Tumorigenesis in mice carrying a truncating Brca1 mutation

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We generated mouse mutants carrying in the Brca1 locus a modification (Brca1tr) that eliminates the C-terminal half of the protein product and obtained results indicating that, depending on genetic background, the missing BRCT and/or other domains are dispensable for survival, but essential for tumor suppression. Most of the apparently hypomorphic Brca1tr/tr mutants developed various tumors. Lymphomas were detected at all ages, whereas sarcomas and carcinomas, including breast cancer, appeared after a long latency. The mammary tumors showed striking variability in histopathological patterns suggesting stochastic engagement of tumorigenic pathways in their progression, to which the apparent hypomorphic Brca1tr/tr mutants developed various tumors. Lymphomas were detected at all ages, whereas sarcomas and carcinomas, including breast cancer, appeared after a long latency. The mammary tumors showed striking variability in histopathological patterns suggesting stochastic engagement of tumorigenic pathways in their progression, to which the apparent hypomorphic Brca1tr/tr mutation was apparently a late participant.

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The human breast cancer susceptibility gene BRCA1 [for review, see Scully and Livingston 2000; Welesh et al. 2000] encodes predominantly a nuclear phosphoprotein of 1863 amino acids [shorter isoforms, some of them confined to the cytoplasm, are translated from minor mRNA species]. With the exception of a RING finger domain located at the N-terminal region and two BRCT domains that are proximal to the C terminus, no other motifs have been recognized. Nevertheless, direct or indirect interactions of unknown physiological significance between BRCA1 segments and various other proteins have been revealed using cell lines or in vitro conditions. Some of these experiments suggested that the C-terminal region of BRCA1 acts as a transactivator [for review, see Monteiro 2000], whereas other data implied that BRCA1 is involved in the maintenance of genomic integrity (see Scully and Livingston 2000). It remains unknown, however, how ablation of BRCA1 function contributes to the pathogenesis of breast cancer.

Initial attempts to generate animal models of BRCA1-associated breast cancer were unsuccessful, as knockouts of the Brca1 murine homolog [1812 amino acids; 58% human–mouse homology] resulted in embryonic lethality of nullizygous embryos, whereas heterozygous mice did not develop mammary or other tumors [for review, see Deng and Scott 2000; for conditional mutants, see Results and Discussion]. In contrast with mutant mice, a patient with breast cancer has been described (Boyd et al. 1995) with inherited homozygosity for a frameshift mutation potentially generating a truncated protein of 900 amino acids [deletion of two A residues and appearance of a stop codon at positions 2800 and 2820, respectively, of the BRCA1 cDNA sequence]. Considering that none of the described Brca1 nullizygous mice could synthesize a truncated Brca1 peptide with sufficient length for nuclear localization, it was still possible to postulate that the domains remaining on a truncated 900-residue BRCA1 protein could be sufficient, if stable, to sustain embryonic development in both humans and mice. To test this hypothesis, we modified the Brca1 locus in mice by mimicking the human AA2800 mutation. Here, we show that the mutant mice, which are viable in particular genetic backgrounds, develop a variety of tumors, including breast cancer.

Results and Discussion

Gene targeting and breeding of mutants

A mutant allele, designated Brca1tr, was generated by introducing with a two-step knock-in targeting approach a 50-bp insertion into exon 11 of a Brca1 locus in mouse 129/Sv embryonic stem [ES] cells [see Fig. 1A–C]. The Brca1tr heterozygous progeny of transmitting male chimeras mated with C57BL/6J females were phenotypically normal and were intercrossed to generate Brca1tr/tr homozygous mutants [Fig. 1D].

The modification of the Brca1tr allele was verified by sequencing cloned PCR products [Fig. 1B]. Moreover, as shown by sequencing RT-PCR products derived from mutant RNA templates, the insertional mutation did not result in splicing abnormalities. The mutation resulted in a frameshift and in the appearance of a stop codon expected to lead to truncation of the protein product after the first 924 amino acids [Fig. 1B]. Therefore, it was not surprising to observe by Northern analysis of embryonic RNA that, because of nonsense-mediated mRNA decay, the mutant transcript [practically indistinguishable in size from wild type; ~7.2 kb] was significantly reduced in amount in comparison with the controls [Fig. 1E]. In contrast, the amount of a splicing variant lacking exon 11 (∆11, 3.9 kb), which is normally approximately fivefold less abundant than the full-length transcript [Mixer et al. 2000], was maintained at approximately wild-type levels [not shown]. However, the short cytoplasmic Brca1 isoform lacking nuclear localization signals that is encoded by ∆11 cannot sustain by itself viability beyond embryonic day 18.5 [see Deng and Scott 2000].

