VOLUME 24 · NUMBER 26 · SEPTEMBER 10 2006

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

# ErbB-3 Predicts Survival in Ovarian Cancer

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Submitted November 6, 2005; accepted May 2, 2006; published online ahead of print at www.jco.org on August 7, 2006.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/06/2426-4317/$20.00

DOI: 10.1200/JCO.2005.04.8397

## ABSTRACT

### Background
HER3 (erbB-3) is a member of the epidermal growth factor receptor (EGFR) family. After dimerization with other members of the EGFR family several signal transduction cascades can be activated, including phosphoinosite 3′-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (ERK1/2). Here, we studied a possible association between HER3 expression and prognosis in patients with ovarian cancer.

### Methods
Tumor tissue of 116 consecutive patients diagnosed with primary epithelial ovarian cancer between 1986 and 1995 was analyzed immunohistochemically for HER3 expression. A possible influence of HER3 expression on survival was studied by multivariate Cox regression adjusting for established clinical prognostic factors.

### Results
A positive HER3 expression was observed in 53.4% of the patients. HER3 expression was associated with decreased survival in proportional hazard modeling, including the International Federation of Gynecology and Obstetrics (FIGO) stage, histologic grade and type, residual disease, and age. After likelihood ratio forward as well as backward selection, only HER3 expression (hazard ratio, 1.71; 95% CI, 1.10 to 2.67; $P = .018$), FIGO stage (hazard ratio, 4.78; 95% CI, 1.89 to 12.08; $P = .001$), residual tumor (hazard ratio, 2.69; 95% CI, 1.40 to 5.17; $P = .003$), and age (hazard ratio, 2.06; 95% CI, 1.17 to 3.65; $P = .013$) were found to be significant. Kaplan-Meier plots demonstrated a clear influence of HER3 expression on survival time. Median survival time was 3.31 years (95% CI, 1.93 to 4.68) for patients with low HER3 expression, compared with only 1.80 years (95% CI, 0.83 to 2.78) for patients with HER3 overexpression (log-rank test $P = .0034$).

### Conclusion
HER3 may represent a new prognostic factor in primary epithelial ovarian cancer. Pending validation, exploration of therapeutic strategies to block HER3 could be warranted.

*J Clin Oncol 24:4317-4323. © 2006 by American Society of Clinical Oncology*

## INTRODUCTION

Ovarian cancer is the leading cause of death from gynecologic malignancy. However, relatively little is known about the molecular ethiology. Alterations in expression or copy number changes have been identified in a number of genes including *BRCA1*, *TP53*, *RB1*, *OPCML*, *PIK3CA*, *HER2* (erbB-2), and *EEF1A2*.[1-7]

Trastuzumab (Herceptin; Genentech Inc, South San Francisco, CA), a humanized monoclonal antibody directed against human epidermal growth factor receptor 2 (HER2; erbB-2), provides clinical benefits for patients diagnosed with advanced breast cancer overexpressing HER2 protein.[8,9] However, in ovarian cancer, the clinical value of single-agent trastuzumab is limited.[10] Only a relatively small fraction of ovarian carcinoma patients overexpress HER2.[10] In addition, only 7.3% of HER2-overexpressing (2+ or 3+ HER2) recurrent ovarian carcinomas showed an objective response to single-agent trastuzumab. Therefore, identification of new therapeutic targets in ovarian cancer is necessary.

Recently, van der Horst et al[11] demonstrated the inhibibitory effect of an antibody directed against HER3 ($\alpha$-HER3$^{ECD}$) on HER3-mediated signaling. Interestingly, inhibition by $\alpha$-HER3$^{ECD}$ was also observed in tumor cell lines resistant to antibodies directed against HER2. Similar to Herceptin, $\alpha$-HER3$^{ECD}$ also caused internalization of its target receptor. The intriguing data of van der

Horst et al reinforce the notion that HER3 could be another key target in cancer drug design.[11] HER3 is a dimerization partner of HER2. HER2/HER3 heterodimers are known to deliver the most potent and long-lasting signal among the possible combinations of the four members of epidermal growth factor receptors HER1 to HER4.[12,13] One of the mechanisms responsible for the potency of HER2/HER3 dimers is the ability to continue signaling after ligand-induced internalization.[14] While HER3 activates other receptor tyrosine kinases, its own tyrosine kinase domain is inactive.[15] This may explain, why until recently,[11] HER3 has not been studied as a therapeutic target.

Little is known about the prognostic relevance of HER3 in ovarian cancer. Therefore, we determined HER3 expression in 116 tumor specimens of patients with primary epithelial ovarian cancer and studied a possible association with survival. Here, we report that HER3 is a prognostic factor, independent from the established histopathologic parameters. Our data suggest that HER3 represents a promising target in the treatment of ovarian cancer.

