Fluctuation of HER2 Expression in Breast Carcinomas during the Menstrual Cycle

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The hormonal milieu at time of tumor surgery seems to have a significant impact on survival in premenopausal breast cancer patients. Indeed, surgery performed during the follicular phase of the menstrual cycle was suggested to correlate with a poor prognosis. To investigate the relationship between prognosis and menstrual cycle at time of surgery, we analyzed the expression of some markers associated with tumor aggressiveness, such as the hormone receptors, HER2, p53, Bcl2, and cathepsin D in breast carcinomas obtained from 198 premenopausal women who underwent surgery during different phases of the menstrual cycle. HER2 overexpression was found to fluctuate in hormone receptor-positive tumors. In actual fact, 20% of the tumors removed during the follicular phase scored HER2-positive, versus 8% of those removed during the luteal phase. Similarly, a number of hormone receptor-positive tumor specimens, obtained from the same patients during follicular and luteal phases, were scored HER2-positive when the sample was removed during the follicular phase and HER2-negative when removed in the luteal phase. Southern blot analysis of the *HER2* gene indicated that, in hormone receptor-positive cases, the overexpression of HER2 is often not associated with gene amplification. The finding that overexpression of the *HER2* gene, associated with tumor aggressiveness, can fluctuate according to the hormonal milieu may explain the increased survival of patients operated during the luteal phase. It is also relevant to the selection and treatment of patients most likely to benefit from anti-HER2 antibody therapy. (*Am J Pathol* 1999, 155:1543–1547)

The timing of breast cancer surgery with respect to the menstrual cycle was suggested to be associated with prognosis,¹ even though there is no general consensus and controversial data have been reported as reviewed

by Hagen and Hrushesky.^{2,3} Nevertheless, different reports indicated that surgery performed during the follicular phase is unfavorable when compared with surgery performed during the luteal phase.⁴ Both lymph node involvement and vascular invasion have been found to be increased in patients undergoing surgery during the first phase of the menstrual cycle.⁵ The biomolecular basis of this finding, to this day, remains unclear. One hypothesis explaining the relationship between surgery timing and prognosis focuses on the mechanical handling of the tumor. It is believed that such manipulation may lead to the spreading of malignant cells that would be influenced both directly and indirectly by the hormonal milieu. Because normal breast cells respond to cyclical hormone fluctuations with changes in gene expression,^{6,7} it is likely that some tumors respond to these stimuli in a similar fashion. A recent small study of 32 premenopausal women indicated that mRNA expression levels of *cathepsin L*, *MMP-9*, and *TP53* were all significantly higher in tumors resected during the follicular and periovulatory phases of the menstrual cycle than those operated on at other times within the cycle.⁸ Furthermore, the number of cells in mitosis in primary breast carcinomas from premenopausal patients has been found to fluctuate according to the hormonal milieu.⁹

In this study, to evaluate whether tumor cells respond to cyclical hormone fluctuations with corresponding changes in marker expression, we analyzed primary breast carcinomas from 198 premenopausal women operated on during different phases of menstrual cycle. The frequency of HER2 expression was found to fluctuate in hormone receptor-positive tumors. This finding was confirmed by testing the expression of this gene on biopsies and surgical specimens from the same patient obtained during the luteal and follicular phases. Furthermore, regarding hormone-induced HER2 regulation, we found that hormone receptor-positive breast carcinomas overexpressing HER2 seldomly showed gene amplification.

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Materials and Methods

Immunohistochemistry

Tissue specimens were obtained from 198 consecutive premenopausal patients operated on in 1978 and 1979 at this institute for primary duct, lobular, or mixed infiltrating breast carcinoma. For each patient, the day of the last menstrual period before surgery was obtained and compared to the date of surgery. The following panel of monoclonal antibodies (mAbs) was applied: anti-c-erbB-2 mAb cB11 (1:60 diluted; Ylem, Avezzano, AQ, Italy), anti-p53 mAb DO7 (1:500 diluted; Novocastra, Newcastle-on-Tyne, UK), anti-bcl2 mAb 100 (1:20 diluted; a kindly gift from Dr. David Mason), anti-progesterone receptor mAb 1A6 (1:20 diluted; DBA, Segrate, MI, Italy), and polyclonal anti-cathepsin D (1:300 diluted; Novocastra).