Despite several attempts, it was impossible to visualize the presence of truncated Brca1, because the only available antibody [rabbit polyclonal B28] recognizing the N-terminal region of the protein generated extremely high background upon immunoblotting. However, using a monoclonal antibody [GH118] recognizing the C-terminal region of Brca1, we showed by Western analysis
either directly or after immunoprecipitation with B28 that, in contrast with wild-type controls, full-length Brca1 was absent from protein extracts of mutant embryos (Fig. 1F).

Breeding of Brca1<sup>tr/tr</sup> heterozygotes indicated that survival of Brca1<sup>tr/tr</sup> homozygous mutants depended on genetic background [details of an extensive genetic analysis with complete data presentation will be published elsewhere; in prep]. Of the progeny that came to term from matings between 129/Sv × C57BL/6J heterozygous hybrids, only ∼4% were homozygous Brca1<sup>tr/tr</sup> mutants. This significant deviation from the expected mendelian frequency (25%), which was aggravated further by backcrossing with C57BL/6J mice, was the consequence of a high incidence of embryonic lethality associated with developmental abnormalities and growth retardation. The latter was previously correlated in Brca1 nullizygotes with hypoproliferation and increase in the expression of p21<sup>WAF1</sup>, a p53 target gene [Hakem et al. 1996]. A similar increase of p21<sup>WAF1</sup> transcripts was observed in Brca1<sup>tr/tr</sup> embryos in comparison with controls (Fig. 1E). Interestingly, rescue from lethality and complete restoration of mendelian ratios was observed by backcrossing with 129/Sv animals [several rounds] or by outcrossing using the MF1 strain of mice. The survivors manifested mild growth retardation, kinky tails, skin pigmentation defects, and male [but not female] infertility due to arrested spermatogenesis.

It remains to be seen whether different Brca1 domains are involved in mechanistically different functions. Clearly, Brca1 is indispensable for early embryos and plays other developmental roles revealed in hypomorphic mutants. Interestingly, in the absence of adverse strain modifiers, the C-terminal half of the protein is dispensable for viability, but crucial for a gender-specific meiotic role and for tumor suppression [see below].

**Brca1<sup>tr/tr</sup> mutants develop a variety of tumors**

Monitoring of a cohort of viable Brca1<sup>tr/tr</sup> mutants showed that tumors appeared in 76 of 89 mice (∼85%), which died or were killed when moribund. Kaplan-Meier cumulative survival curves [not shown] indicated that the time of median tumor-free survival [T<sub>50</sub>] was ∼1.4 years. During the same time period, only seven of 27 control animals (26%) died of spontaneous tumors that appeared at a very progressed age. The difference was statistically highly significant (∼p < 0.0001) indicating that the Brca1<sup>tr/tr</sup> mutation participated in tumorigenesis. Sex had no influence on tumor incidence or survival time. Most of the animals with tumors (∼83%) had a genetic background enriched in MF1 strain component. Because only a few survivors with different backgrounds were monitored, potential strain effects on latency were not analyzed [the data on tumorigenesis are presented altogether, as significant statistical bias could not be introduced].

Overall, 92 tumors were encountered in the mutants (60 animals had a single tumor, and 16 animals had two tumors; Table 1). The tumor spectrum included lymphomas, sarcomas, adenomas/carcinomas, and other types. Interestingly, lymphomas appeared at any age between 1 and 24 mo, whereas nonlymphoid tumors started appearing in animals older than 9 mo [there was also a significant difference between average latencies; T<sub>50</sub> of ∼14 vs. 18 mo].

