer. Two major susceptibility genes have been isolated, and subsequent work provided sufficient management information to allow genetic testing for *BRCA1* and *BRCA2* mutations to become a part of routine practice in many clinical centers. In addition, work has begun on the characterization of genetic variants that, although associated with a lower risk of cancer than germline *BRCA1* and *BRCA2* mutations, are far more common in the population and thus may have a substantial role in defining the risk of cancer. Finally, gene-expression profiling, coupled with the sequencing of most or all of the genes in the human genome, is revolutionizing the study of the biology and the molecular classification of breast and ovarian cancer. Combined with data from projects conducting a genome-wide mutation analysis of all genes implicated in the development of cancer, the importance of which has just been illustrated with the discovery that more than 60 percent of melanomas have mutations in *BRAF* (*v-raf* murine sarcoma viral oncogene homologue B1),<sup>56</sup> and progress in developing effective preventive measures, a marked reduction in mortality from breast and ovarian cancer is a realistic goal for the next decade.

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