Poly(ADP-ribose) Polymerase Gene Expression Status and Genomic Instability in Human Breast Cancer

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ABSTRACT
The poly(ADP-ribose) polymerase gene is involved in DNA repair, cell proliferation, differentiation, and malignant transformation. Because dysregulation of PARP expression might lead to genetic instability in human tumors, we examined PARP gene expression and genetic instability in 35 primary human breast carcinomas. Genetic instability was studied by analyzing nine genetic abnormalities among those most frequently observed in breast tumor DNA, including amplification of proto-oncogenes MYC and ERBB2 and chromosome regions 7p, 11q13, and 20q13, and loss of heterozygosity on chromosome arms 1p, 3p, 7q, and 11p. We found a significant link between strong PARP gene expression and low genetic instability ($\chi^2_{corr} = 5.33; P = 0.012$), pointing to a possible involvement of this gene in DNA repair in human breast tumor cells. A trend toward a link between PARP gene overexpression and amplification at 1q41-q44 (the site of the PARP gene) was also observed, suggesting that dysregulation of PARP expression could be due partly to a gene dosage effect.

INTRODUCTION
Genomic instability is a common feature of cancer cells, but the underlying mechanisms are largely unknown. The main forms of genetic instability in breast tumors are amplification of proto-oncogenes (MYC and ERBB2) and chromosome bands 11q13 and 22q13, mutation of p53, and loss of heterozygosity on chromosomes and chromosome arms 1p, 3p, 6q, 7q, 8p, 11q, 13q, 16q, 17, 18q, and 22q (1). Microsatellite instability, which has recently been demonstrated in tumors from subjects with hereditary nonpolyposis colorectal cancer and those with sporadic forms of colorectal and other cancers, is very rarely seen in breast cancer (2).

p53 is the first gene shown to increase genetic instability when altered (3). High levels of overall LOH and gene amplification have been observed in breast tumors with p53 abnormalities (4, 5). These genetic alterations are likely to reflect a malfunction of cell division. Normal cells arrest in G1 before entering the S-phase in response to DNA damage. Cells lacking normal p53 protein do not arrest in G1, and this may lead to accumulation of unrepaired lesions and an increased mutation frequency. However, p53 is clearly not the only major factor involved in the control of genetic stability, since chromosomal alterations have also been detected in tumors without p53 abnormalities (6).

PARP is another gene involved in DNA repair (for review, see Ref. 7) and other cell responses to DNA damage such as cell cycle disturbances (8) and malignant transformation (9). It encodes a nuclear chromatin-associated enzyme which, in the presence of DNA breaks, covalently attaches ADP-ribose from NAD to various nuclear acceptor proteins, and also transfers additional ADP-ribose units onto this initial adduct to form poly(ADP-ribose) (for review, see Ref. 10). PARP protein may be classified as a posttranslational protein modification enzyme. This enzyme mainly modifies itself (autopolymerization, although other proteins, including histones and topoisomerases, also are known to be acceptors for poly(ADP-ribose) synthesis (heteromodification). The branched homopolymer chains can attain a size of 200–300 residues but are degraded by poly(ADP-ribose) glycohydrolase soon after their synthesis. The molecular function of poly(ADP-ribose) synthesis is not clear, although it seems to be required for DNA repair. Satoh and Lindahl (11) have suggested that attachment of unmodified PARP protein to the damaged site interferes with access to the strand break for DNA repair enzymes. After a short period of protection, auto-poly(ADP-ribose)ylation of the protein would then reduce its affinity for DNA and allow DNA repair enzymes to access the lesions.

Decreased levels of PARP have been proposed as a potential risk factor predisposing to the development of malignancies (12). Several studies have suggested that PARP activity falls with age, and this may render aging cells more susceptible to cytotoxic, degenerative, and mutagenic insults (13, 14). Variations in PARP gene expression with age or other conditions might thus contribute to progressive genetic instability and a variety of degenerative or malignant disorders. It was recently proposed that this transient protection of DNA lesions by the PARP DNA-binding domain could also slow the progression of the replicative fork, leading DNA polymerases to stall on DNA in the vicinity of an unrepaired break. This delay could provide the cell with the time required to repair damage before DNA replication (15).