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**Figure 1. Generation of Brca1 hypomorphic mouse mutants. (A)** Targeting scheme. A partial restriction map [exons 9–13; black rectangles] of the Brca1 locus is shown on top, followed by a diagram of the targeting vector used to insert a tk-neo cassette flanked by loxP sites [open triangles; not to scale] into a unique Nhel site of exon 11 by homologous recombination [large X symbols]. An open rectangle and a wavy line represent a diptheria toxin gene [DT] and the plasmid vector. The tk-neo cassette was removed by transient expression of cre in targeted embryonic stem (ES) cells [generation of a Brca1<sup>tr</sup> allele carrying a single loxP site insert]. [B] Ecorv [RV] EcoRv, [N] Nhel, [P] PacI. The positions of the probes used for Southern analyses and the sizes of the endogenous and targeted DNA fragments recognized by these probes are shown. [β] Structure of exon 11 in the region of insertion in Brca1<sup>tr</sup> as determined by DNA sequencing of PCR and RT-PCR products [the Nhel site at position 2882 was destroyed, whereas a PacI site was introduced in the mutant allele]. The appearance of a stop codon [TAA; asterisk] in the Brca1<sup>tr</sup> sequence is shown. [C] Southern analysis of ES cell DNA digested with EcoRV, to confirm the initial targeting. Because of introduction of an additional EcoRV site by the inserted cassette, the 11-kb EcoRV wild-type fragment detected by probe A [lane 1] is reduced to 5.9 kb in targeted ES clones [lanes 2, 3]. [D] Genotyping by Southern analysis with probe B by using tail DNA digested with EcoRI/Nhel [left] or EcoRI/Paci [right] from wild-type [lane 1], Brca1<sup>+</sup> heterozygous [lanes 2–4], and Brca1<sup>tr/tr</sup> homozygous [lane 5] mice. [E] Northern analysis of total RNA [15 µg per lane] from e11.5 wild-type [lanes 1, 6], Brca1<sup>−/−</sup> heterozygous [lanes 3, 5], and Brca1<sup>tr/tr</sup> homozygous [lanes 2, 4] embryos. The blot was hybridized sequentially [after stripping] with cDNA probes for Brca1, p21<sup>WAF1</sup>, and Gapdh [loading control]. [F] Western analysis to assay for the presence of Brca1 in protein extracts from wild-type [lanes 1, 2] and Brca1<sup>tr/tr</sup> homozygous mutant [lanes 3, 4] embryos by using monoclonal GH118 either directly [left] or after immunoprecipitation with the polyclonal antibody B28 [right; see Materials and Methods].
In ~60% of the cases (19 of 32), the lymphomas were large tumor masses (probably thymic in origin) localized in the anterior mediastinum (Fig. 2A,B), which often involved the heart and lungs and extended into the thoracic soft tissues. In some cases, there was widespread dissemination to abdominal organs (liver, kidney, spleen, and mesenteric lymph nodes) with occasional involvement of mammary glands, gonads, and uterus. Lymphomas of a second type (nodal, 12 of 32; see Fig. 2C), consistently involved massively enlarged spleen and mesenteric lymph nodes and frequently infiltrated additional organs. In two of these cases, lymphoblasts were also present in peripheral blood (leukemia/lymphoma). Some lymphomas were characterized further by immunostaining using antibodies against the T- and B-cell lineage-specific markers CD3 and B220, respectively. Not unexpectedly, six of six examined mediastinal tumors were of T-cell origin, whereas of eight examined nodal lymphomas, five were of T- and three of B-cell origin (see Fig. 2B,C, insets).

Table 1. Tumor spectra

|                      | Brca1tr/tr | Brca1tr/tr/p53+/− | Brca1tr/tr/p53+/− |
|----------------------|------------|------------------|------------------|
| Tumors               | 92 (76 mice) | 9 (8 mice)       | 8 (7 mice)       |
| Lymphomas            | 32 (35%)   | 8 (89%)          | 3 (37%)          |
| Mediastinal          | 19 (12%)   |                  |                  |
| Nodal                | 12 (7%)    |                  |                  |
| Thymic               | 1 (0.6%)   |                  |                  |
| Sarcomas             | 10 (11%)   |                  |                  |
| Angiosarcoma         | 2 (2%)     |                  |                  |
| Spindle cell sarcoma | 8 (7%)     |                  |                  |
| Retrperitoneal       | 7 (6%)     |                  |                  |
| Dermal               | 1 (0.6%)   |                  |                  |
| Adenomas/carcinomas  | 41 (44%)   | 3 (100%)         |                  |
| Breast               | 12 (12%)   |                  |                  |
| Lung                 | 13 (13%)   |                  |                  |
| Liver                | 13 (13%)   |                  |                  |
| Uterus               | 2 (2%)     |                  |                  |
| Colon                | 1 (1%)     |                  |                  |
| Other tumors         | 9 (9%)     |                  |                  |
| Undifferentiated     | 4 (4%)     |                  |                  |
| Hemangiomasis        | 4 (4%)     |                  |                  |
| Ovarian teratoma     | 1 (1%)     |                  |                  |

