

# REPORTS

## Colorectal Carcinoma Invasion Inhibition by CO17-1A/GA733 Antigen and Its Murine Homologue

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**Background:** The gastrointestinal carcinoma antigen GA733 is a potential target for passive and active immunotherapy for patients with colorectal carcinoma. This antigen has been characterized previously as a homophilic adhesion (i.e., adhesion to self) protein, but the functional consequences of homophilic adhesion for tumor growth and invasion are unknown. The availability of a murine homologue of GA733, i.e., murine epithelial glycoprotein (mEGP), allows for functional analysis of cell adhesion as it relates to tumor growth and invasion, both *in vitro* and *in vivo*. **Methods:** CT-26 murine colorectal carcinoma cells were transfected with complementary DNAs encoding either the human or the murine antigen. GA733- or mEGP-producing cells were evaluated for homophilic adhesion, growth on plastic surfaces, colony formation in soft agar, and invasion through a reconstructed basement membrane (Matrigel). mEGP-producing cells were also examined for their capacity to metastasize in mice. Reported *P* values are two-sided. **Results:** Compared with control cells, mEGP-producing cells showed significantly lower growth rates, colony formation, and invasion through Matrigel *in vitro* (all *P* values <.05). Compared with vector-only transfected cells and parental cells, mEGP-producing cells showed a reduction in metastatic potential in syngeneic immunodeficient

and immunocompetent mice (all *P* values <.05). In contrast to mEGP-transfected cells, GA733-transfected cells did not exhibit significantly reduced growth or colony formation *in vitro* (all *P* values >.05). However, GA733-transfected cells did show reduced invasion through Matrigel compared with vector-only transfected cells or parental cells (all *P* values <.05). **Conclusion:** The adhesion proteins GA733 and mEGP inhibit invasion of tumor cells. [J Natl Cancer Inst 1998; 90:691-7]

The 40-kd glycoprotein defined by monoclonal antibodies (MAbs) CO17-1A and GA733 (1,2) is a suitable target for active immunotherapy for gastrointestinal cancers. The antigen (referred to hereafter as GA733 antigen) is highly expressed on these tumors (3-7) and also on some normal tissues, albeit at lower density (1,8-10).

The results of a randomized phase II trial with MAb CO17-1A in colorectal carcinoma (CRC) patients have demonstrated a significant increase in survival of MAb-treated patients versus control patients (11). The antigen has shown promise in approaches to active immunotherapy using anti-idiotypic antibodies (11-16) or viral vectors (17,18).

The GA733 antigen has been characterized previously as a homophilic adhesion protein (19). With the use of murine fibroblastic L cells transfected with the human GA733 complementary DNA (cDNA), this protein was shown to mediate homophilic cell-cell adhesion through a calcium-independent mechanism (19). However, the functional consequences of GA733 antigen-mediated adhesion on growth rate and invasion have not been investigated. A better understanding of GA733 antigen function(s) may have important implications for immunologic targeting of the GA733 antigen in cancer patients.

In this study, the role of the GA733 antigen and its murine homologue, mu-

rine epithelial glycoprotein (mEGP) (20), in tumor cell growth and invasion was investigated by use of murine CT-26 CRC cells stably transfected with cDNAs encoding either the human or the murine antigen. Using a syngeneic host, we also determined the metastatic potential of mEGP-producing cells relative to that of parental CT-26 cells.

## Materials and Methods

### Plasmids and Cell Lines

The mammalian expression vector for mEGP (pcDNA3-mEGP) was constructed by subcloning the *EcoRI* fragment from the pGEM-4Z-mEGP plasmid (provided by Dr. W. M. Kuehl, NCI-Navy Medical Oncology, Bethesda, MD) into the *EcoRI* site of the pcDNA3 vector (Invitrogen Corp., San Diego, CA). Expression of mEGP was placed under the control of the constitutive cytomegalovirus promoter from the pcDNA3 plasmid. Murine BALB/c CT-26 CRC cells were transfected with pcDNA3-mEGP or the pcDNA3 control plasmid by use of a calcium phosphate transfection kit (5'→3' Inc., Boulder, CO). Transfected cells were grown continuously in selection medium containing 1 mg/mL of G418 (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD), and colonies of transfectants were isolated by use of cloning cylinders. Fifty clones were isolated, and one representative clone that had a growth rate similar to that of the other mEGP-producing clones was retained for detailed study. The cell line carrying the mEGP cDNA was designated CT-26-mEGP, while the vector-only transfected cell line was called CT-26-pcDNA3. CT-26 cells transfected with the full-length human GA733-2 cDNA (CT-26-GA733) or vector only (CT-26-ASEN) have been described (21). The human CRC cell line SW1116 was obtained from the American Type Culture Collection (Rockville, MD). The human melanoma cell line WM115 has been described (22). All murine tumor cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM), and human CRC and melanoma cell lines

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were cultured in Leibovitz's L-15 medium (Life Technologies Inc.); both media were supplemented with fetal calf serum at 10% and 2%, respectively.

## Mice

Six- to 8-week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA) and nude mice (nu/nu, BALB/c background; Taconic, Merton, NY) were used in metastases experiments. The studies in mice were performed in accordance with institutional guidelines.

## Antigens

Recombinant GA733-2E protein extracellular domain and recombinant mEGP extracellular domain were produced by baculovirus (23,24). Full-length recombinant GA733-2F protein was produced in baculovirus essentially as described for GA733-2E (24), except that a full-length GA733-2 cDNA was used (25). Recombinant GA733-2E and GA733-2F proteins were purified from either recombinant baculovirus-infected insect cell supernatants or cell extracts, respectively, by immunoaffinity column chromatography with the use of coupled MAB GA733 (23). Recombinant mEGP was purified by high-performance liquid chromatography (24).

## Antibodies

Rabbit anti-GA733-2E protein and anti-mEGP antibodies directed against baculovirus-derived extracellular domain proteins were prepared by immunizing New Zealand rabbits with 52  $\mu\text{g}$  of recombinant antigen in Freund's complete adjuvant (first injection) and 16  $\mu\text{g}$  of antigen in incomplete adjuvant (second and third injections). Antibodies were isolated from immune rabbit sera on Sepharose columns coupled with recombinant extracellular GA733-2E or mEGP, and their specificity was determined as previously described (24). The care and use of rabbits to prepare antisera for this study followed institutional guidelines. Normal rabbit immunoglobulin (Ig) (Organon Teknika Co., Durham, NC) was used as a negative control.

## Immunofluorescence Microscopy

Cells were grown on glass coverslips placed in 24-well tissue culture dishes (Corning Costar Corp., Cambridge, MA). The cells were fixed with 1% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) for 15 minutes, washed with phosphate-buffered saline, and incubated first with 1:200 diluted rabbit anti-mEGP serum and then with biotinylated goat antibody directed against rabbit IgG (25  $\mu\text{g}/\text{mL}$ ; Cappel, West Chester, PA), followed by staining with ExtraAvidin-fluorescein isothiocyanate conjugate (10  $\mu\text{g}/\text{mL}$ ; Sigma Chemical Co.). All antibodies were diluted in 1% bovine serum albumin (BSA)-phosphate-buffered saline. The preparations were analyzed with a TCS 4D confocal microscope (Leica Inc., Heidelberg, Germany) with the use of an excitation wavelength of 488 nm at 100 $\times$  magnification.

## Growth Assay

All growth assays were performed with CT-26 cells adapted to continuous growth in 10% fetal calf serum (26). In brief, cells were seeded at densities of

60, 90, 180, and 360 cells/cm<sup>2</sup> in 10-cm<sup>2</sup> tissue culture plates precoated with 1% gelatin. Eight days later, the cells were fixed with 10% formaldehyde in saline, stained with eosin solution, and counted by use of phase-contrast microscopy (100 $\times$  magnification); the population doubling (PD) time (hours) was calculated by use of the following formula: PD time (hours) = hours of incubation/log<sub>2</sub> (mean number of cells per colony).

## Adhesion Assay

Tumor cell lines at a density of  $1 \times 10^5$  cells/100  $\mu\text{L}$  in serum-free medium were allowed to adhere for 20–25 minutes at 37 °C to different concentrations of antigens (0.5–8  $\mu\text{g}/10 \mu\text{L}$ ) coated onto nitrocellulose-precoated Petri dishes (27). Plates were washed gently with phosphate-buffered saline, and the total number of adherent cells per field (in triplicate) was determined with the use of phase-contrast microscopy (100 $\times$  magnification).

## Colony Formation Assay

Tumor cell lines were seeded at densities of 600–3600 cells/10 cm<sup>2</sup> onto plates precoated with 1% gelatin. Eight days later, cells were fixed with 10% formaldehyde in phosphate-buffered saline and stained with eosin solution, and the total number of colonies per plate was counted. Only colonies of four or more cells were included.

For the determination of colony size, single-cell suspensions of different tumor cell lines ( $1 \times 10^4$  cells per 3.5-cm<sup>2</sup> well of six-well plate; Becton Dickinson Labware, Franklin Lakes, NJ) were grown in 0.2% agar containing 10% fetal calf serum in IMDM on top of 0.5% agar. The cells were incubated for 14 days at 37 °C in an atmosphere of 5% CO<sub>2</sub>. The percentage of colonies of various sizes (10–25, 26–40, 41–55, and 56–70  $\mu\text{m}$ ) was determined for each cell line by counting 100 colonies per plate by use of a phase-contrast microscope (100 $\times$  magnification).

## Cell Invasion Through Matrigel

Invasion chambers (24-well Biocoat cell culture insert; 8- $\mu\text{m}$  pore size; Becton Dickinson Labware) were coated with 25  $\mu\text{g}$  of Matrigel and dried (26). Before use, the Matrigel-coated membrane was rehydrated with 0.5 mL of warm Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Inc.) containing 0.1% BSA for 2 hours. For generation of conditioned medium, confluent 3T3 cells were incubated for 20–24 hours in the presence of serum-free DMEM. The conditioned medium was removed, and 0.5 mL of medium was added per invasion chamber as a source of chemoattractant. The CT-26, CT-26-mEGP, and CT-26-GA733 cells were diluted to a density of  $2 \times 10^5/\text{mL}$  with 0.1% BSA-DMEM, and 250  $\mu\text{L}$  from each cell suspension was added to the chamber. The chambers were incubated for 18–20 hours at 37 °C in an atmosphere of 5% CO<sub>2</sub>. At the end of the incubation period, the cells on the upper surface of the membrane were removed with a cotton swab and washed with DMEM. The cells on the lower surface were dried, fixed in methanol, stained with Giemsa, and counted under the microscope (100 $\times$  magnification). The percentage of invasion was determined as the number of cells on the lower surface of the Matrigel-

coated membrane (minus the number of cells on the lower surface of the uncoated membrane) relative to the total number of cells added per well, multiplied by 100.

## Experimental Metastases

The metastatic potential of different cell lines to the liver in BALB/c and nude (nu/nu, BALB/c background) mice was determined by use of an intrasplenic injection assay (28,29). In brief, mice were anesthetized with ketamine (60–70 mg/kg body weight) and xylazine (5–7 mg/kg) given intraperitoneally. Spleens were exposed through a short incision, and  $3 \times 10^5$  tumor cells suspended in 100  $\mu\text{L}$  of saline were injected into the lower pole. The spleen was then returned to the abdominal cavity, and the wound was closed by sutures. Metastatic potential was evaluated 27–30 days after cell injection by counting the number of surface nodules present on the liver of each animal.

