Amplification and Overexpression of HER-2/neu (c-erbB2) in Endometrial Cancers: Correlation with Overall Survival

Bahman Saffari, Lovell A. Jones, Adel El-Naggar, Juan C. Felix, Jay George, and Michael F. Press

ABSTRACT

Few molecular genetic alterations have been identified in endometrial cancers that are associated with poor clinical outcome. Overexpression of HER-2/neu, transforming growth factor α, and p53 proteins have all been associated with poor prognosis in women with endometrial cancer. In this study, the level of HER-2/neu gene amplification and expression was characterized in 92 endometrial cancers. Fluorescence in situ hybridization (FISH) was used to characterize HER-2/neu gene copy number, and immunohistochemistry was used to characterize expression. Forty-seven of the 90 (52%) endometrial cancers were characterized as showing moderate or high immunostaining. HER-2/neu gene amplification was detected in 17 of 81 (21%) cases. Immunohistochemical staining and FISH results were both available for 80 cases. Twenty of these cases showed both moderate or high immunostaining and gene amplification. Clinical follow-up information was available for 76 women in this study. Women whose endometrial cancer exhibited HER-2/neu gene amplification by FISH had a shorter overall survival than women whose endometrial cancer lacked amplification (P = 0.018). Likewise, tumors with moderate or high HER-2/neu immunostaining were associated with a lower cumulative overall survival than tumors with low immunostaining by log rank analysis (P < 0.0001). Multivariate analysis of survival rates revealed HER-2/neu overexpression to be an independent predictor of overall survival (P = 0.0163). Among those patients with HER-2/neu overexpression, adjuvant chemotherapy or radiation therapy was associated with an improved overall survival (P = 0.039). However, among those women whose tumor lacked overexpression, overall survival was not improved by adjuvant treatment.

INTRODUCTION

Endometrial cancer is the most common cancer of the female genital tract in the United States, with approximately 33,000 new cases diagnosed annually (1). Relatively few studies have been performed to identify molecular genetic alterations in endometrial cancers. Some proto-oncogenes and tumor suppressor genes have been reported to be altered. Activating point mutations in c-Ki-ras (2) appear to be an early event in tumorigenesis because the altered form of the gene is uniformly present in endometrial carcinoma, as well as in adjacent atypical hyperplasia (3). Missense single base substitutions have been observed in the p53 tumor suppressor gene of endometrial carcinomas (4). Increased expression of HER-2/neu oncoprotein (5–12), transforming growth factor α protein (11), and macrophage colony-stimulating factor proto-oncogene mRNA (13) have also been described. Various chromosomal alterations including isochromosome of 1q10; deletion of 6q21–25 (14); and loss of heterozygosity for 3p, 9q, 10q, and 17p (15) suggest that tumor suppressor genes may be located at these sites. Recently, it was reported that genomic instability in the form of microsatellite instability also occurs in endometrial carcinoma, which may contribute to the transformed phenotype (16, 17). Molecular genetic alterations have not only been identified in endometrial carcinomas, but some of them are of potential prognostic utility. These include p53 (18), transforming growth factor α (11), and HER-2/neu proteins (9, 19), which have been associated with poor clinical outcome.

The HER-2/neu proto-oncogene encodes a Mr, 185,000 transmembrane tyrosine kinase receptor, the aberrant overexpression of which has been implicated in the induction of a malignant phenotype (20–22). The molecular mechanism responsible for kinase activation is thought to involve receptor homodimerization or heterodimerization. It has been postulated that overexpression of HER-2/neu oncoprotein leads to autoactivation of the tyrosine kinase domain, activation of signal transduction pathways, and cellular transformation or cellular proliferation (23). HER-2/neu overexpression has been used to predict both disease-free and overall survival in patients with various cancers including those of breast, ovary, and salivary gland (24, 25). Twenty-five to 30% of breast and ovarian cancers show HER-2/neu overexpression and/or amplification. Women whose tumors harbor these alterations, independent of other indicators of clinical outcome such as nodal involvement, have lower rates of 5-year disease-free and overall survival. Although several studies have examined the association of HER-2/neu overexpression with prognostic indicators such as clinical stage, histological type, grade, and differentiation markers, as well as clinical outcome in endometrial cancer patients (5–12), the results obtained from these studies are not in agreement with respect to clinical outcome (9, 11, 12, 19). Because the prognostic value of HER-2/neu is not established, we evaluated HER-2/neu gene amplification and overexpression in a series of endometrial cancers and compared the results with established prognostic markers and with overall survival. In addition, the response of the patients to adjuvant therapies was compared to HER-2/neu expression level of the endometrial cancer.