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Of 10 soft tissue sarcomas, two (one hepatic and one splenic) were angiosarcomas arising in a background of hemangiomas (Fig. 2D), whereas the remaining eight were widely metastatic spindle cell sarcomas (Fig. 2E). The 41 detected primary epithelial tumors included a single colorectal cancer (Fig. 2H), 2 endometrial adenocarcino-
mas [Fig. 2I], 13 tubulopapillary bronchioloalveolar lung neoplasms (Fig. 2F), 13 liver neoplasms (11 adenomas and 2 frank hepatocellular carcinomas, Fig. 2G), and 12 breast carcinomas described separately below.

To ascertain whether lack or haploinsufficiency of p53 could affect Brca1tr/tr-associated tumorigenesis, we generated by limited breeding and then monitored eight Brca1tr/tr/p53+/− and seven Brca1tr/tr/p53−/− double mutants (Table 1). The animals carrying the Brca1tr/tr mutation in p53 null background died of lymphomas within a period of <4 mo. Importantly, the T50 for the Brca1tr/tr/p53−/− lymphomas [98 d] was significantly shorter than the T50 of the same tumor type appearing in single Brca1tr/tr or p53−/− mutants (P < 0.0001). Analogous observations made with a few Brca1tr/tr/p53−/− double mutants also indicated an acceleration in tumorigenesis (T50 238 d).

Three previously described Brca1Δ223−763/p53−/− double mutants (Cressman et al. 1999) developed lymphomas by the age of 3 mo (one of these animals also developed a hemangiosarcoma). However, because of the double nullizygosity and the fact that lymphomas and sarcomas appear rapidly in p53−/− single mutants, a conclusion that the absence of Brca1 function had contributed to tumor development could not be reached from these data.

Compared with the situation in humans, the spectrum of tumors developing in Brca1tr/tr mice has only partial similarities and is wider, extending to nonepithelial types. Nevertheless, Brca1, first identified as a breast and ovarian cancer susceptibility gene, could have a broad although not ubiquitous tumor suppressing function (carcinomas at additional sites have been reported in some patients).

Mammary tumors in Brca1tr/tr mutants

Twelve mutant animals [11 females and, remarkably, one male] ranging in age between 9 and 23 mo [median of 15 mo] developed often palpable mammary tumors [Fig. 3A] of strikingly heterogeneous histological patterns [see Table 2].

In general, preneoplastic lesions in mouse mammary glands appear as focal epithelial hyperplasias either in alveoli [hyperplastic alveolar nodules [HANs]] or in terminal ductules [ductal hyperplasias [DHs]]. They can then progress to a stage of atypia similar to that of human ductal carcinoma in situ [DCIS], with potential for further development to invasive carcinoma [Medina 1996].

Most of the histologically diverse Brca1tr/tr breast carcinomas could not be easily classified as belonging to previously described tumor types. Spontaneous mouse mammary adenocarcinomas, induced in their majority by mouse mammary tumor virus [MMTV], have been classified mainly into types A and B [Dunn 1959]. Dunn type A microalveolar tumors consist of single layers of small cuboidal cells surrounding cavities, whereas ductal adenocarcinomas lacking acinar features and appearing as cysts, papillary projections, cords, tubular structures, or solid tumors are grouped in the B category. However, MMTV long terminal repeat–driven transgenic oncogenes mostly generate mammary carcinomas with distinctive patterns. For example, tumors induced by a myc transgene have large cells with pleomorphic nuclei and dark blue cytoplasm [hematoxylin and eosin staining]; ras–associated, usually papillary tumors, have small cells with relatively uniform nuclei and red cytoplasm; and neu–induced solid nodular tumors have cells of intermediate size with pleomorphic nuclei and pale pink cytoplasm [see Cardiff et al. 2000]. An additional mammary tumor type, adenocanthoma, is quite common in mice exposed to chemical carcinogens [see, e.g., Medina et al. 1980].