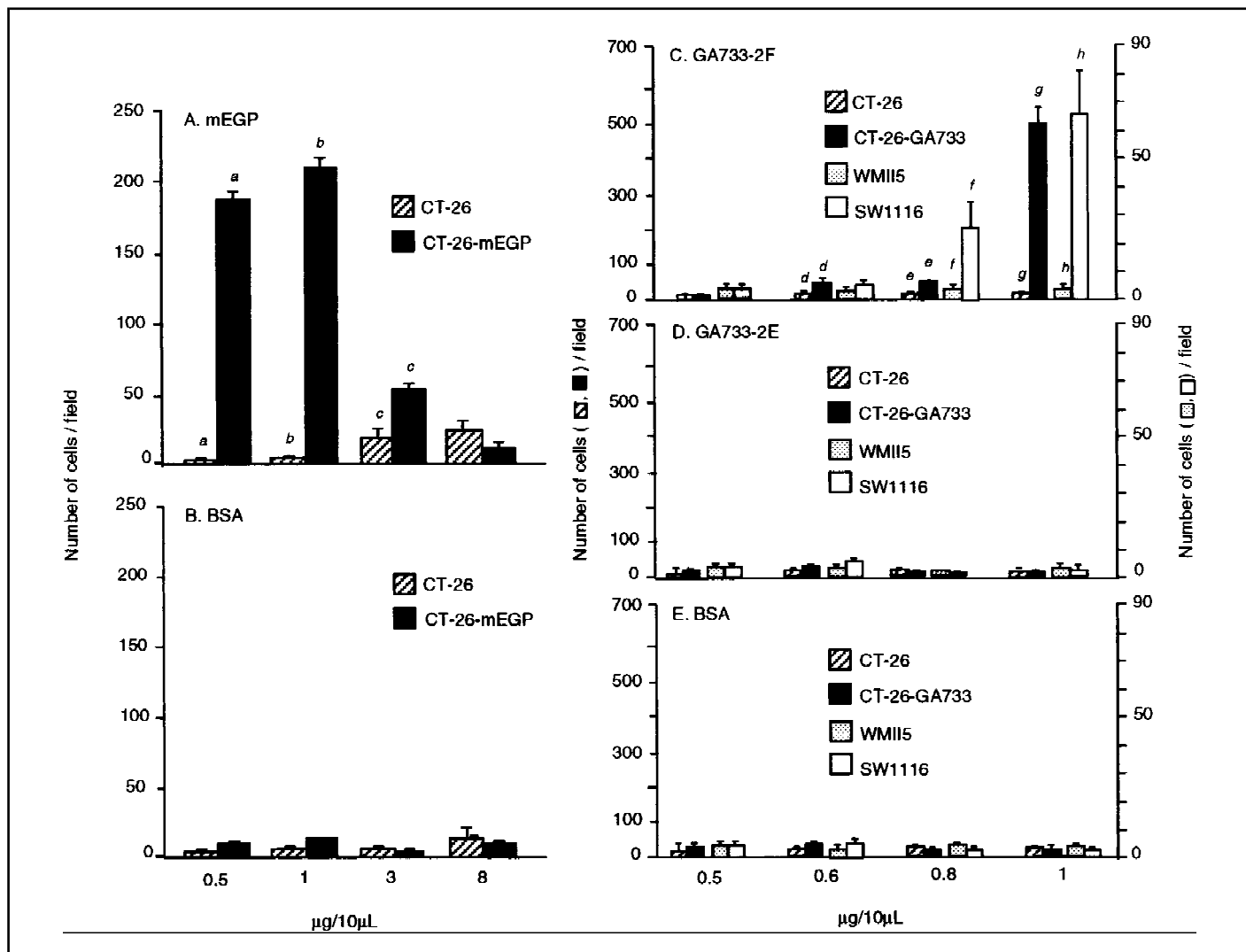
## Statistical Analysis

Experimental and control values obtained in growth rate determinations (see "Results" section) and adherence assays (Fig. 1) were compared by Student's *t* test. Statistical significance was determined for each experiment. Analysis of variance was performed for the results presented in Figs. 2–4, followed by post-hoc multiple pairwise comparisons performed by Tukey's method. All tests were two-tailed and considered significant for *P* < .05. All observations had normal distribution where testable. Normal distribution could not be tested for groups of replicates with two or more identical values (Fig. 1).

## Results

### mEGP and CO17-1A/GA733 as Homophilic Adhesion Proteins

The pcDNA3-mEGP-transfected murine colon carcinoma cell line CT-26-mEGP specifically expressed mEGP. Immunofluorescence microscopy showed that the antigen was evenly distributed over the entire cell surface, including cell-cell contact sites (data not shown). The vector-only transfected cells, CT-26-pcDNA3, and CT-26-GA733 cells grew as monolayers, whereas the mEGP-producing cells formed multilayer aggregates (data not shown). CT-26-mEGP cells, but not CT-26 cells, significantly and specifically adhered to 0.5–3  $\mu\text{g}$  of recombinant mEGP extracellular domain (Fig. 1, A and B). Like the parental CT-26 cells, CT-26-pcDNA3 cells did not significantly adhere to recombinant mEGP (data not shown). Adherence of CT-26-mEGP cells was almost completely blocked (>95%) by preincubation with 5  $\mu\text{g}/\text{mL}$  anti-mEGP rabbit antibodies, but not by normal rabbit IgG (data not



**Fig. 1.** Homophilic adhesion of murine epithelial glycoprotein (mEGP) and GA733 antigen. **A)** CT-26-mEGP (solid bars) and CT-26 control (hatched bars) cells were allowed to adhere for 20–25 minutes at 37 °C to different concentrations (0.5–8 µg/10 µL) of mEGP coated on nitrocellulose-precoated Petri dishes. Values labeled with identical letters are significantly different from each other as determined by Student's *t* test (*a* and *b*,  $P = .0001$ ; *c*,  $P = .032$ ). **B)** Absence of adhesion of CT-26-mEGP cells to bovine serum albumin (BSA). **C)** CT-26 control cells (hatched bars), CT-26-GA733 cells (solid bars), WMI15 cells

(stippled bars), and SW1116 cells (open bars) were allowed to adhere to different concentrations (0.5–1 µg/10 µL) of GA733-2F. Values with identical letters are significantly different from each other as determined by Student's *t* test (*d*,  $P = .029$ ; *e* and *f*,  $P = .001$ ; *g*,  $P = .045$ ; *h*,  $P = .042$ ). **D)** Absence of adhesion of CT-26-GA733 cells to GA733-2E. **E)** Absence of adhesion of CT-26-GA733 cells to BSA. The total number of adherent cells per field (triplicates) was determined under a microscope. Bars indicate the standard error of triplicate determinations.

shown). CT-26-GA733 cells significantly and specifically adhered to 0.6–1 µg of the full-length GA733-2F protein, but not to 0.5–1 µg of recombinant GA733-2E extracellular domain (Fig. 1, C–E). Because mEGP appeared to show a prozone effect (i.e., inhibition of molecule interactions at concentrations >1 µg mEGP) (Fig. 1, A), we did not test adherence of CT-26-GA733 cells to greater than 1 µg of GA733-2E. Similarly, the GA733 antigen-positive human colon cancer cells SW1116 significantly and specifically adhered to 0.8 and 1 µg of GA733-2F protein, but not to 0.5–1 µg of GA733-2E protein (Fig. 1, C–E).

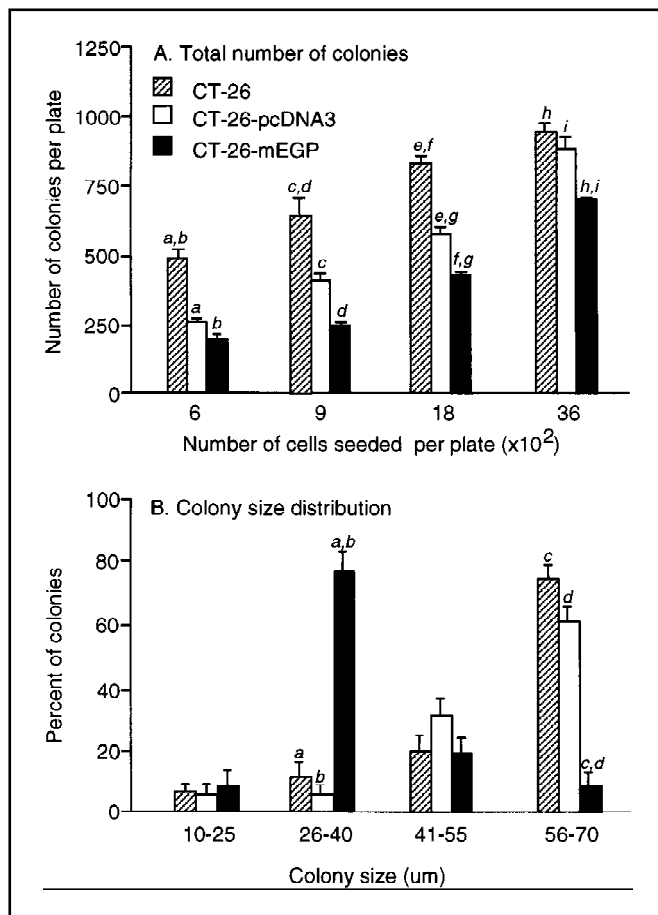
### Expression of mEGP and GA733 Antigens and Inhibition of CT-26 Cell Growth on Plastic Surfaces and in Soft Agar

The population doubling (PD) time (see "Materials and Methods" section) for CT-26-mEGP cells was significantly ( $P = .01$ ) higher than that for CT-26-pcDNA3 cells (PD time =  $20.58 \pm 0.71$  hours versus  $15.65 \pm 0.34$  hours [means  $\pm$  standard error of four different experiments], respectively). In contrast, the PD time for CT-26-GA733 cells was not significantly ( $P = .22$ ) different from that for CT-26-ASEN cells ( $16.05 \pm 0.13$

hours and  $15.16 \pm 0.46$  hours, respectively).

The CT-26-mEGP cells showed a moderate, but significant, decrease in colony-forming ability in soft agar compared with the colony-forming ability of either CT-26 or CT-26-pcDNA3 cells at densities of 180 or 360 cells per cm<sup>2</sup> (on 10-cm<sup>2</sup> plates), although CT-26-pcDNA3 cells were also inhibited at densities of 180 cells per cm<sup>2</sup> but not at densities of 360 cells per cm<sup>2</sup> (Fig. 2, A). In contrast, CT-26-GA733 cells were able to form colonies similar ( $P > .05$ ) to CT-26 or CT-26-ASEN cells (range of number [mean  $\pm$  standard error] of colonies per plate at

**Fig. 2.** Inhibition of colony-forming ability of CT-26 cells after transfection with murine epithelial glycoprotein (mEGP) complementary DNA. CT-26 cells (hatched bars), CT-26-pcDNA3 cells (open bars), and CT-26-mEGP cells (solid bars) were seeded in soft agar (see "Materials and Methods" section). **A.** Total number of colonies per 10-cm<sup>2</sup> plate was determined after day 8. **B.** Percentage of colonies formed was determined after 14 days in culture. Bars indicate the standard error of triplicate determinations. Values with identical letters differ significantly ( $P < .05$ ) from each other as determined by analysis of variance and Tukey's multiple pairwise comparisons tests.



densities of 60–360 cells per cm<sup>2</sup>: CT-26-GA733,  $217 \pm 17$  to  $617 \pm 20$ ; CT-26-ASEN,  $133 \pm 30$  to  $619 \pm 16$ , in agreement with a previous study performed with L cells transfected with GA733-2 cDNA (19).