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The abbreviations used are: LOH, loss of heterozygosity; AI, allelic imbalance; PARP, poly(ADP-ribose) polymerase.
In this study, we analyzed the involvement of the PARP gene in the genetic instability of human breast tumors. We detected an association between a low level of PARP gene expression and genetic instability measured as amplification and LOH at a number of different chromosome loci.

PATIENTS AND METHODS

Tissue and Blood Samples

Thirty-five primary breast tumor samples were obtained at the Centre René Huguenin (Saint-Cloud, France), and five samples of normal breast tissue were obtained at the Hôpital St. Louis (Paris, France). None of the 35 patients with primary breast cancer had undergone radiation therapy or chemotherapy.

Immediately following surgery, the tissue samples were placed in liquid nitrogen until extraction of high molecular weight DNA and RNA. A blood sample was also taken from each patient.

DNA Analysis

DNA was extracted from tumor tissue and blood leukocytes of each patient by using standard methods (16).

LOH expression and genetic instability measured as amplification and PARP gene in the genetic instability of human breast tumors. We

Determination of Allelic Loss. Normal DNA samples which were polymorphic at a given locus were considered informative, whereas homozygotes were considered uninformative. Only cases of constitutional heterozygosity were used in the evaluation of allelic loss. The signal intensity of the polymorphic alleles was determined by visual examination and confirmed by means of densitometry. The loading of the paired normal and tumor DNA samples from each patient was taken into account in judging allelic loss in the tumor DNA. The loading of DNA in each lane was evaluated by using control probes on other chromosomes. LOH was considered to occur when the intensity (peak area corresponding to the hybridizing signal) of one allele in tumor DNA was less than 50% of that in corresponding normal leukocyte DNA. The observed partially retained allele is due to either contaminating normal tissue or tumor heterogeneity.

Determination of Allelic Gain. Restriction enzyme-digested tumor DNA was compared with matching leukocyte DNA in the same agarose gels. Blots of these gels were first hybridized with the proto-oncogene probes. Rehybridization of the same blots with the c-mos (pHM2A) and β-globin (JW151) probes was used to check the amount of DNA transferred to the nylon membranes. The proto-oncogene and control gene autoradiographs were examined visually and/or using densitometry. Only signals with an intensity of two copies or more were considered to represent allelic gain.

RNA Analysis

RNA was extracted from normal and tumoral breast tissue by using the LiCl/urea method (19).

Northern Blot Analysis. Ten µg RNA were fractionated by electrophoresis on 1.2% agarose gels containing 6% formaldehyde and analyzed by blot hybridization after transfer onto nylon membrane filters (Hybond N; Amersham). The filters were hybridized with a nick-translated 32P-labeled PARP probe in 50% formamide at 42°C. Membranes were washed under stringent conditions in 0.1× SSPE, 0.1% SDS at 50°C, and subjected to autoradiography for various periods at ~80°C. Membranes were rehybridized with a 36B4 cDNA probe corresponding to a ubiquitous RNA. The signal obtained was used to check the amount of RNA loaded on the gel in each experiment. The 36B4 signal also showed that the RNA samples were not extensively degraded.

DNA Probes. The cDNA probes used for Northern blots were as follows: (a) the PARP probe was a 3.1-kb PstI fragment (20) and (b) a 0.7-kb PstI fragment of the 36B4 cDNA, described by Masiakowski et al. (21).

Evaluation of PARP Expression. Relative intensities of the mRNA bands were assessed by visual examination and confirmed by means of densitometry, taking the ubiquitous 36B4 bands into account; intensities were assigned a score of + (low), ++ (moderate), or +++ (high). Expression was quantified by serial dilution of tumor RNA to obtain a Northern blot hybridization signal similar to that obtained with normal breast tissue.

Acknowledgments

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tumors showed 2-5-fold increases in tumors scored ++ and mRNA Expression PARP

The largest increase in expression was more than 6-fold increases in tumors scored + with normal breast tissue. Densitometric analysis of these 20 overexpressing PARP mRNA in 57% (20/35) of tumor samples than in corresponding normal breast tissue. Densitometric analysis of these 20 overexpressing

Levels of PARP scripts were tested for in the 35 human breast carcinomas. Specification of the blot with the 36B4 probe showed that similar amounts of RNA were loaded in each case. The three normal breast tissues contained low levels of PARP mRNA (scored +). The breast tumor tissues contained levels of PARP mRNA ranging from low (scored +; Lanes 5 and 8) to moderate (scored ++; Lanes 4 and 6) and high (scored +++; Lanes 7 and 9).