Whereas rare mammary adenocarcinomas developing in p53 nullizygous mice have been described as Dunn type B [see Harvey et al. 1993], only one of 12 Brca1tr/tr tumors that we have examined showed a mixture of Dunn type A and B patterns, in combination with myc-
like cytological features (Fig. 3H; Table 2, case 7a). Overall, the variable histological features of breast tumors in Brca1tr/tr mice encompassed a range of growth patterns that included solid, papillary, cribriform, tubular, acinar, mucinous, adenoacanthomatous, and sarcomatous forms (see Fig. 3). Frequently, combinations of these dissimilar patterns were present within a single tumor (Fig. 3D). Some tumors showed stromal desmoplasia (Fig. 3C). The degree of nuclear atypia varied between tumors, whereas the infiltration patterns ranged from circumscribed, expansive lesions with pushing borders to raggedly infiltrating, highly invasive tumors. A single male breast tumor belonged to the latter category and showed uniquely an infiltrative pattern bearing a striking resemblance to human invasive lobular carcinoma (Fig. 3F). In some cases, HAN or foci of DCIS adjacent to tumors were detected (Fig. 3K), but in most animals a background of extensive proliferative breast disease was not observed. The heterogeneity in tumor histopathology was paralleled to some extent with variability of immunophenotypes for estrogen and progesterone receptors and neu (Table 2). On the other hand, all of the breast carcinomas examined were positive for cyclin D1 and p21Waf1 expression and also showed p53 immunoreactivity with only one exception (Table 2).

It is likely that the Brca1<sup>tr/tr</sup> mutation was involved in the development of at least one of two mammary carcinomas detected in Brca1<sup>tr/tr</sup>/p53<sup>+/−</sup> double mutants (Table 2), because the histological pattern was not observed previously in rare mouse breast tumors associated with haploinsufficiency or loss of p53 (Harvey et al. 1993), whereas the latency was only 6 mo (p53<sup>+/−</sup> mice do not develop tumors of any kind before the age of 11 mo; for review, see Attardi and Jacks 1999). Histopathologically diverse breast carcinomas, including tubular and solid adenocarcinomas, were detected previously by microscopic examination of mammary tissue in five of 23 conditional mouse mutants between 10 and 13 mo of age, after Cre-mediated deletion of Brca1 exon 11 specifically in mammary epithelial cells (Xu et al. 1999). However, in contrast with these microscopic carcinomas, eight of 12 mammary tumors that were encountered in Brca1<sup>tr/tr</sup> mice were large, palpable masses. In addition, other phenotypic differences were noted that

### Table 2. Mammary carcinomas

| Case | Age (days) | Size (mm) | Pattern | Borders | Nuclear grade | Immunohistochemistry |
|------|-----------|-----------|---------|---------|---------------|---------------------|
|      |           |           |         |         |               | ER PR neu cD1 p21 p53 |
|      |           |           |         |         |               | A. Brca1<sup>tr/tr</sup> |
| 1    | 280       | 13        | Solid with few microglandular elements and focal adenoacanthoma | Invasive | Int./high | − − − + + + |
| 2    | 334       | 14        | Peripherally solid and centrally cystic with stromal desmoplasia | Highly invasive | High | − − − + + + |
| 3    | 354       | 12        | Papillary, cribriform, and cystic | Pushing margins | Int./high | + + − + + + |
| 4    | 422       | 1         | Adenoacanthoma | Pushing margins | Low | ± ± ± + + − |
| 5    | 440       | 8         | Infiltrating lobular pattern (male animal) | Highly invasive | Low | − − + ± + |
| 6    | 447       | 7         | Papillary | Highly invasive | High | ± − + + + + |
| 7a   | 474       | 12        | Ductal carcinoma: Dunn type B pattern (tubular, alveolar, papillary, and large cell ["myc-like"] elements) in combination with Dunn type A pattern (focal acinar growth), also multifocal DCIS at tumor borders | Invasive | Int./high | ± + + + ± ± |
| 7b   | 494       | 8         | Predominantly cystic in combination with solid ["neu-like"], glandular, and focally mucinous patterns | Invasive | Intermediate | ± + − + + + |
| 9    | 525       | <0.5      | DCIS | Highly invasive | High | − − − + +++ |
| 10   | 589       | 17        | Poorly differentiated adenoacanthoma with extensive sarcomatous metaplasia | Highly invasive | High | − − − + + + |
| 11   | 654       | <0.5      | DCIS | Invasive | Intermediate | − − − + + + |
| 12   | 680       | 2         | Ductal tubular | Invasive | Intermediate | − − − + + + |
| B. Brca1<sup>tr/tr</sup>/p53<sup>+/−</sup> |
| 13   | 179       | 16        | Solid and glandular | Invasive | Very high | − − − ± ± ± |
| 14   | 386       | 10        | Solid and focally cystic with focal adenoacanthoma | Invasive | High | − − − + ± ± |