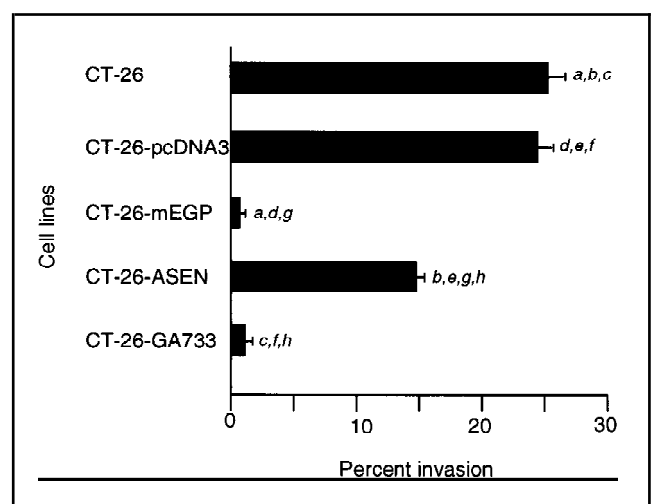
Compared with CT-26 or CT-26-pcDNA3 control cells, CT-26-mEGP cells formed significantly higher numbers of smaller, tightly bound colonies and lower numbers of larger, loosely bound colonies (Fig. 2, B). Similar results were obtained with CT-26-GA733 cells (data not shown), in agreement with the previously reported observations in mouse L cells transfected with this cDNA (19).

### Expression of mEGP and GA733 Protein and Inhibition or Invasion of CT-26 Cells Through a Reconstructed Basement Membrane

CT-26-mEGP cells were significantly less invasive through Matrigel-coated basement membrane *in vitro* than either CT-26 or CT-26-pcDNA3 cells (Fig. 3). There was no significant difference in the invasive capacity of parental CT-26 cells

compared with pcDNA3 vector-only transfected control cells (Fig. 3). Compared with CT-26 parental cells, CT-26-GA733 cells also showed significantly reduced invasion through the membrane; this difference was less pronounced when compared with vector-only transfected CT-26-ASEN cells (Fig. 3). Both experi-

**Fig. 3.** Inhibition of CT-26 cell invasion through reconstructed Matrigel-coated basement membrane after transfection with murine epithelial glycoprotein (mEGP) or GA733 complementary DNAs. Transfected or untransfected CT-26 cells ( $5 \times 10^4$ ) were added to prepared chambers (see "Materials and Methods" section). Percent invasion of cells through Matrigel-coated membrane reconstruct was determined as the number of cells on the lower surface of the Matrigel-coated membrane (minus the number of cells on the lower surface of the uncoated membrane) relative to the total number of cells added to each well, multiplied by 100. Bars indicate the standard error of triplicate determinations. Values with identical letters differ significantly ( $P < .05$ ) from each other as determined by analysis of variance and Tukey's multiple pairwise comparisons tests.



ments were repeated with similar results (data not shown).

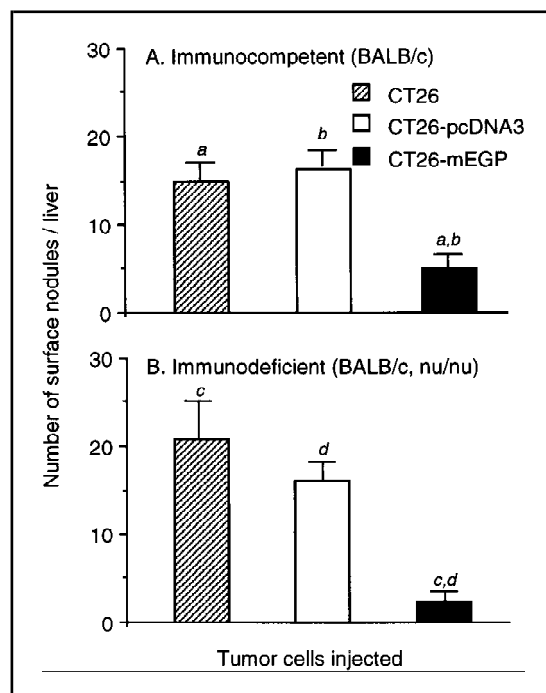
### CT-26 Cell Expression of mEGP and Inhibition of Metastasis in BALB/c and Nude Mice

The capacity of CT-26-mEGP cells to metastasize from the spleen to the liver of immunocompetent BALB/c mice was significantly reduced compared with that of either CT-26 or CT-26-pcDNA3 cells (Fig. 4, A). Identical results were obtained in immunodeficient nude mice (nu/nu, BALB/c background; Fig. 4, B), which suggests that the effect of mEGP on metastasis by CT-26 cells is independent of immune effector mechanisms that may be induced against the ectopic protein. Experiments concerning metastasis of human GA733 antigen-producing cells in mice were not conducted because the tumor and normal tissue antigens were mismatched. In light of the possible interactions of homophilic adhesion molecules on tumor and normal tissues during the metastatic process, it was important that both proteins were identical in the *in vivo* experiments.

### Discussion

We have demonstrated here that mEGP is a homophilic adhesion protein, in agreement with the adhesion function reported previously for the human homologue, the gastrointestinal carcinoma-associated antigen GA733 (19). The demonstration of similar functions of the two proteins is not surprising because

**Fig. 4.** Inhibition of spontaneous metastases by murine epithelial glycoprotein (mEGP) complementary DNA-transfected CT-26 cells in immunocompetent BALB/c mice (A) and immunodeficient nude mice (nu/nu, BALB/c background) (B). CT-26 cells (hatched bars), CT-26-pcDNA3 vector-only control cells (open bars), and CT-26-mEGP cells (solid bars) were injected into the spleen of BALB/c mice (four mice per group,  $3 \times 10^5$  cells per mouse) and nude mice (six mice per group,  $10^6$  cells per mouse). Nude BALB/c and BALB/c mice were killed on days 30 and 27, respectively, and the number of tumor nodules per liver was determined. Bars indicate the standard error of means of each group of mice. Values with identical letters differ significantly ( $P < .05$ ) from each other as determined by analysis of variance and Tukey's multiple pairwise comparisons tests.



they show 82% amino acid sequence identity (20) and similar tissue distribution in mice and humans (24). The human GA733 antigen is expressed on normal tissue of the gastrointestinal tract, lung, kidney, and breast. In addition, mEGP is expressed on spleen tissue (1,8-10,20,24). However, there were differences in the homophilic adhesion properties of the two proteins. The CT-26 mEGP-transfected colon carcinoma cells bound strongly to the extracellular domain of a recombinant mEGP, whereas the same cells producing the human antigen bound the full-length recombinant human protein (GA733-2F), but not the extracellular domain (GA733-2E). Furthermore, the human and murine proteins differed in their effects on cell growth. CT-26-mEGP cells formed multilayer aggregates, whereas parental CT-26 cells and CT-26-GA733 cells grew exclusively as monolayers. Similarly, CT-26-mEGP transfectants, but not CT-26-GA733 transfectants, were less able to form colonies in soft agar than either parental CT-26 cells or vector-only transfected CT-26 cells. However, both proteins significantly decreased invasion of CT-26 cells through Matrigel-coated membranes.

The observed differences in the adhesion properties and anchorage-dependent and anchorage-independent growth of the murine colon carcinoma cells transfected with mEGP or GA733-2 cDNAs may be

due to differences in the amino acid sequences of the two proteins, differences in antigen density between CT-26-mEGP and CT-26-GA733 cells, or differences in the environmental conditions to which the proteins were exposed. Thus, it is possible that the human antigen needs to be produced by human cells to have full functional activity. To address this issue would require producing the GA733 antigen in a suitable GA733 antigen-negative human cell line. Currently, no GA733 antigen-negative human colon carcinoma cell lines exist (30).

We showed that mEGP expression by transfected CT-26 colon carcinoma cells significantly inhibited metastasis of the cells from the spleen to the liver (Fig. 4). The decrease in cell metastasis may be a reflection of decreased cell growth at the primary site, as suggested by the inhibitory effect of mEGP on CT-26 cell growth *in vitro*. However, we cannot exclude the possibility that mEGP expression affected steps of the metastatic process after extravasation or detachment of tumor cells from the spleen, followed by cell migration to the liver through the peritoneal cavity. Thus, the clumping or clustering of CT-26-mEGP cells, which we observed *in vitro*, may inhibit cancer cell dispersion and limit their migration capacity through the circulation or through the peritoneal cavity and reduce cell passage through liver capillary beds.

A similar mechanism was proposed to explain decreased metastases in nude mice by human breast carcinoma cells overproducing the homophilic adhesion molecule E-cadherin (31). In that study, E-cadherin expression exclusively reduced the metastatic potential of the cells but had no effect on cell growth and tumorigenicity. However, other investigators (32-34) reported a decrease in anchorage-independent growth and tumorigenicity by human tumor cells after transfection with E-cadherin cDNA.

In addition to E-cadherin, other adhesion molecules, such as the cell adhesion molecule C-CAM1 (35),  $\alpha_2\beta_1$  integrin (36), and CD44H (37), have been shown to decrease tumorigenicity of tumor cells after cDNA transfection. Furthermore, the level of expression of all three proteins in human tumors, *in situ*, directly correlated with a differentiated phenotype exhibited by the cells. Conversely, poorly differentiated cells showed low or no expression of C-CAM1,  $\alpha_2\beta_1$  integrin, and CD44H proteins.

mEGP has the unique property of decreasing not only colon carcinoma growth *in vitro* but also the metastatic capability of the cells *in vivo*. Because mEGP inhibits metastasis in immunocompetent mice (Fig. 4), it is plausible that the GA733 antigen inhibits metastasis of human colon carcinoma cells *in vivo* (possibly in concert with other adhesion molecules). However, unlike E-cadherin, C-CAM1,  $\alpha_2\beta_1$  integrin, and CD44H, which demonstrate decreased expression with increasing stage of tumor progression (31-37), the human GA733 antigen is equally well expressed on both primary and metastatic CRC cells *in vivo* (7). Thus, the role of GA733 antigen as an inhibitor of tumor cell invasion and metastasis in humans is unclear.