Immunostaining was performed by a sensitive peroxidase-streptavidin method on Bouin-fixed, paraffin-embedded material. Consecutive sections were processed (cut, deparaffinized, and rehydrated) and pretreated with a heat-induced epitope retrieval method.¹⁰ After washes in phosphate-buffered saline (PBS), immunostaining was performed using automated immunostainer (Dako Tech-Mate 1000). The primary antibody was replaced with a nonimmune serum, from the same species in which the primary antibody was produced, or an unrelated monoclonal antibody as a negative control. Appropriate cases with known reactivity for each antibody applied were used as positive controls. Sections were considered positive when more than 5–10% of the tumor cells were labeled for p53, hormone receptor, Bcl2, and cathepsin D, while sections were scored positive for HER2 when they showed a strong membrane labeling.

Immunostaining was also performed on eight hormone receptor-positive (both ER- and PgR-positive) primary breast carcinoma specimens from women included in a controlled clinical trial in 1998 (ECTO) who underwent core or needle biopsies during the luteal phase and, about 15 days later, surgery during the follicular phase, or *vice versa*.

Southern Blot Analysis

Total genomic DNA was extracted from 23 frozen primary breast carcinoma specimens, using a kit for genomic DNA extraction (Talent, Trieste, Italy). These specimens, stored at -80° , were obtained from premenopausal patients who had undergone surgery between 1990 and 1995 and were selected from a series of about 100 cases for HER2-positivity by immunohistochemistry. Extraction involves an initial lysis by means of a cationic detergent followed by a chloroform extraction. DNA samples were treated with the restriction enzyme *EcoRI*, fractionated by electrophoresis in a 1% agarose gel, transferred to nylon Hybond-N membranes (Amersham, Little Chalfont, UK), and cross-linked by UV irradiation. Prehybridization was performed at 42°C for at least 5–6 hours with 50% (v/v) formamide, 5 $\times$ Denhardt's solution, 1% sodium dodecyl sulfate (SDS), and 10 μg of salmon sperm DNA per ml.

Hybridization with a [^{32}P]-c-erbB-2 coding region probe was carried out at 42°C for 18 hours in the same solution as used for prehybridization. Unbound probe was washed from the membranes by treating twice at room temperature for 30 minutes with 2 $\times$ standard saline citrate/0.1% SDS.

Statistical Methods

All variables used in the analysis were categorical. Relationships between variables were studied, in a two-by-two setting, by means of contingency tables evaluated with the χ^2 test.

Results

Biochemical analysis of the estrogen receptor expression as well as immunohistochemical analysis of progesterone receptor expression in relation to menstrual cycle phase of the patient at time of surgery revealed a similar frequency of positive cases, irrespective of whether tumors were operated on during the follicular or luteal phase (59% *versus* 67% of ER positivity, 67% *versus* 69% of PgR positivity, respectively). The majority of samples (more than 80%) positive for the estrogen receptor were also found to be positive for progesterone receptor expression, in accordance with previous reports.¹¹ The lack of fluctuation in tumor cell of hormone receptor expression, with respect to the menstrual cycle, indicates that negative receptor expression in tumors does not reflect receptor down-modulation by hormones.