MATERIALS AND METHODS

Patient Information. The research protocol for this investigation was reviewed and approved by the Institutional Research Board (Protocol No. 059251). Racial or ethnic group was identified as white in 43, as black in 8, and as “Hispanic” in 5 women. Racial origins of Hispanic patients were not specified. Age at the time of diagnosis ranged from 29 to 87 years, with a mean of 60 years. Clinical information including age, sex, date of diagnosis, stage of disease, treatment, presence of recurrent disease, date of death, and cause of death were recorded for 76 of the 92 cases. The remaining 16 cases did not contain enough information to assess the clinical outcome. Clinical follow-up ranged from 3 to 123 months, with a mean of 67 months. Two patients were treated with chemotherapy, and 30 patients were treated with radiation therapy. The laboratory analyses were performed masked to the clinical information (Table 1).

Tumor Tissue. Frozen tumor tissue from 90 endometrial cancers was analyzed for HER-2/neu gene expression by immunohistochemistry, and 81
Frozen tissues were analyzed for gene amplification by FISH. The neoplasms were accessioned between 1978 and 1989. Histopathological review of frozen tissue sections confirmed that tumor cells were present in all specimens included in this study. The histopathological profiles of tumors analyzed are described in Table 1.

**Immunohistochemistry.** The immunohistochemical staining method, as described previously (24–27), involved the sequential application of three antibodies to tissue sections as follows: (a) primary rabbit HER-2/neu antibody; (b) a secondary or bridging goat antirabbit IgG antiserum (1:75 dilution; Zymed, Inc.); and (c) a rabbit peroxidase antiperoxidase antiserum (1:75 dilution; Dako, Carpinteria, CA). The antiserum had no cross-reactivity to epidermal growth factor receptor (24).

The tissue sections were incubated for 1 h with primary antibody and for 0.5 h each with secondary and tertiary antibodies at room temperature. After treatment with a tertiary antiserum, the sections were incubated for 0.5 h each with primary antibody and with secondary and tertiary antibodies at room temperature. After treatment with each antibody, the tissue sections were washed with PBS. The immunoperoxidase were identified microscopically after incubation with the chromo- 

Membrane staining was interpreted as HER-2/neu oncoprotein expression. The amount of staining was scored in a blinded fashion as negative (no immunostaining), trace positive (weakly immunostained tumor with membrane staining at the level of detectability), moderate immunostaining (distinct membrane staining in the majority of tumor cells), or strong immunostaining (intense staining). The heterogeneous staining was observed in cases with radiation therapy or chemotherapy before hysterectomy and in cases with marked morphological heterogeneity among the tumor cells, especially the malignant Müllerian mixed tumors. Among malignant Müllerian mixed tumors, the carcinomatous component had membrane staining, and the sarcomatous component lacked staining.

**FISH.** The gene copy level of HER-2/neu and α satellite (pericentromeric) DNA for chromosome 17 was identified in the nuclei of tumor cells by FISH. Because the HER-2/neu gene is located on chromosome 17 (28), the α satellite (pericentromeric) DNA was selected as an internal control for aneuploidy of chromosome 17. By comparing the number of copies of these two chromosomal markers, aneuploidy of chromosome 17 could be excluded as a source of increased HER-2/neu gene copy number. The α satellite DNA was also used as an internal control to correct for differences that might arise due to tissue sectioning artifacts.