Currently, we can only speculate on the possible consequences of immunologic targeting of the GA733 or mEGP antigens *in vivo*. Antibody blockade of either antigen may actually disperse tumor cell clumps, resulting in increased metastases. For example, treatment of noninvasive canine kidney cells *in vitro* with MAb directed against E-cadherin rendered GA733-producing cells invasive (34). However, this outcome seems unlikely for the GA733 antigen because MAb CO17-1A was reported to significantly enhance survival of CRC patients

in a phase II randomized control study (11). Furthermore, a recombinant adenovirus expressing the GA733 antigen significantly enhanced survival of mice bearing established CT-26-GA733 tumors in the presence of GA733 antigen-specific antibodies (18).

The effects of the GA733 antigen on growth and invasion of the transfected tumor cells *in vitro* are surprising in light of the beneficial effects of MAbs to the antigen in experimental animals and patients. These effects are most likely due to the high expression of the antigen on both primary and metastatic tumor cells (7). Thus, it is possible that the antigen can serve as an excellent target for active immunotherapy and still inhibit tumor invasion in patients.

Our results demonstrate the importance of including murine homologues of human tumor-associated antigens in functional studies. By examination of the functions of mEGP in a relevant murine tumor model, potential functions of the human GA733 antigen that would otherwise remain undetected were revealed. Our findings contribute to our understanding of the growth behavior of CRC cells and may have important consequences for immunotherapeutic targeting of the GA733 antigen in CRC patients.

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## Notes

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## Analysis of Loss of Heterozygosity in 399 Premalignant Breast Lesions at 15 Genetic Loci

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**Background:** Usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS) are risk factors for invasive breast cancer (IBC), suggesting that these lesions may be direct precursors of IBC. To identify genetic changes that may be important in the early development of precursor lesions and their progression to malignant or invasive disease, we examined 399 putative precursors (211 UDH, 51 ADH, 81 non-comedo DCIS, and 56 comedo DCIS) for loss of heterozygosity (LOH) at 15 polymorphic genetic loci known to exhibit high rates of loss in IBC. We also assessed the sharing of LOH by putative precursors and synchronous cancers. **Methods:** The polymerase chain reaction was used to analyze DNA from microdissected archival specimens. **Results and Conclusions:** In hyperplasias from noncancerous breasts (i.e., without DCIS and/or IBC in analyses of hyperplasias), LOH at any given locus was rare (range, 0%–15%), although 37% of UDH and 42% of ADH lesions showed loss for at least one locus, suggesting that the development of hyperplasias can involve many different tumor suppressor genes. In DCIS from noncancerous breasts (i.e., without IBC in analyses of DCIS), LOH was common, with 70% of noncomedo lesions and 79% of comedo lesions showing at least one loss. In DCIS, substantial rates of loss (up to 37%) were observed at loci on chromosomes 16q, 17p, and 17q, suggesting that inactivated tumor suppressor genes in these regions may be important in the development of noninvasive breast cancer. When DCIS lesions from cancerous and noncancerous breasts were compared, substan-

tially more LOH was observed in the cancerous breasts at a few loci (on chromosomes 2p, 11p, and 17q), suggesting that genetic alterations in these regions may be important in the progression to invasive disease. Among specimens harvested from cancerous breasts, 37% of UDH, 45% of ADH, 77% of noncomedo DCIS, and 80% of comedo DCIS lesions shared LOH with synchronous cancers at one locus or more, supporting the idea that the putative precursors and the cancers are genetically related. [*J Natl Cancer Inst* 1998;90:697-703]

Breast cancer is the most common and second most lethal cancer in women in Western cultures. In the United States alone in 1997, there were an estimated 180 200 new cases and 43 900 deaths from breast cancer (1). Because this disease is so common and so difficult to treat once it has developed, there is growing interest in studying precursor lesions of invasive breast cancer (IBC), which may be preventable or easier to control.

Epidemiologic studies have identified several putative precursors, including usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS). Although DCIS lesions have some malignant properties (e.g., loss of growth control), they lack the ability to invade and metastasize and, in this sense, are premalignant or precursor lesions. Women whose biopsy specimens in the past contained UDH, ADH, or DCIS showed approximately twofold, fourfold, and 10-fold increased relative risks, respectively, of eventually developing IBC (2-7). Understanding the biology of precursors may provide insights into preventing their development or progression. Unfortunately, very little is known about their biologic characteristics, particularly at the genetic level.

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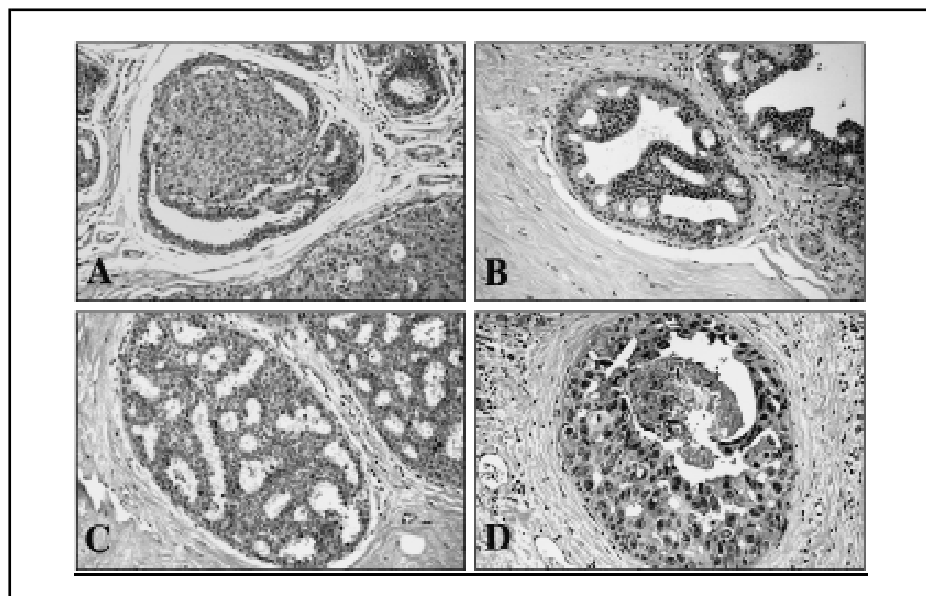
We were the first to report loss of heterozygosity (LOH) in hyperplastic breast disease (8), suggesting that “hyperplasias” are really benign neoplasms whose development involves the inactivation of tumor suppressor genes. In the same pilot study, we also observed LOH in a large proportion of DCIS lesions, consistent with one earlier report of LOH in noninvasive breast cancer (9). The majority of hyperplasias and DCIS lesions in our pilot study shared their LOH phenotypes with malignant/invasive disease in the same breasts, providing novel biologic evidence that they are genetically and perhaps evolutionarily related.

This study is a more comprehensive follow-up analysis of LOH at 15 genetic loci in 399 putative precursor lesions. The specific loci evaluated all showed high rates of loss in previous studies of IBC. The majority of lesions were from noncancerous breasts, allowing us to look for alterations that might be important in early lesion development. We also evaluated a smaller number of putative precursors from cancerous breasts, to begin to identify changes that might be important in the progression to malignant or invasive disease and to assess further the genetic relatedness of precursor and malignant lesions by determining how often they shared LOH phenotypes.

## Materials and Methods

### Tissue Samples

Histologic slides from routine, archival (formalin-fixed and paraffin-embedded), clinical cases were screened microscopically for adequate amounts of normal (control) tissue, putative precursor lesions, and IBC. In approximately 75% of cases, normal tissue consisted of benign breast tissue (terminal-duct lobular units [TDLUs], large ducts, and stroma) within the same specimens. Skin or lymph nodes from the same specimens were used as normal controls in the remaining cases. On the basis of data suggesting that closely adjacent breast cancer and morphologically normal TDLUs may occasionally share LOH for certain loci (10), we may be slightly underestimating rates of LOH for some markers in cases where adjacent TDLUs were the only source of normal tissue available. The precursor lesions we evaluated included florid examples of UDH, ADH, noncomedo DCIS (ncDCIS), and comedo DCIS (cDCIS) (Fig. 1). The majority of UDH (n = 163) and ADH (n = 26) lesions were harvested from noncancerous breasts (i.e., without DCIS or IBC). The majority of ncDCIS (n = 67) and cDCIS (n = 42) lesions were harvested from breasts without IBC. A smaller number of UDH (n = 48) and ADH (n = 25) lesions were isolated from cancerous breasts containing either DCIS or IBC. Similarly, a limited number of ncDCIS (n = 14) and cDCIS (n



**Fig. 1.** Representative photomicrographs of the putative precursor lesions evaluated in this study, including florid usual ductal hyperplasia (A), atypical ductal hyperplasia (B), noncomedo ductal carcinoma *in situ* (C), and comedo ductal carcinoma *in situ* (D). Hematoxylin–eosin, original magnification  $\times 200$ .

= 14) lesions were taken from cancerous breasts with synchronous IBC. In total, 399 putative precursor lesions were evaluated. We were unable to obtain sufficient quantities of two other putative precursors (atypical lobular hyperplasia and lobular carcinoma *in situ*), and they were excluded from the analysis.

This study, which involved the use of archival tissue from human subjects, was approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio.

### Sample Preparation

Alternating 3- $\mu\text{m}$  and 10- $\mu\text{m}$  histologic sections were cut from selected formalin-fixed, paraffin-embedded tissue blocks and were float-mounted onto glass slides. Areas of interest on the hematoxylin–eosin-stained 3- $\mu\text{m}$  slides were outlined with a felt-tip pen and used as a template to guide the independent microdissection of corresponding regions on the unstained 10- $\mu\text{m}$  slides. Microdissection was performed manually with a single-edge razor blade on a light box viewed under a dissecting microscope and was precise enough to ensure that 75% or more of the cellularity of each sample was derived from the targeted tissue. Cellular enrichment was about 90% in the majority of samples.

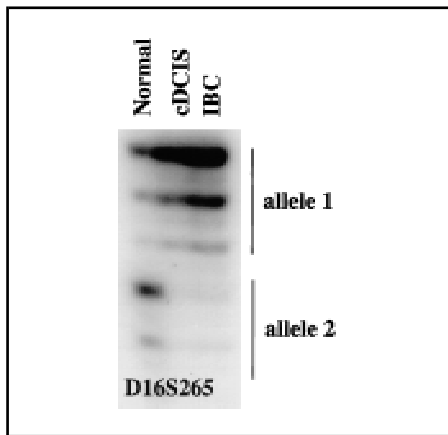
DNA was liberated from the samples by a modification of the method of Wright and Manos (11). Briefly, paraffin and lipids were first extracted by adding 0.4 mL of octane to 1.5-mL microcentrifuge tubes containing the samples. The residual cell material was then incubated for 3 hours at room temperature with 50  $\mu\text{L}$  of a solution containing 0.01 M Tris–HCl (pH 8.5), 0.001 M EDTA, 0.045% Nonidet P-40, 0.045% Tween-20, and 1.0 mg/mL proteinase K (i.e., lysis buffer). The mixture was heated to 95  $^{\circ}\text{C}$  for 15 minutes to inactivate the protease and was stored at  $-80^{\circ}\text{C}$  until use.