Tumors in different mammary glands are described in case 7a and b. Immunostaining is indicated as positive (+), strongly positive (+++), weakly positive (±), or negative (−). When an immunophenotype was not determined, the space is left blank. [ER] estrogen receptor; [PR] progesterone receptor; [cD1] cyclin D1; [int.] intermediate; [DCIS] ductal carcinoma in situ; [HAN] hyperplastic alveolar nodule.
can be potentially attributed to structural dissimilarities between the conditional and Brca1 hypomorphic mutations. Thus, ablation of the Brca1 exon 11 resulted in mammary gland underdevelopment, increased apoptosis, and abnormalities in involution [Xu et al. 1999], whereas the mammary glands of Brca1 hypomorphic mutants, if not affected by tumors, were normal. In both types of mutants, however, reduction of p53 dosage had a similar impact in accelerating progression.

How do cellular changes elicited by the absence of Brca1 function participate in tumor pathogenesis? Although this key mechanistic question continues to remain open, the genetic evidence that we have provided is compatible with a view of opportunistic participation in tumorigenesis. Thus, we speculate that the pre-existing Brca1 lesion remains dormant until a randomly and progressively occurring combinatorial engagement of other deranged pathways seizes by chance the lack of Brca1 action as a fitting component in triggering progression toward full-fledged malignancy. Perhaps, the histologic heterogeneity that we have observed in mammary tumors reflects the potential of a Brca1 lesion to become a late participant in variable combinatorial sets of tumorigenic pathways.

Materials and methods

Targeted mutagenesis

The targeting vector [Fig. 1A] consisted of a cloned 8.5-kb 129/Sv DNA fragment carrying exons 9–12 of Brca1 that was interrupted by the insertion of a dual selection marker cassette (ik-neo) flanked by loxP sites into a unique NheI site of exon 11 [position 2882 of the mouse cDNA sequence; GenBank accession no. US2446]. A diphtheria toxin A gene cassette was included in the construct as a negative selection marker against random integration. To avoid potential transcriptional interference, we electroporated cells of independently targeted 129/Sv ES cell clones with a Pgk-cre phosphamid for transient expression of the recombinase, to excise the loxP-flanked selection marker cassette. Gancyclovir-resistant clones analyzed for successful deletion were then used for gene targeting. Male chimeras were then generated from ES cell clones with a tk-neo selection marker cassette. Gancyclovir-resistant mice used in some of the experiments were obtained from the Jackson Laboratories.

Molecular and biochemical analyses

For genotyping by Southern analysis, DNA was prepared from yolk sacs of embryos or the tail tip of 10-day-old mice. Northern blots were hybridized with cDNA probes for Brca1 (exons 16–24), p21Waf1, and Gapdh [loading control].

Two antibodies raised against GST fusion proteins representing different regions of murine Brca1 were used for protein analysis by standard protocols: the mouse monoclonal antibody GH118 [raised against residues 1336–1821] and the rabbit polyclonal antibody B28 [raised against residues 1–231].

Histological analysis

Mice showing overt pathological signs were killed and underwent autopsy. All major organs were processed for histology. Paraffin blocks were sectioned at 5 µm and stained with hematoxylin and eosin. Immunohistochemistry was performed with primary antibodies against estrogen receptor, progesterone receptor, and p21Waf1 [Santa Cruz Biotechnology]; c-neu and p53 (Oncogene Research Products); cyclin D1 [Novocastra Laboratories]; B220 [Pharminingen]; CD3 [Dako]; cytokeratin [Chemicon]; and vimentin [Research Diagnostics].

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