Frequency analysis of HER2, p53, Bcl2, and cathepsin D marker of gene expression in breast carcinomas in the hormone receptor (either estrogen or progesterone receptor) positive *versus* negative subgroups revealed that, except for cathepsin D, all markers studied were expressed more in hormone receptor-negative than in hormone receptor-positive cases. Indeed, overexpression of HER2 was found in 13% of PgR-positive cases *versus* 42% in PgR-negative cases and in 18% of ER-positive cases *versus* 31% of ER-negative cases. Because the major hormonal change occurring during the menstrual cycle regards the fluctuation of progesterone level, we report the marker expression during the menstrual cycle according to the PgR expression. HER2 overexpression in PgR-positive cases was detected in 20% of tumor samples (10/50) obtained from patients operated on in the first menstrual phase (1–14 days) and in only 8% of tumor samples (7/84) obtained during the second phase (14–28 days) (Table 1). The χ^2 test indicates a *P* value of 0.05. A four-category analysis of the menstrual cycle (menses, follicular, luteal, and premenses phases) also revealed statistically significant differences in the frequency of HER2 overexpression, varied from 25% to 7% (*P* = 0.04) (Figure 1). In PgR-negative cases, a small nonsignificant increase in the percentage of cases that overexpressed this marker was observed in tumor samples obtained in the late phase of the menstrual cycle (Figure 1). Similar differences in the frequency of HER2 positivity was observed in ER-positive tumors (20% of

Table 1. Variation in Marker Expression with Respect to the Menstrual Cycle Phase in Progesterone Receptor-Positive and Progesterone Receptor-Negative Breast Carcinomas

Markers	No. of cases/total (%)					
	PgR-pos.			PgR-neg.		
	First phase* [follicular]	Second phase† [luteal]	P‡	First phase* [follicular]	Second phase† [luteal]	P‡
HER2-pos.	10/50 (20)	7/84 (8)	0.05	9/24 (38)	18/39 (46)	n.s.
p53-pos.	5/50 (10)	10/84 (12)	n.s.	8/24 (33)	17/39 (43)	n.s.
Bcl2-neg.	12/50 (24)	14/84 (17)	n.s.	18/24 (75)	26/39 (67)	n.s.
Cath. D-pos.	28/49 (57)	52/84 (62)	n.s.	13/24 (54)	17/39 (43)	n.s.

Data are based on analysis by immunohistochemistry of 198 histological tumor samples. n.s., not significant.

*1–14 days of the menstrual cycle.

†15–28 days of the menstrual cycle.

‡Evaluated by χ^2 analysis.

HER positivity during the follicular phase *versus* 11% in the luteal phase). The frequencies of other markers such as p53, Bcl2, and cathepsin D, in both hormone receptor-positive and -negative cases, were independent of the menstrual phase during which the tumor was surgically removed.

The HER2 overexpression was then analyzed in eight cases of biopsies and corresponding primary breast carcinomas, positive for both estrogen and progesterone receptors, obtained from premenopausal patients who had undergone core or needle biopsies about 15 days before surgery. Two cases were scored HER2-positive on the specimen removed during the follicular phase and HER2-negative on the specimen removed during the luteal phase (Figure 2). Another six cases were found to be either HER2-negative (four cases) or HER2-positive (two cases; one shown in Figure 2) on both samples, independent of menstrual phase at removal.

HER2 gene amplification was then analyzed by Southern blot in 23 primary breast carcinomas. Of these, 11 cases were HER2-positive and hormone receptor-positive, whereas 12 cases were HER2-positive and hormone receptor-negative. *HER2* gene amplification was found in nine of 12 hormone receptor-negative cases (75%) and in only two of 11 (18%) of hormone receptor-positive cases. Therefore, according to hormone receptor expression, a

significant difference in gene amplification frequency was found ($P = 0.02$) (Figure 3).

Discussion

The frequency of expression of p53, Bcl2, and cathepsin D in both hormone receptor-positive and -negative cases was found to be independent of menstrual phase during which the tumor was surgically removed. In contrast, the expression of HER2, a 185-kd transmembrane growth factor receptor, amplified and/or overexpressed in approximately 30% of breast carcinomas and associated with poor prognosis, was found to fluctuate in hormone receptor-positive tumors according to the menstrual cycle at surgery. Indeed, an increase in the expression of HER2 was observed in hormone receptor-positive tumors resected during the follicular phase compared to those removed during the later phase of the cycle.