Statistical Analysis

Differences were analyzed for statistical significance by using the χ² test with Yates' correction to adjust for the continuity of the χ² distribution when appropriate. Differences between the two populations were judged to be significant at a confidence level greater than 95% (P < 0.05).

RESULTS

DNA Status of the 1q41-q44 Region (Site of the PARP Gene)

The 35 tumors had been tested previously with 13 polymorphic DNA markers spanning the entire long arm of chromosome 1 (22). Of these 35 tumors, 16 (46%) showed allele gain, 4 (11%) LOH, and 15 (43%) a normal DNA profile at the 1q41-q44 region. All but two of the altered tumors showed allelic gain or LOH at all informative loci tested on 1q, indicating polysomy and/or deletion of the entire long arm of chromosome 1. The two other tumors showed partial polysomy of the 1q41-q44 region.

PARP mRNA Expression

PARP Expression in Normal Breast Tissue. PARP mRNA, standardized by using the 36B4 control probe, was present at low levels (scored +) in all five normal breast specimens studied (Fig. 1).

PARP Expression in Breast Tumor Tissue. PARP transcripts were tested for in the 35 human breast carcinomas. Levels of PARP mRNA varied from low (scored +) in 15 tumors to moderate (scored ++), in 10 tumors and high (scored ++++) in 10 tumors (Fig. 1). PARP mRNA levels were higher in 57% (20/35) of tumor samples than in corresponding normal breast tissue. Densitometric analysis of these 20 overexpressing tumors showed 2-5-fold increases in tumors scored ++ and more than 6-fold increases in tumors scored +++ compared with normal breast tissue. The largest increase in expression was 12-fold.

Table 1 Relationship between the number of 1q44-q41 copies and PARP mRNA levels in breast tumor tissues

<table>
<thead>
<tr>
<th>PARP mRNA level</th>
<th>Amplified</th>
<th>Deleted</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>5</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>++</td>
<td>4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>+++</td>
<td>7</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2 Frequency of allelic imbalance at different chromosomal regions in human primary breast tumors

<table>
<thead>
<tr>
<th>Chromosomal region</th>
<th>Al status</th>
<th>% of Al</th>
</tr>
</thead>
<tbody>
<tr>
<td>1pter-p32</td>
<td>LOH</td>
<td>60% (21/35)</td>
</tr>
<tr>
<td>3p24</td>
<td>LOH</td>
<td>27% (3/11)</td>
</tr>
<tr>
<td>7p22-p14</td>
<td>LOH + AG</td>
<td>53% (17/32)</td>
</tr>
<tr>
<td>7q31</td>
<td>LOH</td>
<td>47% (16/34)</td>
</tr>
<tr>
<td>8q24</td>
<td>AG</td>
<td>20% (7/35)</td>
</tr>
<tr>
<td>11p15.5</td>
<td>LOH</td>
<td>67% (6/9)</td>
</tr>
<tr>
<td>11q13</td>
<td>AG</td>
<td>20% (7/35)</td>
</tr>
<tr>
<td>17q11.2-q12</td>
<td>AG</td>
<td>14% (5/35)</td>
</tr>
<tr>
<td>20q13</td>
<td>AG</td>
<td>19% (5/26)</td>
</tr>
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</table>

Numbers in parentheses, cases with allelic imbalance/informative cases.
AG, allelic gain.

Relationship between the PARP mRNA Level and the 1q41-q44 Copy Number

We observed a trend toward a link between the number of 1q41-q44 copies and the PARP mRNA level (Table 1). Seven (70%) of 10 tumors strongly overexpressing the PARP gene (tumors scored ++++) were amplified in the 1q41-q44 region compared with 36% (9/25) of the tumors with lower PARP expression (tumors scored + or ++).