### Analysis of LOH

Samples were independently evaluated for LOH at each of 15 highly polymorphic microsatellite loci

known—from our own studies (8) or from the literature—to have high rates of loss (i.e.,  $>25\%$ ) in fully developed IBC. The antisense primers used for each locus were 5'-end labeled in a standard polynucleotide kinase reaction with 3000 Ci/mmol [ $\gamma$ - $^{32}\text{P}$ ]adenosine triphosphate ([ $\gamma$ - $^{32}\text{P}$ ]ATP) and a molar ratio of [ $\gamma$ - $^{32}\text{P}$ ]ATP to primer of 18 : 1. Locus-specific polymerase chain reaction (PCR) assays were performed in a total volume of 15  $\mu\text{L}$  containing 1.5 mM  $\text{MgCl}_2$ , 1 mM spermidine base, 0.75 U *Taq* DNA polymerase, 100  $\mu\text{M}$  each of the four standard nucleoside triphosphates, 100 nM each primer, and 5  $\mu\text{L}$  tissue lysate (diluted 1 : 15 in lysis buffer without proteinase K). Thirty PCR cycles (template denaturation at 94  $^{\circ}\text{C}$  for 30 seconds, primer annealing at 55  $^{\circ}\text{C}$  for 1 minute, and primer extension at 72  $^{\circ}\text{C}$  for 1 minute) were carried out in a 96-well thermocycler (Techne PHC3; Techne, Inc., Princeton, NJ). The amplified DNA was diluted 1 : 1 with stop solution (97% formamide, 1% EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol) and denatured at 85  $^{\circ}\text{C}$  for 2 minutes. Three microliters of denatured DNA from each sample was loaded onto 7% polyacrylamide gels (ratio of acrylamide to bisacrylamide = 19 : 1) containing 32% formamide and 34% urea and was fractionated over a period of 2.5 hours at 60 W. The gels were then transferred onto Whatman 3MM paper (WR Bals-ton, Ltd., Maidstone, U.K.), covered with plastic wrap, equilibrated in a 20% methanol–20% acetic acid solution, and dried at 80  $^{\circ}\text{C}$ . Dried gels were exposed to Fuji x-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan) with an intensifying screen at  $-80^{\circ}\text{C}$  (usually 16 hours).

Genetically informative samples showed two distinguishable alleles (see example in Fig. 2). On average, about 90% of samples were informative for each locus. Only 5%–10% of cases for each locus showed evidence of microsatellite instability, and such cases were omitted from further analysis. The intensity ratio of the two allelic bands of DNA from normal tissue relative to DNA from lesions in the same case was obtained from digitized data col-



**Fig. 2.** Representative example of loss of heterozygosity, as evaluated in this study, for marker D16S265 on chromosome 16q21. Relative to normal tissue in the same breast, adjacent comedo ductal carcinoma *in situ* (cDCIS) and invasive breast cancer (IBC) show shared loss of allele 2.

lected with a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA) and analyzed with ImageQuant software (Molecular Dynamics, Inc.). A conservative ratio of greater than or equal to 1.5:1 (ratio of normal tissue to tumor) was used to define LOH in this study. Our 75% or more level of target cell enrichment is adequate to meet or exceed this ratio in cases with pervasive LOH. In addition, all cases showing LOH in the original assay were re-examined twice, and only those showing losses in all three assays were considered as positive for LOH.

### Statistical Methods

Morphologically similar precursor lesions from noncancerous breasts and cancerous breasts were compared to highlight increases in LOH at specific loci that might be important in the progression to cancer. The significance of differences in these comparisons was assessed by use of Fisher's exact tests (two-sided).

## Results

### LOH in Precursors From Noncancerous Breasts

The primary purpose of this part of the study was to assess putative precursors from noncancerous breasts to identify high rates of LOH at specific loci that might be particularly important in the early development of these lesions. Table 1 summarizes the rates of LOH observed at 15 genetic loci in the lesions from noncancerous breasts. Noncancerous breasts were defined as those without synchronous DCIS and/or IBC in analyses involving UDH and ADH. In analyses of DCIS, noncancerous breasts were defined as those without synchronous IBC.

LOH was relatively rare at individual loci in UDH (range, 0%–12%;  $n = 163$ )

and ADH (range, 0%–15%;  $n = 26$ ) from noncancerous breasts. In contrast, substantial rates of loss were observed at three loci in ncDCIS from noncancerous breasts ( $n = 67$ ), including D16S265 (35%), D17S960 (31%), and NF1 (27%). cDCIS from noncancerous breasts ( $n = 42$ ) showed relatively high rates of loss at four loci, including D11S1328 (39%), D16S265 (30%), D17S960 (37%), and D17S787 (22%).

### LOH in Precursors From Cancerous Breasts

We also conducted a preliminary study of LOH in putative precursors from cancerous breasts, which were defined as breasts containing synchronous DCIS and/or IBC in analyses of UDH and ADH and as breasts containing synchronous IBC in analyses of DCIS. The rationale was to identify large increases, relative to the LOH in similar lesions from noncancerous breasts, that might be particularly important in the evolution from benign to malignant or invasive disease. Table 1 also summarizes the rates of LOH observed at 15 genetic loci in lesions from cancerous breasts.

Only one marker, D11S988, showed substantial increases in LOH in UDH (from 12% to 20%) and ADH (from 15% to 38%), although these increases did not reach statistical significance ( $P = .31$  for both comparisons). Notable increases were observed at the same locus in ncDCIS (from 18% to 75%;  $P = .004$ ) and cDCIS (from 19% to 43%;  $P = .32$ ). Additional loci showed large increases in LOH in ncDCIS, including TPO (from 16% to 36%;  $P = .20$ ) and D14S62 (from 16% to 36%;  $P = .21$ ). Several markers also showed large increases in cDCIS, including TPO (from 0% to 40%;  $P = .003$ ), D2S362 (from 9% to 31%;  $P = .17$ ), D6S417 (from 11% to 29%;  $P = .27$ ), D11S988 (from 19% to 43%;  $P = .32$ ), and D17S597 (from 7% to 44%;  $P = .02$ ). The failure of some of these increases to reach statistical significance may be partially due to the small numbers of cases in some of these subsets.

### Overall Extent and Sharing of LOH

Table 2 summarizes the combined extent of LOH in putative precursors from noncancerous and cancerous breasts and the degree to which precursors from can-

cerous breasts shared their LOH phenotypes with synchronous cancer. Again, cancerous breasts were defined as those containing synchronous DCIS and/or IBC in analyses involving hyperplastic breast disease, while they were defined as breasts containing IBC in analyses of DCIS.

Although LOH was uncommon at individual loci in UDH from noncancerous breasts, 37% of these lesions ( $n = 163$ ) showed loss for at least one locus (range, 1–4). Similarly, while LOH was relatively rare at individual loci in ADH from noncancerous breasts, 42% of these lesions ( $n = 26$ ) showed loss for at least one locus (range, 1–4). At least one LOH (range, 1–6) was observed in 70% of ncDCIS lesions ( $n = 67$ ), and at least one LOH (range, 1–5) was observed in 79% of cDCIS lesions ( $n = 42$ ) from noncancerous breasts.

The combined rates of LOH at one locus or more were not significantly higher in putative precursors from cancerous breasts compared with noncancerous breasts, including UDH (40% versus 37%;  $P = .87$ ), ADH (44% versus 42%;  $P = 1.0$ ), ncDCIS (93% versus 70%;  $P = .10$ ), and cDCIS (79% versus 79%;  $P = 1.0$ ).

UDH and ADH from cancerous breasts shared at least one LOH (range, 1–3) with adjacent cancer in 37% ( $n = 19$ ) and 45% ( $n = 11$ ) of cases with LOH, respectively. Seventy-seven percent of ncDCIS lesions with LOH from cancerous breasts ( $n = 13$ ) shared at least one loss (range, 1–3) with synchronous IBC. Eighty percent of cDCIS lesions with LOH from cancerous breasts ( $n = 11$ ) shared at least one loss (range, 1–5) with adjacent IBC.

## Discussion

The idea that benign breast disease may be a precursor of breast cancer was recognized as far back as the early 19th century (12). More recent historic studies (13–17) suggested that hyperplasias and *in situ* carcinomas, in particular, might be direct precursors of IBC because they appeared to lie on a histologic continuum and because they commonly coexisted. Additional indirect evidence came from epidemiologic studies (2–6,18–27) during the past 30 years, showing that women with benign breast disease had an increased relative risk of

**Table 1.** Rates of loss of heterozygosity (LOH) in putative precursor lesions taken from breasts without (–) and with (+) synchronous cancer\*

Marker/locus	UDH		ADH		ncDCIS		cDCIS	
	–CA	+CA†	–CA	+CA‡	–IBC	+IBC	–IBC	+IBC
TPO/2pter	7 (108)	8 (36)	6 (16)	7 (14)	16 (44)	36 (11)	0 (27)	40 (10)§
D2S362/2q35	4 (131)	2 (43)	0 (20)	6 (17)	6 (50)	15 (13)	9 (32)	31 (13)
D4S192/4q25	1 (111)	5 (39)	0 (20)	0 (17)	2 (41)	0 (12)	7 (29)	9 (11)
D6S417/6qter	5 (87)	10 (28)	6 (18)	9 (11)	17 (42)	0 (6)	11 (27)	29 (7)
D8S264/8p	5 (129)	7 (27)	0 (21)	5 (19)	23 (43)	11 (9)	16 (31)	20 (10)
D9S157/9p	10 (111)	7 (28)	8 (13)	0 (12)	10 (39)	13 (8)	11 (28)	0 (5)
D11S988/11p15	12 (115)	20 (20)	15 (20)	38 (8)	18 (38)	75 (8)¶	19 (27)	43 (7)
D11S1328/11q23	3 (104)	3 (40)	0 (16)	10 (19)	21 (39)	18 (11)	39 (28)	14 (7)
D13S137/13q13	8 (125)	3 (29)	13 (15)	9 (11)	17 (41)	0 (6)	13 (24)	33 (9)
D14S62/14q24	6 (110)	6 (32)	0 (16)	12 (16)	16 (43)	36 (11)	18 (34)	18 (11)
D16S265/16q21	1 (127)	5 (38)	10 (20)	13 (15)	35 (46)	33 (12)	30 (30)	7 (14)
D17S960/17p13	6 (64)	0 (28)	11 (19)	8 (13)	31 (32)	25 (8)	37 (27)	29 (7)
NF1/17q11	5 (82)	8 (24)	14 (14)	10 (10)	27 (26)	18 (11)	15 (20)	0 (6)
D17S597/17q21	3 (117)	5 (36)	5 (19)	6 (17)	20 (41)	18 (11)	7 (29)	44 (9)¶¶
D17S787/17q25	10 (111)	5 (38)	5 (19)	10 (19)	19 (43)	15 (13)	22 (32)	40 (10)

\*In analyses of hyperplastic lesions (i.e., usual ductal hyperplasia [UDH] and atypical ductal hyperplasia [ADH]), cancerous breasts (CA) were defined as those containing ductal carcinoma *in situ* (DCIS) and/or invasive breast cancer (IBC); in analyses of DCIS, cancerous breasts were defined as those containing IBC. Values in columns = % LOH (number of cases). ncDCIS = noncomedo DCIS; cDCIS = comedo DCIS.

†Includes DCIS (n = 32) and IBC (n = 16).

‡Includes DCIS (n = 13) and IBC (n = 12).