Cyclical hormone fluctuations regulate cell proliferation and a variety of differentiated functions in target tissues such as uterus, vagina, pituitary, and mammary epithelium. Such effects are due in large part to hormone-induced alterations in gene transcription.^{12,13} The *HER2* gene promoter is known to contain hormone-responsive elements,¹⁴ but it is unlikely that the expression of an amplified gene can be still regulated by the hormonal milieu. It is known that a small proportion of breast carcinomas overexpress HER2 because of deregulation of gene expression, not gene amplification.¹⁵ Indeed, our data indicate that *HER2* gene was found to be amplified in most hormone receptor-negative cases, whereas the amplification showed a rather low frequency in hormone receptor-positive tumor. Overexpression of HER2 in hormone receptor-positive breast carcinomas seems therefore to be due mostly to gene regulation, and fluctuation of expression according to the hormonal milieu might be due to the hormone responsiveness of the promoter. Alternatively, the changes in expression that were observed at the protein level might be due to changes in HER2 protein half-life according to the hormonal stimulation of the tumor cells.

Another receptor of the HER family, the epidermal growth factor (EGF) receptor, was also found to be not amplified but overexpressed in some breast carcinomas.

Figure 1. Frequency of HER2 overexpression according to the menstrual phase at time of surgery. Total series (▲, 198 cases); hormone receptor-positive cases (■, 134 cases, including 8, 42, 29, 55 cases in the four menstrual phases, respectively); hormone receptor-negative cases (●, 64 cases, including 6, 18, 15, 25 cases in the four menstrual phases, respectively).

Figure 2. Immunohistochemical analysis of HER2 expression in two cases of breast carcinomas. **Top:** Case 1 removed during follicular phase (A; magnification $\times 220$), and luteal phase (B; magnification $\times 180$). **Middle:** Case 2 removed during follicular phase (C; magnification $\times 95$, inset $\times 180$) and luteal phase (D; magnification $\times 95$). **Bottom:** Case 3 removed during follicular phase (E; magnification $\times 180$) and luteal phase (F; magnification $\times 180$).

Similar to the HER2 fluctuations found in hormone receptor-positive breast tumors during the menstrual cycle, the levels of EGF receptor have been reported to fluctuate during the cycle. Indeed, the EGF receptor level was found to be significantly higher in women undergoing surgery during the follicular phase of the menstrual cycle.¹⁶

Because the major change during the menstrual cycle is the progesterone level, it is likely that the progesterone receptor is the most relevant to the cell response to the hormonal fluctuation occurring during the menstrual cy-

cle. Because of the small number of cases expressing only one of the two hormone receptors, it was impossible to analyze whether the HER2 fluctuation is associated with the expression of both receptors, or whether one hormone receptor is sufficient to render the tumor cell responsive to the hormonal fluctuation during the menstrual phase. In our retrospective series the menstrual phase was assigned on the basis of patients' recall of the date of the last menstrual period. Therefore data on the correlation between estrogen or progesterone levels and HER2 fluctuation are not available. The analysis of a

Figure 3. Southern blot analysis of DNA isolated from the indicated sources. Ten micrograms of DNA was digested with *EcoRI* and hybridized with full-length *c-erbB2* cDNA. **Lanes: 1, 3, and 5,** tumors ER negative; **2 and 4,** tumors ER positive. Phage λ *HindIII* size markers in kilobases are indicated to the right.

series of patients with information about the hormonal level would give more definitive information on the role of hormone level on HER2 expression. In fact, because an increase in HER2 positivity was not observed in all tumors removed during the follicular phase, it is likely that other parameters such as hormone level and molecular profile of the tumor are involved in the responsiveness to hormonal fluctuation.