Four-μm-thick frozen tissue sections were allowed to air-dry for 30 min at room temperature. Tissue sections were placed in 100% methanol at –20°C for 30 min and subsequently in a 4:1 solution of methanol:acetic acid for 20 min. The fixed tissues were washed in running tap water for 5 min, dehydrated through a graded series of ethanol (70–100%), treated with RNase in 2X SSC (1 X SSC = 0.15 M NaCl, 0.015 trisodium citrate)-0.1% triton solution at 37°C for 1 h, washed three times in 2X SSC, and dehydrated through a graded series of ethanol. Tissue sections were denatured in 60% formamide-2X SSC at 78°C for 9 min and then hybridized overnight at 37°C with a solution containing cosm id probes for HER-2/neu (Oncor, Inc., Gaithersburg, MD) and chromosome 17 centromere (Oncor, Inc.), labeled, respectively, with biotin and digoxigenin. After washing the sections in posthybridization washing solution and 2X SSC, the probes were detected with avidin-FITC and rhodamine-labeled antidigoxigenin antibody. The tissue sections were washed with 1X phosphate-buffered detergent (Oncor, Inc.), and the signal was amplified by successive incubations with antiavidin antibody, FITC-linked avidin, and rhodamine-labeled antidigoxigenin antibody. The nuclei were counterstained with 4-6-diamino-2-phenylindole. The staining was visualized with a Zeiss fluorescence microscope. Using criteria established for Southern hybridization and confirmed as appropriate for FISH, a ratio of 2:1 or greater between the HER-2/neu signals and chromosome 17 centromere signals was considered to be consistent with HER-2/neu gene amplification (24, 25, 29–31).

**Statistical Methods.** The clinical measurement of primary interest was time to death. Kaplan-Meier product-limit estimates of survival time were plotted (32, 33). The Cox proportional hazard model and log rank test were used to test for statistical significance (34–37). Associations between variables were measured by standard methods for contingency tables (38). All P values reported are 2-sided.

### RESULTS

**Expression of HER-2/neu in Endometrial Cancers.** Ninety endometrial cancers were examined for HER-2/neu expression by immunohistochemistry. Forty-eight % (43 of 90) of the cases showed no or weak membrane staining (Fig. 1D). Moderate or strong HER-2/neu membrane staining was observed in 35 and 12 cases, respectively. Most of the cases with moderate or strong immunostaining had a relatively uniform pattern of membrane staining; tumor cells within a given case showed a similar amount of staining (Fig. 1, E and F). However, a few cases did demonstrate a heterogenous pattern of HER-2/neu immunostaining. The heterogeneous staining was observed in cases with radiation therapy or chemotherapy before hysterectomy and in cases with marked morphological heterogeneity among the tumor cells, especially the malignant Müllerian mixed tumors. Among malignant Müllerian mixed tumors, the carcinomatous component had membrane staining, and the sarcomatous component lacked staining.

**HER-2/neu Gene Amplification.** FISH was used to identify HER-2/neu and α satellite DNA for chromosome 17 (centromere) in interphase tumor cell nuclei. Twenty-one % of cases (2 of 82) showed amplification as defined by the HER-2/neu signal/α satellite DNA signal ratio ≥2 (Fig. 1, A–C). Three cases were amplified 5-fold or greater, and the remaining 14 cases were amplified 2–4-fold (Fig. 1, B and C). All the cases that showed amplification exhibited a distinctive “clustering” of HER-2/neu signals within interphase nuclei.