§Statistically significant difference (two-sided Fisher's exact test, P = .003) in rates of LOH at TPO, comparing cDCIS (–IBC) with cDCIS (+IBC).

¶Statistically significant difference (two-sided Fisher's exact test, P = .004) in rates of LOH at D11S988, comparing ncDCIS (–IBC) with ncDCIS (+IBC).

¶¶Statistically significant difference (two-sided Fisher's exact test, P = .02) in rates of LOH at D17S597, comparing cDCIS (–IBC) with cDCIS (+IBC).

developing IBC. The most enlightening of these studies, published in a series of articles (2,6,22,24,28) by Dupont, Page, and their colleagues, was from a large prospective study that assigned risk to specific types of epithelial lesions and established reproducible histologic criteria for identifying them. Other studies (3–5) confirmed the methodologic approach and findings, and the combined results suggest that women with usual hyperplasias, atypical hyperplasias, and *in situ* carcinomas have approximately twofold, fourfold, and 10-fold increased relative risks, respectively, of eventually developing IBC.

Because of the high incidence of IBC and our limited success in treating it, there is growing interest in understanding more about its precursors. Preventing the development of and predicting the behavior of precursor lesions will almost certainly be based on an understanding of the biologic alterations underlying their formation and progression. Research in this area has been very active (29,30). Our study used LOH to assess the role that tumor suppressor genes might play in precursor evolution. When evaluated by restriction fragment length polymorphism analysis, as in this study, LOH may result from a loss of tumor suppressor genes, from allelic imbalance due to (onco)gene amplification, or simply from nonspecific ge-

nomically instability. However, when high rates of loss (i.e., >20%) are repeatedly observed at specific loci, they usually indicate the approximate locations of inactivated tumor suppressor genes, as illustrated by the important role that this approach played in the discovery of RB (31,32), p53 (also known as TP53) (33), NF1 (34,35), and BRCA1 (36,37).

In this study, LOH was observed in 37% of UDH lesions and in 42% of ADH lesions from noncancerous breasts, which confirms and augments our original observation of LOH in hyperplastic breast

disease (8) and supports the premise that inactivated tumor suppressor genes play an important role in the early development of these lesions. Rates of loss were relatively low (range, 0%–15%) at individual loci in hyperplasias, suggesting that the evolution of hyperplastic lesions is genetically heterogeneous. At least three other studies (8,38,39) have reported LOH involving seven chromosomal regions, including five of the same regions that we studied, in about 50 cumulative cases of UDH from noncancerous breasts. Similarly, at least three

**Table 2.** Combined extent of loss of heterozygosity (LOH) in putative precursors of breast cancer and sharing of LOH with adjacent cancerous lesions

Category*	% with LOH at ≥1 locus		% sharing, with adjacent cancer, of LOH at ≥1 locus (range‡; total No.)
	Noncancerous breasts (range†; total No.)	Cancerous breasts (range†; total No.)	
UDH	37 (1–4; 163)	40 (1–3; 48§)	37 (1–3; 19  )
ADH	42 (1–4; 26)	44 (1–4; 25¶)	45 (1–3; 11#)
ncDCIS	70 (1–6; 67)	93 (1–4; 14**)	77 (1–3; 13**)
cDCIS	79 (1–5; 42)	79 (1–6; 14**)	80 (1–5; 11**)

\*UDH = usual ductal hyperplasia; ADH = atypical ductal hyperplasia; ncDCIS = noncomedo ductal carcinoma *in situ*; cDCIS = comedo ductal carcinoma *in situ*.

†Range refers to the smallest (i.e., 1) and largest number of loci showing LOH in lesions of each category.

‡Range refers to the smallest (i.e., 1) and largest number of loci with sharing of LOH between synchronous cancers and lesions of each category.

§Includes cases from breasts with synchronous DCIS (n = 32) and invasive breast cancer (IBC) (n = 16).

||Includes cases from breasts with synchronous DCIS (n = 11) and IBC (n = 8).

¶Includes cases from breasts with synchronous DCIS (n = 13) and IBC (n = 12).

#Includes cases from breasts with synchronous DCIS (n = 6) and IBC (n = 5).

\*\*Restricted to breasts with synchronous IBC.

studies (40–42) have reported LOH at five chromosomal regions, including three of the same regions that we analyzed, in about 30 cumulative cases of ADH from noncancerous breasts. For LOH to be detectable in hyperplasias by genetic marker analysis, it must be present in the majority of cells. Thus, these data and results from other studies—showing nonrandom inactivation of the X chromosome (43,44) and aneuploidy (45–47)—clearly demonstrate that hyperplasias are clonal neoplasms and indicate the need for more appropriate terminology (e.g., typical and atypical mammary adenomas, etc.).

As might be predicted, LOH was more common in DCIS than in hyperplasias at nearly all loci, which is consistent with the notion that DCIS represents a later stage in malignant evolution. Overall, 70% of ncDCIS and 79% of cDCIS lesions from noncancerous breasts (i.e., without IBC) showed losses involving at least one locus (range, 1–6). In addition, certain loci showed very high rates of loss, suggesting that suppressor genes in these regions may be particularly important in the early development of DCIS. In ncDCIS, these “hot spots” included D16S265 on 16q (35%), D17S960 on 17p (31%), and NF1 on 17q (27%). In cDCIS, hot spots were more common and included D11S1328 on 11q (39%), D16S265 (30%), D17S960 (37%), and D17S787 (distal to NF1) on 17q (22%). Candidate genes that might be responsible for some of these losses include cyclin D1 on 11q (48), E-cadherin on 16q (49), p53 on 17p (50), and NF1 (51) and BRCA1 (36) on 17q. Of these, p53 (52,53), NF1 (34,35), and BRCA1 (36) have shown a substantial relationship between mutation and loss in several types of cancer, including invasive breast cancer, but the significance of these genes in precursor lesions remains speculative until detailed mutational analyses are performed. There are no cloned candidate genes for most of the other loci showing losses.

Since the initial study of LOH in DCIS by Radford et al. (9), there have been at least 10 other reports (54–63) involving 27 chromosomal regions in DCIS from noncancerous breasts. Our results are generally consistent with these studies, and there is a consensus that loci on 16q, 17p, and 17q show the highest rates of loss. We observed the highest rates of LOH at

many of the same loci in both ncDCIS and cDCIS from noncancerous breasts, suggesting that the early development of these lesions may have important genetic similarities. In contrast, we observed higher rates of LOH at more loci in cDCIS than in ncDCIS from cancerous breasts, suggesting that cDCIS may be genetically more unstable and likely to acquire additional defects resulting in progression to an invasive phenotype.

In a preliminary comparison of morphologically similar putative precursor lesions from noncancerous breasts and cancerous breasts, we observed substantial increases in LOH at several loci in cancerous breasts, suggesting that these loci may harbor genes that are especially important in the progression to cancer. One locus, D11S988 on 11p, showed prominent increases for UDH (from 12% to 20%), ADH (from 15% to 38%), ncDCIS (from 18% to 75%), and cDCIS (from 19% to 43%), although only the change for ncDCIS reached statistical significance ( $P = .004$ ). Significant increases were noted at two other loci for cDCIS, including TPO on 2p (from 0% to 40%;  $P = .003$ ) and D17S597 on 17q (from 7% to 44%;  $P = .02$ ). Many other loci in DCIS showed prominent increases in LOH that failed to reach statistical significance, which may be partially due to the small numbers of cases and the large numbers of comparisons being made in some of these subsets. In ncDCIS, these large but nonsignificant increases included losses at TPO (from 16% to 36%) and D14S62 (from 16% to 36%). In cDCIS, they included losses at D2S362 (from 9% to 31%), D6S417 (from 11% to 29%), D13S137 (from 13% to 33%), and D17S787 (from 22% to 40%). Cloned genes that may be playing a role in some of these escalating losses include the estrogen receptor gene (64) near D6S417, cyclin D1 (48) near D11S988, RB (32) and BRCA2 (65) near D13S137, and BRCA1 (36) near D17S597.

Our early pilot study (8), showing a high degree of sharing of LOH phenotypes between hyperplastic breast disease and synchronous cancer, gave the first genetic evidence to our knowledge supporting the notion that hyperplasias may be direct precursors of breast cancer. This observation was sustained and augmented in the present study, which found that 37% of UDH and 45% of ADH lesions

with LOH shared at least one loss (range, 1–3) with synchronous cancer, and is not incompatible with the bilateral relative risk for developing breast cancer associated with hyperplasias (2–5), given that these lesions are often multifocal and bilateral (17). We also observed that the majority of ncDCIS lesions (77%) and cDCIS lesions (80%) shared their LOH phenotypes with synchronous IBC, consistent with our earlier pilot study (8), which has been confirmed by others (55,56,59,66,67), and supporting the idea that DCIS is a direct precursor of IBC.

This study also evaluated several breasts ( $n = 53$ ) with multiple UDH lesions (range, 2–5) and found that only 15% of the lesions within the same breast shared their LOH phenotypes, suggesting that they are usually independent neoplasms. Furthermore, in the subset of cases with multiple UDH lesions in cancerous breasts ( $n = 6$ ), only one or two UDH lesions shared LOH with the synchronous cancer, suggesting that multiple UDH lesions in the same breast may have different capacities to progress to cancer.

Premalignant or precursor breast lesions are currently defined by histologic features evident under light microscopy, and their adverse clinical significance is imprecisely estimated on the basis of indirect epidemiologic evidence. The results from this study and from similar studies suggest that, while lesions within these categories may look alike, they possess distinct genotypic abnormalities that may have important prognostic implications. Identifying low-risk or high-risk genotypes may enable us to match treatment to risk more appropriately in patients who have premalignant disease. Understanding more about the genetics of precursors may also lead to safe and effective strategies to prevent their development and progression.

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## Oral Clodronate and Reduction in Loss of Bone Mineral Density in Women With Operable Primary Breast Cancer

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**Background:** Women with primary breast cancer who receive systemic therapy may experience ovarian failure or early menopause, leading to a loss of bone mineral density (BMD). Loss of BMD may be reduced by use of bisphosphonates, compounds that inhibit the action of osteoclasts (cells that absorb or remove bone tissue). We have conducted a double-blind, randomized, two-center trial to evaluate BMD in women with primary breast cancer who were given the bisphosphonate clodronate (1600 mg/day orally) or placebo for 2 years. **Methods:** From August 31, 1990, through March 31, 1996, more than 300 eligible patients had been accrued, randomly assigned to study treatment, given the appropriate primary surgical care and systemic (chemotherapy and/or tamoxifen) therapy, and had completed follow-up for at least 1 year. BMD in the lumbar spine and in the hip, including the trochanteric area, was measured by use of dual-energy x-ray absorptiometry at the beginning of treatment and after 1 and 2 years of treatment. Changes in BMD were calculated as percent changes from the initial readings. Treatment effects for clodronate versus placebo (i.e., mean percent changes in BMD with clodronate minus mean percent changes in BMD with placebo) at 1 and 2 years for individual sites were calculated. **Results:** After 1 year, the treatment effects for clodronate versus placebo in the lumbar spine, the total hip, and the trochanter, respectively, were as follows: +2.38% (95% confidence interval [CI] = 1.36–3.41), +0.74%

(95% CI = -0.13–1.60), and +1.29% (95% CI = 0.24–2.34). After 2 years, the corresponding treatment effects were +1.72% (95% CI = 0.12–3.34), +1.85% (95% CI = 0.51–3.20), and +2.30% (95% CI = 0.66–3.94), respectively. **Conclusions:** Oral clodronate appears to reduce the loss of BMD in patients who receive treatment for primary breast cancer. [J Natl Cancer Inst 1998;90:704–8]

Reduced bone density, with a subsequent risk of osteoporotic bone fractures, is likely to become an increasingly important clinical problem in the very large numbers of women treated for primary breast cancer. Premenopausal women who receive adjuvant chemotherapy may be at special risk of increased bone loss because of early menopause (1) or as a result of adjuvant ovarian ablation (2). Tamoxifen, although preventing bone loss in postmenopausal women by an agonistic estrogenic effect (3), appears to cause bone loss in premenopausal women, presumably by an antagonistic antiestrogenic effect on bone (4).