AI at Different Chromosomal Regions in Breast Tumors

In addition to the 1q41-q44 region, we analyzed normal DNA (peripheral blood leukocytes) and autologous tumor DNA from the same 35 breast cancer patients in the chromosomal regions 1pter-p32, 3p24, 7p22-p14, 7q31, 8q24 (MYC), 11p15.5, 11q13 (CCND1), 17q11.2-q12 (ERBB2), and 20q13. The incidence of LOH and allelic gain is summarized in Table 2. In spite of the small number of samples, the observed frequencies of genetic alterations were in keeping with results previously published by our group and others (23, 24).

Relationship between the PARP mRNA Level and Genetic Instability

To determine whether PARP expression is associated with genetic instability, as measured in terms of overall Al, we calculated an Al score. For each tumor, we computed the number of chromosomal regions with Al as a percentage of all informative regions. All tumors were informative for five or more chromosomal regions. The Al score ranged from 0 to 71% according to the tumor.

We observed a low level of genetic instability in most PARP-overexpressing tumors (Table 3). In particular, a corre-
PARP Expression and Genomic Instability in Breast Cancer

Table 3 Relationship between the PARP mRNA level and genetic instability in breast tumors

<table>
<thead>
<tr>
<th>mRNA level</th>
<th>&lt;50%</th>
<th>≥50%</th>
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<tbody>
<tr>
<td>+</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>++</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>+ +</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

*High PARP mRNA levels (scored +++) correlated with Al score below 50% (χ² corr = 5.33; P = 0.012).

Discussion

A large number of studies using a variety of experimental systems have led to the view that the nuclear enzyme PARP is involved in malignant transformation. To date, however, little is known of the expression of this gene in fresh tumors. In this work, PARP gene expression was increased in more than 50% of the breast tumor biopsies studied, pointing to a role of PARP in breast carcinogenesis. The genetic mechanisms underlying ectopic expression of this gene remain to be elucidated. We have shown previously that amplification of the 1q41-q44 region is one of the most frequent genetic alterations in human breast tumors (22). The data presented here show that the majority of breast tumors strongly expressing PARP have amplifications in the 1q41-q44 region, suggesting that expression of this gene could be disrupted by a gene dosage effect (Table 1). Indeed, the proportion of samples scoring +++ for PARP mRNA was higher among 1q41-q44-amplified tumors than in normal or deleted tumors [7/16 (44%) versus 3/19 (16%)]. This trend toward increased PARP gene expression due to extra 1q41-q44 copies requires confirmation in a larger population.

Acquisition of one or several extra copies of chromosome region 1q41-q44 does not seem to be the only mechanism underlying increased expression of the PARP gene in breast cancer. Indeed, we also found that PARP mRNA was overexpressed in several tumors in which the PARP gene was unaltered. In these tumors, PARP gene overexpression could alternatively be linked to another genetic mechanism. For example, alteration of parental imprinting is a potential mechanism by which genes expressed in low amounts may be activated, because of parentally programmed suppression of expression (25).

These data further suggest that the PARP gene is a major component of the response to DNA damage in human breast tumor cells: all tumors with strong PARP gene expression kept relative genetic stability. This was not, however, the case of tumors with moderate PARP expression (Table 3). Bürkle et al. (26) have shown that inhibitors of poly(ADP-ribosylation), such as 3-aminobenzoamide, increase the frequency of carcinogen-induced methotrexate resistance and, in parallel, increase dihydrofolate reductase gene amplification in cell culture systems. They concluded that poly(ADP-ribosylation) would act as a negative regulatory factor in the induction of DNA amplification. We postulated that genetic instability (DNA amplification and LOH) would develop if PARP expression could not be increased (or sufficiently increased). This was supported by the possibility that amplification of the 1q41-q44 region is an early primary event in breast carcinogenesis, because it is a clonal chromosome abnormality in near-diploid breast karyotypes (27, 28) and is also found in benign breast tumors (29). Amplification of the 1q41-q44 region, observed in the early stage of breast tumor development, might be aimed at inhibiting nascent genetic instability via PARP overexpression.

We have identified a second major factor which may be involved in the control of breast tumor cell genetic stability, the first being the p53 gene. Additional studies will be required to determine the respective role of p53 and PARP genes in DNA repair in human breast tumor cells.

Acknowledgments

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References

13. Jackowski, G., and Kun, E. Age-dependent variation of rates of polyadenosine-diphosphoribose synthesis by cardiocyte nuclei and the


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