### Comparison of HER-2/neu Gene Amplification to Immunostaining

Eighty-eight % of cases (14 of 16) with gene amplification by FISH also showed HER-2/neu protein overexpression by immu-

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*Table 1 Relationship of HER-2/neu proto-oncogene levels and immunostaining to known prognostic indicators*

<table>
<thead>
<tr>
<th>HER-2/neu Immunostaining</th>
<th>HER-2/neu gene copy status</th>
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<tbody>
<tr>
<td><strong>Stage</strong></td>
<td><strong>Total no. of cases (%)</strong></td>
</tr>
<tr>
<td>I</td>
<td>26 (52)</td>
</tr>
<tr>
<td>II</td>
<td>4 (57)</td>
</tr>
<tr>
<td>III</td>
<td>4 (50)</td>
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<tr>
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<td>II</td>
<td>19 (51)</td>
</tr>
<tr>
<td>III</td>
<td>7 (41)</td>
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<tr>
<td><strong>Histological type</strong></td>
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<tr>
<td>Adenosquamous</td>
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<td>MMMT</td>
<td>2 (25)</td>
</tr>
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<td>Serous</td>
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</table>

*P = 0.011*

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*The abbreviation used is: FISH, fluorescence in situ hybridization.*

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Fig. 1. HER-2/neu gene copy level and expression in endometrial cancers. A–C, FISH of HER-2/neu gene in endometrial cancers. A, endometrial cancer showing no increase in the number of HER-2/neu gene copies in interphase nuclei of tumor cells, as revealed by the number of yellow-green FITC fluorescent signals (e.g., arrowheads). Red rhodamine fluorescent signals correspond to chromosome 17 pericentromeric α satellite DNA. Nuclei are counterstained with 4'–6-diamino-2-phenylindole and appear blue. B, another endometrial cancer showing an increase in the number of HER-2/neu signals, averaging 4–5 copies per tumor cell nucleus, with 1–2 chromosome 17 α satellite DNA signals. Notice that the HER-2/neu gene signals are arranged in groups or clusters in a limited area of the nucleus consistent with localization in homogeneous staining regions of chromosomes. C, many HER-2/neu signals clustered in the nuclei of this endometrial carcinoma, which, like B, demonstrates gene amplification. D–F, endometrial cancers were subgrouped into low, moderate, and high expression groups based on the level of HER-2/neu immunostaining, with low immunostaining considered to be the "normal" level of expression and moderate/strong membrane immunostaining of tumor cells considered to be overexpression. D, endometrial carcinoma illustrating low expression as assessed by weak HER-2/neu immunostaining. E, endometrial carcinoma with overexpression, as demonstrated by moderately increased immunostaining. F, endometrial carcinoma with overexpression, as demonstrated by markedly increased (strong) membrane immunostaining of tumor cells. ×1600.

Eighteen % of cases (14 of 80) showed both overexpression and amplification of the gene. Forty-one % of cases (33 of 80) lacked gene amplification and overexpression, whereas 39% of cases (31 of 80) showed overexpression but lacked gene amplification.

Association of HER-2/neu Immunostaining and Gene Amplification to Stage, Grade, and Histological Type. Gene amplification was associated with histological type ($P = 0.011$) of endometrial cancer, with 50% of carcinosarcomas (malignant Müllerian mixed tumors) showing amplification. There was no association between...
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Fig. 2. Kaplan-Meier actuarial survival curves for women with endometrial cancer. Women whose endometrial cancer had gene amplification or moderate/high HER-2/neu immunostaining had lower cumulative survival rates than women whose tumors lacked these alterations. A, cumulative survival of all women stratified by low (••••••) versus moderate/high (— — — — ) immunostaining. The difference in survival is statistically significant (P < 0.0001). B, overall survival rates for patients with (— — — — ) versus women without (••••••) gene amplification. The difference in survival between these groups is also statistically significant (P = 0.018). C, cumulative survival of women with endometrial cancer stratified by HER-2/neu expression (•••••• = low, — — — — — — = high) and treatment (Tx). No Tx, no treatment; n, total of women in each category at the time of diagnosis. Twenty women had no treatment and low HER-2/neu immunostaining of their tumors; 13 women had adjuvant therapy and low HER-2/neu immunostaining of their tumors; 14 women had moderate/high immunostaining of their tumors and also had adjuvant therapy; and 27 women had moderate/high immunostaining of their tumors and had no adjuvant therapy. a, statistically significant difference observed in the overall survival of women not receiving any adjuvant therapy when they were stratified according to the immunostaining of their endometrial cancer (P < 0.00001); b, statistically significant difference (P = 0.039) observed in the overall survival of women whose endometrial cancers had moderate or high immunostaining if they were stratified by treatment versus no treatment with adjuvant therapies. All P values for the survival curve comparisons were generated with log rank statistics.

stage or grade and amplification (Table 1). In contrast, gene expression by immunostaining showed no statistically significant association with known prognostic indicators including stage, grade, and histological type of endometrial cancer (Table 1), although all four stage IV endometrial cancers had moderate to strong immunostaining.