Use of estrogen replacement therapy has generally been considered unsafe in women who have had breast cancer (5), although the risk of activation of occult malignant disease has not been proven (6,7). There are clinical trials under way to evaluate the risks of using estrogen replacement therapy in patients with cancer and, in the meantime, its use to prevent bone loss should be restricted to these trials.

Bisphosphonates, such as clodronate and pamidronate, are agents that inhibit osteoclasts in bone and reduce bone turnover. Bisphosphonates are widely used in breast cancer to treat hypercalcemia (8). In women with metastatic breast cancer, clodronate has been shown to reduce the osteolytic complications of metastases, such as hypercalcemia, bone pain, and vertebral fracture (9). Furthermore, in patients with non-osseous metastases from breast cancer, clodronate will reduce the risk of developing bone metastases (10). In healthy women, bisphosphonates have been shown to be effective in reducing bone loss (11–13).

These observations encouraged us to undertake a double-blind clinical trial involving more than 1000 women treated for primary breast cancer. The clinical

trial was designed to evaluate the effects of clodronate on the risk of developing bone metastases and, within this trial, to evaluate nonmetastatic changes in bone mineral density (BMD) in at least 300 patients. Herein, we report the sequential measurement of BMD in these patients.

## Patients and Methods

The primary objective of the adjuvant clodronate trial is to evaluate the effect of clodronate on the incidence of bone metastases in patients with primary breast cancer. This multicenter, double-blind trial has accrued more than 1000 patients with histologically or cytologically confirmed primary operable breast cancer. These patients have been randomly assigned to receive orally four capsules per day of either 400 mg clodronate (Bonefos®; Leiras OY, Helsinki, Finland) (i.e., 1600 mg clodronate/day) or an identical placebo, to be taken over a 2-year period at least half an hour before or after eating, with recorded dose modifications and compliance.

Participants in the main trial were randomly assigned to receive clodronate or placebo within 6 weeks (later amended to 12 weeks) of primary diagnosis (histologic or cytologic). At the same time, most patients started systemic chemotherapy and/or endocrine therapy. Table 1 lists the characteristics of the 311 patients reported in this study who had repeat bone density measurements and indicates the types of systemic therapy they received. The chemotherapy used at the Royal Marsden Hospital (Sutton, Surrey, U.K.) was mainly a mitoxantrone and methotrexate (±mitomycin C) combination (clodronate group, 64 patients; placebo group, 64 patients; total = 65% of the patients treated). At the Tom Baker Cancer Centre, University of Calgary (Alberta, Canada), the combinations were either cyclophosphamide, methotrexate, and 5-fluorouracil (clodronate group, 10 patients; placebo group, 14 patients; total = 21% of the patients treated) or an anthracycline combination, such as doxorubicin and cyclophosphamide or epirubicin, cyclophosphamide, and 5-fluorouracil (clodronate group, 12 patients; placebo group, 13 patients; total = 22% of the patients treated at Calgary). The use and type of chemotherapy were evenly distributed between the clodronate and the placebo groups. Tamoxifen was given to most participants in the BMD study at the Royal Marsden Hospital (clodronate group, 98 patients; placebo group, 88 patients; total = 95% of

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See "Notes" following "References."

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**Table 1.** Patient characteristics

Characteristic	RMH*		Calgary†		Both centers		Total
	Clodronate	Placebo	Clodronate	Placebo	Clodronate	Placebo	
No. of patients	103	93	53	62	156	155	311
Median age, y (range)	54 (35–74)	56 (27–75)	53 (29–73)	51 (33–76)	53.5 (29–76)	54 (27–76)	54 (27–76)
Median height, cm (range)	162.0 (150.0–181.0)	163.5 (147.0–111.8)	161.3 (150.5–178.2)	162.0 (143.5–175.3)	162.0 (150.0–181.0)	162.0 (143.5–183.0)	162.0 (147.0–183.0)
Median weight, kg (range)	64.0 (44.7–110.4)	64.5 (43.0–111.8)	67.1 (40.6–112.5)	68.45 (47.4–175.3)	64.7 (40.6–112.5)	67.0 (43.0–111.8)	65.7 (40.6–112.5)
Median BMI‡ (range)	24.24 (17.68–42.07)	25.04 (17.90–45.97)	25.56 (17.81–41.32)	25.78 (19.67–40.51)	24.47 (17.68–45.07)	25.29 (17.90–45.94)	25.05 (17.68–45.94)
Menopausal status§							
Premenopausal	39	34	17	28	56	62	118
Perimenopausal	9	6	8	3	17	9	26
Postmenopausal	55	53	28	31	83	84	167
Systemic therapy							
None	0	0	5	4	5	4	9
Tamoxifen	32	24	26	31	58	55	113
Chemotherapy	5	5	21	25	26	30	56
Chemotherapy + tamoxifen	66	64	1	2	67	66	133

\*RMH = Royal Marsden Hospital (Sutton, Surrey, U.K.).

†Calgary = Tom Baker Cancer Centre, University of Calgary, Alberta, Canada.

‡BMI = body mass index, defined as weight in kg divided by height in square meters.

§See "Patients and Methods" section for definitions of categories.

||See "Patients and Methods" section for details concerning systemic therapy.

the patients treated) and at Calgary (clodronate group, 27 patients; placebo group, 33 patients; total = 52% of the patients treated). This therapy was similarly distributed between the treatment groups.

The menopausal status of the participants was defined according to the date of their last menstrual period (LMP) and was categorized as premenopausal (LMP <3 months), perimenopausal (LMP between 3 and 12 months), or postmenopausal (LMP >12 months). Patients who had had a bilateral oophorectomy were classified as postmenopausal. Patients who had had a hysterectomy without bilateral oophorectomy were classified according to their age (<50 years as premenopausal, from 50 to 54 years as perimenopausal, and ≥55 years as postmenopausal).

It was estimated that the BMD study needed 300 patients to detect clinically significant differences in BMD between the clodronate and the placebo groups. Unselected recruitment from the main trial to the BMD study began August 31, 1990, at the Royal Marsden Hospital and April 1, 1992, at Calgary (when dual-energy x-ray absorptiometry [DEXA] became available), and continued until March 31, 1996. Measurement of BMD was approved by the Royal Marsden Hospital and the Tom Baker Cancer Centre research ethics committees and was included in the information and consent mechanism for the main trial. All participants in the main trial were considered potentially eligible for the BMD study, subject to consent and to the limited availability of DEXA within the study period. During the recruitment period, a total of 703 patients were randomly assigned to receive clodronate or placebo in the main trial, and treatment allocation remained blinded throughout the study (clodronate, 350 patients; placebo, 353 patients). Among the 703

patients, 414 gave their consent for participation in the BMD study, and DEXA was available for baseline BMD measurements (clodronate group, 208 patients; placebo group, 206 patients).

At the time of this interim analysis, 328 patients (clodronate group, 167 patients; placebo group, 161 patients) had had follow-up BMD measurements. (DEXA was not always available, or participants did not always agree to repeat measurements.) Clinical records for 17 of the patients (clodronate group, 11 patients; placebo group, six patients) had not been monitored at the time of the analysis; thus, they were not included, leaving a total of 311 patients (clodronate group, 156 patients; placebo group, 155 patients) for inclusion in the analysis (Fig. 1).

All participants had operable breast cancer that was clinically staged as T1–T3 (14), and they had appropriate metastatic staging prior to primary treatment. The staging protocol included a clinical assessment, baseline blood tests (including routine hematology and biochemistry), and a chest x ray. Other investigations, which included other radiologic examinations, bone scans, and magnetic resonance imaging, were undertaken if clinically indicated, and patients with evidence of metastases were excluded from the main trial and the BMD study. Estrogen receptor status was not available for the participants in this study.

BMD was assessed by DEXA, using an Hologic QDR 1000 densitometer (Vertec Scientific Ltd, Reading, U.K.), at the start of treatment and at 1 year and 2 years later. BMD measurements of the lumbar spine included the first through fourth lumbar vertebrae (L1–L4), with the exclusion of vertebrae affected by fracture or marked osteoarthritis on both the initial and the repeat scans. Measurements at the

hip involved the total hip, including the trochanteric area. All bone density scans were analyzed at the Bone Metabolism Unit of the University of Sheffield by reviewers who were blinded to the randomly allocated treatment assignments. All clinical, prescribing, and investigative data have been monitored blindly by Leiras OY, according to U.S. Food and Drug Administration requirements. The data have been kept under annual review by an external data monitoring committee.

In a quality-control exercise, all of the BMD scans were reviewed, blinded to the randomly allocated treatment assignments. When the areas covered by the baseline scans and the follow-up scans were not within the limits of repeatability, those scans were identified for a later review and were excluded from the current analysis. In this way, data from 26 patients were excluded from the spinal analysis, and data from 29 patients were excluded from the hip analysis.

Toxic effects and adverse events related to drug therapy including chemotherapy, endocrine therapy, and clodronate/placebo treatment were recorded at outpatient clinics by use of standard data forms that were completed by the clinicians.

### Statistical Analysis

Analyses were based on the percent change in BMD from baseline in the spine and the hip (and the trochanter) at 1 and 2 years on an intent-to-treat basis by use of simple paired *t* tests.