The finding that overexpression of the *HER2* gene, which is associated with proliferative and metastatic potential, can fluctuate in hormone receptor-positive breast carcinomas according to the hormonal milieu may explain, at least partially, the improved survival of breast cancer patients operated on during the luteal phase of the menstrual cycle.² In our small series, no significant differences in overall survival after 20 years of follow-up was observed in patients operated on in the two different phases of the menstrual cycle. It is noteworthy that in the majority of the studies in which a statistical significance in survival was observed, series of thousands of patients were analyzed.¹⁻³

Our finding might also be relevant for therapeutic purposes. Indeed, the possible fluctuation of the oncoprotein must be taken into consideration when patients most likely to benefit from anti-HER2 antibody therapy are being selected and treated.^{17,18} Furthermore, hormonal treatment of the patient before immunotherapy should be considered to increase HER2 expression on the tumor.

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References

- Veronesi U, Luini A, Mariani L: Effect of menstrual phase on surgical treatment of breast cancer. *Lancet* 1994, 343:1544-1546
- Hagen AA, Hrushesky WJ: Menstrual timing of breast cancer surgery. *Am J Surg* 1998, 175:245-261
- Hrushesky WJ: Menstrual cycle timing of breast cancer resection. *Recent Results Cancer Res* 1996, 140:27-40
- Goldhirsch A, Gelber RD, Castiglione M, O'Neill A, Thürlimann B, Rudenstam CM, Lindtner J, Collins J, Forbes J, Crivellari D, Coates, Cavalli F, Simoncini E, Fey MF, Pagani O, Price K, Senn HJ: Menstrual cycle and timing of breast surgery in premenopausal node-positive breast cancer: results of the International Breast Cancer Study Group (IBCSG) Trial VI. *Ann Oncol* 1997, 8:751-756
- Badwe RA, Bettelheim R, Millis RR, Gregory W, Richards MA, Fentiman IS: Cyclical tumour variations in premenopausal women with early breast cancer. *Eur J Cancer* 1995, 31A:2181-2184
- Rodgers WH, Matrisian LM, Giudice LC, Dsupin B, Cannon P, Svitek C, Gorstein F, Osteen KG: Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. *J Clin Invest* 1994, 94:946-953
- Ferguson JE, Schor AM, Howell A, Ferguson MW, Yu D, Hamada J, Zhang H, Nicolson GL, Hung MC: Changes in the extracellular matrix of the normal human breast during the menstrual cycle. *Cell Tissue Res* 1992, 268:167-177
- Saad Z, Bramwell VH, Wilson SM, O'Malley FP, Jeacock J, Chambers AF: Expression of genes that contribute to proliferative and metastatic ability in breast cancer resected during various menstrual phases. *Lancet* 1998, 351:1170-1173
- Ménard S, Casalini P, Agresti R, Pilotti S, Balsari A: Proliferation of breast carcinoma during menstrual phases. *Lancet* 1998, 352:148-149
- Cattoretti G, Becker MHG, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J: Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. *J Pathol* 1992, 168:357-363
- Clarke RB, Howell A, Potten CS, Anderson E: Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 1997, 57:4987-4991
- Landers JP, Spelsberg TC: New concepts in steroid hormone action: transcription factors, proto-oncogenes, and the cascade model for steroid regulation of gene expression. *Crit Rev Eukaryot Gene Expr* 1992, 2:19-63
- Stancel GM, Baker VV, Hyder SM, Kirkland JL, Loose-Mitchell DS: Oncogenes and uterine function. *Oxf Rev Reprod Biol* 1993, 15:1-42
- Dati C, Antoniotti S, Taverna D, Perroteau I, De Bortoli M: Inhibition of c-erbB-2 oncogene expression by estrogens in human breast cancer cells. *Oncogene* 1990, 5:1001-1006
- Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SC, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989, 244:707-712
- Oliver DJ, Ingram DM: Timing of surgery during the menstrual cycle for breast cancer: possible role of growth factors. *Eur J Cancer* 1995, 31A:325-328
- Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L: Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996, 14:737-744
- Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J: Recombinant humanized anti-HER2 antibody (Herceptin(TM)) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res* 1998, 58:2825-2831