Correlation of HER-2/neu Immunostaining and Gene Amplification with Overall Survival. Clinical follow-up for women in this study showed that 53% (40 of 76) were alive with no evidence of disease at the time of last follow-up, 40% (30 of 76) died of their disease, 3% (2 of 76) died of other causes, and 5% (4 of 76) were alive with the disease at the time of last follow-up. In this group, both HER-2/neu overexpression (P < 0.0001) and gene amplification (P = 0.018) were associated with lower overall survival rates by log rank statistical analysis (Fig. 2, A and B).

In the low expression group, only 4 of 34 (12%) patients died of their disease, whereas in the high expression group, 26 of 41 patients (63%) died of their disease during the period of follow-up. Multivariate analysis using the Cox proportional hazard model of survival rates (taking into account the stage, grade, and histological subtype of the tumors) revealed that HER-2/neu overexpression was an independent indicator of poor clinical outcome (P = 0.0163).

Improved Overall Survival among Women with Elevated Levels of HER-2/neu Immunostaining Who Received Adjuvant Therapy. In this retrospective study, those women whose endometrial cancers showed low HER-2/neu immunostaining and who received any adjuvant therapy did not show a statistically significant difference in overall survival in comparison to women with low immunostaining tumors who did not receive adjuvant therapy (P = 0.37; Fig. 2C). Among the 41 women with moderate or strong HER-2/neu immunostaining tumors, 14 women received adjuvant therapy, and 27 women were not treated. In women with moderate or strong HER-2/neu immunostaining tumors, 6 of 14 (43%) women who received adjuvant treatment died of their disease, whereas 20 of 27 (74%) patients without treatment died of their disease. Women in the high immunostaining group who received treatment had better overall survival rates (P = 0.039) than women who did not receive adjuvant therapy (Fig. 2C).

DISCUSSION

HER-2/neu proto-oncogene is amplified and overexpressed in a variety of adenocarcinomas including those of the breast, ovary, and salivary gland (24, 25). Both amplification and overexpression, observed in approximately 25% of these carcinomas, are associated with poor prognosis (24, 25).

In the current study, frozen tissue was used to characterize both expression and amplification of the HER-2/neu gene. Using an antibody, known to be sensitive in both frozen (24) and formalin-fixed (27) tissue, the overexpression rate (demonstrated as moderate or strong immunostaining) in this group of endometrial cancers was...
52%, whereas the gene amplification rate by FISH analysis was only 21%. This group of endometrial cancers showed an exceptionally high rate of HER-2/neu overexpression without gene amplification (39%), suggesting that endometrial cancers, like breast, ovarian, and salivary gland carcinomas, may have overexpression due to a mechanism other than amplification. The overall concordance rate between HER-2/neu gene amplification and overexpression was 88%, with 14 cases exhibiting both gene amplification and overexpression. Two cases had amplification but lacked overexpression.

Nine of the endometrial neoplasms included in this study were malignant müllerian mixed tumors or carcinosarcomas, a tumor type with both malignant epithelial and malignant stromal cells, which is therefore thought to arise from pluripotential cells capable of differentiating in either an epithelial or mesenchymal direction. It was of interest that these tumors showed HER-2/neu expression only in the carcinoma cells and not in the sarcoma cells, and 4 of 8 (50%) cases showed gene amplification.