The baseline BMD at each of the sites was checked for normality by use of the Shapiro–Wilk test. Bone densities were found to be normally distributed. After adjustment for outlier values, the per-

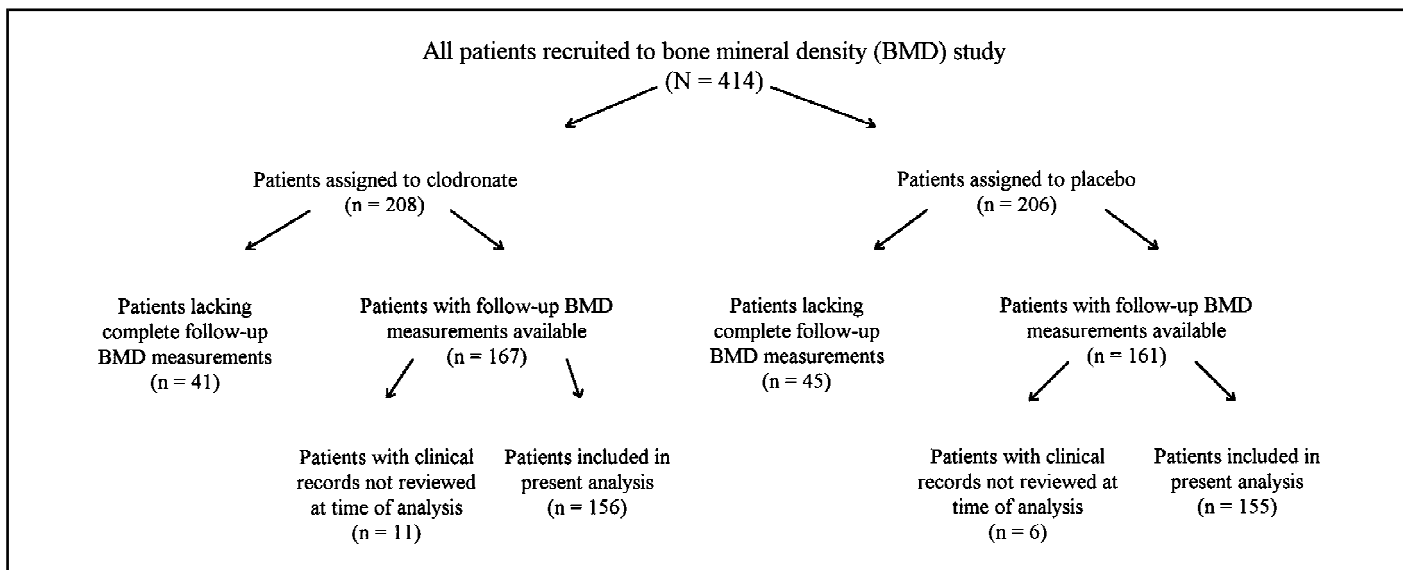


Fig. 1. Study scheme.

cent changes were also shown to follow a normal distribution (Shapiro–Wilk test), and parametric statistics were used in the analysis. Mean percent changes were calculated for various subgroups, and differences were assessed by means of the paired *t* test and by analysis of variance. All reported *P* values are two-sided. The treatment effect was defined as the difference in the mean percent change between the clodronate and the placebo groups.

## Results

Of the 1079 patients (clodronate group, 538 patients; placebo group, 541 patients) entered in the bone metastasis trial, a total of 311 (clodronate group, 156 patients; placebo group, 155 patients) had repeat bone density measurements carried out within the first 2 years of follow-up. The median age, height, weight, and menopausal status were well matched for both treatment groups and for both recruiting centers (Table 1). The type of primary adjuvant or neoadjuvant systemic treatment was well matched between the clodronate and the placebo treatment groups, but substantial differences existed between the two recruiting centers, reflecting differences in breast cancer treatment protocols at the two hospitals.

For all patients at 1 year, the placebo group had a loss of 2.2% in BMD in the lumbar spine, whereas the clodronate group had a small gain of 0.18%, giving a treatment effect for clodronate of +2.38% (95% confidence interval [CI] = 1.36–3.41; *P* < .001) (Table 2). Similarly, at 1 year, in the hip, the placebo group had a loss of 0.34%, whereas the clodronate group had a gain of 0.40%, giving a treat-

Table 2. Changes in bone mineral density (BMD) and treatment effect for patients receiving clodronate (*n* = 156) versus patients receiving placebo (*n* = 155)

Site	Change in BMD		Treatment effect for clodronate*		<i>P</i> †
	Clodronate	Placebo	Mean	95% CI‡	
Lumbar spine					
1 y	0.18%	-2.20%	2.38%	1.36–3.41	<.001
2 y	-0.16%	-1.88%	1.72%	0.12–3.34	.04
Total hip					
1 y	0.40%	-0.34%	0.74%	-0.13–1.60	.09
2 y	1.13%	-0.72%	1.85%	0.51–3.20	.008
Trochanter					
1 y	0.55%	-0.74%	1.29%	0.24–2.34	.02
2 y	0.67%	-1.63%	2.30%	0.66–3.94	.007

\*See "Patients and Methods" section for details on calculating treatment effect.

†Two-sided.

‡CI = confidence interval.

ment effect of 0.74% (95% CI = -0.13–1.60; *P* = .09) (Table 2). At 1 year, in the trochanter, patients who received placebo had a mean loss of 0.74% in BMD, whereas patients who received clodronate had a mean gain of 0.55%, giving a treatment effect of +1.29% (95% CI = 0.24–2.34; *P* = .02) (Table 2). After 2 years, the treatment effect for clodronate in spinal BMD was +1.72% (95% CI = 0.12–3.34; *P* = .04); in hip BMD, it was +1.85% (95% CI = 0.51–3.20; *P* = .008); in trochanteric BMD, it was +2.30% (95% CI = 0.66–3.94; *P* = .007) (Table 2). These results indicated an overall treatment effect for clodronate in preventing loss of BMD in patients after primary treatment for breast cancer (Table 2).

Analysis of changes in BMD for all

subgroups of patients, either by center, menopausal status, or adjuvant treatment with tamoxifen or placebo, showed a similar beneficial treatment effect for clodronate (Table 3).

Premenopausal and perimenopausal women on placebo were at increased risk of loss of BMD, especially in the first year, and clodronate appeared to be effective in minimizing this loss (Table 3). Although most patients were receiving adjuvant chemotherapy and tamoxifen, the total number of patients was small. Therefore, it is not possible to undertake treatment subgroup analyses in premenopausal and postmenopausal women.

Apart from an increased incidence of diarrhea, toxicity related to clodronate medication was very low compared with the toxicity for other treatments, as evi-

**Table 3.** Changes in bone mineral density (BMD) and treatment effect for clodronate versus placebo in lumbar spine according to patient characteristics

Characteristics*	% change in spinal BMD				P§
	Clodronate	Placebo	Treatment effect†		
			Mean	95% CI‡	
All patients (n = 311)					
1 y	0.18%	-2.20%	2.38%	1.36-3.41	<.001
2 y	-0.16%	-1.88%	1.72%	0.12-3.34	.04
RMH   (n = 196)					
1 y	0.23%	-2.54%	2.31%	1.14-3.48	<.001
2 y	-0.58%	-2.32%	1.75%	-0.12-3.62	.07
Calgary¶ (n = 115)					
1 y	0.91%	-1.72%	2.63%	0.70-4.56	.009
2 y	0.76%	-1.28%	2.05%	-0.88-4.97	.2
Premenopausal (n = 118)					
1 y	-1.57%	-4.04%	2.48%	0.91-4.05	.003
2 y	-3.99%	-3.94%	-0.05%	-2.40-2.30	.9
Perimenopausal (n = 26)					
1 y	-0.32%	-4.40%	4.08%	0.98-7.18	.02
2 y	-1.33%	-5.63%	4.30%	-0.23-8.83	.2
Postmenopausal (n = 167)					
1 y	1.63%	-0.37%	1.99%	0.71-3.27	.003
2 y	2.00%	0.09%	1.86%	0.09-3.62	.04
Tamoxifen (n = 245)					
1 y	0.58%	-1.98%	2.56%	1.50-3.62	
2 y	0.33%	-1.16%	1.48%	-0.12-3.09	.07
No tamoxifen (n = 66)					
1 y	-1.37%	-2.95%	1.58%	-1.18-4.33	.3
2 y	-2.62%	-4.39%	1.78%	-2.66-6.22	.4
Chemotherapy (n = 189)					
1 y	-1.03%	-3.04%	2.01%	0.69-3.32	.003
2 y	-1.36%	-3.07%	1.72%	-0.26-3.70	.09
No chemotherapy (n = 122)					
1 y	1.85%	-0.84%	2.70%	1.23-4.16	<.001
2 y	2.21%	0.60%	1.61%	-0.60-3.83	.2

\*See "Patients and Methods" section for definitions of categories of menopausal status.

†See "Patients and Methods" section for details on calculating treatment effect.

‡CI = confidence interval.

§Two-sided.

||RMH = Royal Marsden Hospital.

¶Calgary = Tom Baker Cancer Centre, University of Calgary.

**Table 4.** Toxic effects reported by patients receiving clodronate and placebo\*

Toxic effect	Clodronate, 1600 mg/day (n = 156)†	Placebo (n = 155)‡
Fatigue	19	19
Hot flushes	42	46
Diarrhea‡	27	8
Nausea and vomiting (combined)	43	38
Arthralgia	21	28
Somnolence	29	34
Alopecia	25	25
Erythematous rash	14	14
Premature termination of therapy		
Adverse events	16	12
Noncompliance	12	16
Total	28	28

\*Includes effects caused by endocrine therapy and chemotherapy.

†Values in column = numbers of patients reporting each type of toxic effect.

‡Statistically significant difference between the two groups; two-sided  $P < .0001$ .

ceiving tamoxifen, clodronate has been reported to cause an increase in BMD (15). Therefore, we established a study to measure BMD by DEXA in an unselected subgroup of patients from a large trial involving women with primary operable breast cancer.

The main results from this trial indicate that patients treated for primary operable breast cancer have evidence of bone loss, as estimated by DEXA measurements of BMD. Premenopausal patients were at increased risk of bone loss, presumably because most of these women were receiving adjuvant chemotherapy, with the associated development of ovarian failure and early menopause. Furthermore, many patients received tamoxifen, which has an antiestrogenic effect on bone in premenopausal women, causing a loss of BMD (4). In premenopausal patients in this study, clodronate significantly reduced the loss of BMD at 1 year, although this effect did not persist at 2 years.

Our results confirm similar findings from another small study (16) that demonstrated that clodronate will reduce the loss of BMD caused by ovarian failure following adjuvant chemotherapy in premenopausal women with primary breast cancer. A similar result has been reported for another bisphosphonate, risedronate (17).

In contrast, postmenopausal patients on placebo in our study had relatively little bone loss, probably because most of these women were receiving adjuvant tamoxifen, which has been shown to reduce bone loss in postmenopausal women (3). In postmenopausal women on clodronate, there was a statistically significant increase in spinal BMD at 1 and 2 years.

In conclusion, we have confirmed in this trial that the use of clodronate is safe and effective in preventing bone loss in patients who have been treated for primary operable breast cancer. The extent of this clinical problem of bone loss has not yet been fully evaluated. However, it is likely that there will be an increase in the risk of osteoporosis with future improvements in systemic therapy for breast cancer, together with a likely increase in the loss of BMD due to wider use of current cancer treatments. The cost benefits of widespread use of bisphosphonates as an intervention for patients receiving systemic treatment for primary breast cancer will need to be evaluated.

dent from findings in the placebo group (Table 4). There was no statistically significant difference in the incidence of premature termination of medication between the two treatment groups.

## Discussion

In healthy postmenopausal women, both clodronate and another bisphosphonate (i.e., alendronate) have been shown to reduce the loss of BMD (11,12). With alendronate, this reduction in loss of BMD was associated with a 50% reduction in the risk of vertebral and nonvertebral osteoporotic fractures (13). Whether this treatment would be similarly effective in premenopausal and postmenopausal women who have had breast cancer and were receiving adjuvant chemotherapy and/or tamoxifen was unknown. In postmenopausal women re-

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## Notes

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