HER-2/neu gene amplification is an alteration associated with neoplastic phenotype. Although HER-2/neu is expressed in normal, cyclic, and postmenopausal endometrial epithelium at low levels, the gene is not amplified and is not overexpressed (26).

Extent of disease at diagnosis (high stage), high nuclear grade, adverse histopathology, lymphovascular space invasion, and DNA aneuploidy are associated with poor prognosis in endometrial carcinomas (12). Recent studies have suggested that oncogenes or tumor suppressor genes are not only altered in this disease but may also be of prognostic significance. Several studies have characterized HER-2/neu amplification and overexpression in endometrial carcinomas (5–12, 39). The reported frequency of overexpression analyzed by immunohistochemistry varied from 2 to 36% (5–12). One of these studies, using the TAB250 antibody without protease pretreatment of tissue sections, found an exceptionally low level of immunostaining (2%). This antibody is not very sensitive in paraffin-embedded tissue without protease pretreatment of the tissue to improve antigen recognition (27); however, this factor alone is unlikely to account for such a low level of detection. A few studies involve analysis of small sample sizes varying from 16 to 49 specimens; it is, therefore, difficult to draw conclusions regarding frequency of amplification/overexpression and correlation with clinical outcome (7, 8, 10, 39). Five studies of immunohistochemical staining for HER-2/neu involved 78–247 cases and demonstrated 9–18% overexpression with 1 of these 5 studies (11), showing no correlation between overexpression and poor clinical outcome. One study (12) shows a wide separation of overall survival curves, but this difference does not achieve statistical significance ($P = 0.08$). Two reports from the same group show a correlation with advanced disease stage (6) and presence of persistent and recurrent disease (19). The largest study of HER-2/neu in endometrial cancer showed a strong correlation between overexpression identified by immunohistochemistry and poor overall survival (9).

Our study not only showed a statistically significant association between overexpression and shortened overall survival but a similar association for gene amplification and overall survival. HER-2/neu gene amplification has been demonstrated in one previous study of 16 endometrial carcinomas using Southern hybridization; however, no internal control gene was used to confirm that similar amounts of DNA were loaded into each lane and to control for aneusomy as a source of increased gene copy level (39). In that study (39), amplification was identified in 11 of the 16 endometrial carcinomas; all 11 experienced recurrent disease, and 4 of them died of their disease within 2 years of diagnosis. The other five women whose tumor lacked amplification were alive without disease 31 months after diagnosis.

Our study group is probably biased toward large endometrial cancers because of the requirement for storage of an adequate sample of frozen tissue above and beyond that needed for diagnostic evaluation. An additional factor is the tendency for patients with more aggressive disease to be referred to cancer centers for treatment. This impression is supported by the relatively large proportion of our study group that experienced a recurrence or death from disease. Nevertheless, in our study, the association between HER-2/neu overexpression and poor survival was observed in all women included in the study, as well as in the subgroup of women who had received no adjuvant treatment (Fig. 2C). Women whose endometrial cancers had overexpression showed an improved overall survival if they had received adjuvant chemotherapy or radiation therapy compared to those women who did not receive such therapies (Fig. 2C). Although the number of study subjects was small, the survival of women whose tumors lacked overexpression was not improved by adjuvant therapies. These preliminary findings in a small number of women with endometrial cancer suggest that HER-2/neu expression levels may be useful in selecting adjuvant therapy for women with endometrial cancer. HER-2/neu overexpression appears to be a useful marker in selecting women with breast cancer for high-dose adjuvant chemotherapy (40). More prospective clinical trials are needed to confirm this observation.

In summary, our findings demonstrate that HER-2/neu is both amplified and overexpressed in endometrial cancers. Alteration of HER-2/neu is a potential prognostic marker of poor outcome and may have clinical utility in selecting patients for adjuvant therapies.

**ACKNOWLEDGMENTS**

The authors thank Drs. John Morrison (Division of Biometry, Department of Preventive Medicine, University of Southern California) for assistance with the statistical analysis of the data and Jian-Yuan Zhou (Department of Pathology, University of Southern California) for assistance with the FISH technique.

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