## PATIENTS AND METHODS

### *Patients, Tissue Specimens, and Pathologic Data*

Tumor tissue was collected from 116 patients with primary epithelial ovarian cancer who underwent surgery from 1986 to 1995 at the Department of Gynecology, University of Mainz (Table 1). Patients with benign and nonepithelial tumors, borderline tumors, and recurrent disease were excluded from the study. Tissue specimens were taken intraoperatively, formalin fixed, and paraffin embedded. Treatment of patients with ovarian carcinoma was primarily by surgery. Patients were treated by total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy. In those with International Federation of Gynecology and Obstetrics (FIGO) stages III and IV, which could not be completely removed, a debulking operation was performed, to reduce the remaining tumor to a diameter not exceeding 2 cm. Patients with FIGO stages Ib, Ic, II, III, and IV usually received postoperative chemotherapy with six courses of carboplatin (350 mg/m$^2$) or cisplatin (50 mg/m$^2$) and cyclophosphamide (1,000 mg/m$^2$). The first cycle of chemotherapy was given within 3 to 4 weeks after primary surgery. Histologic typing was performed according to the WHO criteria. Epithelial tumors were subdivided into serous and nonserous carcinomas. Histologic grade of malignancy ranged from G1 (well differentiated) to G3 (poorly differentiated). Grading was performed by the following criteria: tumor architecture, amount of solid tumor, nuclear pleomorphism, nucleus cytoplasm ratio, number of nucleoli, and mitoses. Tumor staging was done according to FIGO guidelines. Since residual disease is a strong prognostic factor, we differentiated between patients with macroscopically complete removal of all tumor tissue and patients with $\leq$ 2 and greater than 2 cm of tumor diameter left after surgery.

**Table 1.** Characteristics of Patients With Primary Epithelial Ovarian Cancer

| | Primary Epithelial Ovarian Carcinomas (N = 116) | | |
|---|---|---|---|
| | No. Assessable | % | Not Assessable |
| FIGO stage | | | 0 |
| I | 22 | 19.0 | |
| II | 7 | 6.0 | |
| III | 68 | 58.6 | |
| IV | 19 | 16.4 | |
| Histologic grade | | | 0 |
| I | 15 | 12.9 | |
| II | 51 | 44.0 | |
| III | 50 | 43.1 | |
| Histologic type | | | 0 |
| Serous | 73 | 62.9 | |
| Nonserous type | 43 | 37.1 | |
| Residual disease | | | 1 |
| No residual tumor | 44 | 38.3 | |
| < 2 cm residual tumor | 35 | 30.4 | |
| > 2 cm residual tumor | 36 | 31.3 | |
| Age at surgery, years | | | 0 |
| ≥ 55 | 24 | 20.7 | |
| < 55 | 92 | 79.3 | |
| Mean | 59.05 | | |
| Standard deviation | 13.08 | | |

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

### *Immunohistochemical Determination of HER3 and HER2*

Immunostaining was performed as described previously[16] with modifications. Specimens on the slides were deparaffinized by rinsing in xylol (10 minutes) and absolute ethanol (5 minutes) followed by hydration in distilled water.[7] The wet slides were cooked twice for 10 minutes each in citrate buffer[16] in a microwave. After cooling down to room temperature, the slides were rinsed twice in triethanolamine-buffered saline (TBS) buffer.[16] Subsequently, the tissue slices were incubated with an overlayer of hydrogen peroxide (3%) for 20 minutes at room temperature and rinsed three times with TBS buffer. The following incubations were performed in a wet chamber at 37°C. The slices were incubated with 3% BSA for 20 minutes followed by the addition of the primary antibody (c-erbB-3, C-17, rabbit polyclonal antibody; Santa Cruz Biotechnology, Heidelberg, Germany; dilution: 1:25 in TBS buffer) and incubation for 1 hour at room temperature. Thereafter, they were rinsed three times with TBS buffer for 5 minutes each and incubated with a biotinylated secondary antibody (biotinylated rabbit immunoglobulin G antibody from the Rabbit UniTect ABC Kit, #XHC02; Oncogene Research Products, Dianova, Hamburg, Germany; dilution: 1:50 in TBS buffer) for 30 minutes at room temperature. Subsequently, the slices were rinsed again in TBS buffer and overlayed with streptavidin and biotinylated horseradish peroxidase[7] and incubated for 30 minutes at room temperature. On rinsing with TBS buffer (three times for 5 minutes each) they were stained with diaminobenzidine substrate (D4168; Sigma, Munich, Germany) for 5 minutes at room temperature. Finally, slides were carefully rinsed with tap water (10 minutes) and counter-stained with hemalaun (1:10 diluted) for 45 seconds. On rinsing with tap water (10 minutes), they were dehydrated and embedded. Adenocarcinomas of the colon were used as positive and negative controls and were included into each immunostaining experiment. The stained slices were evaluated using the Rajkumar score,[17] resulting from the product of the score for the fraction of positively stained tumor cells (ranging from 0 to 4: 0 = < 10% of positively stained tumor cells; 1 = 10% to 25%; 2 = 26% to 50%; 3 = 51% to 75%; 4 = > 75%) and the score for staining intensity (0 to 3). Slices with Rajkumar scores ≥ 8 were classified as "HER3 high," in contrast to slices with scores lower than 8 ("HER3 low"). All investigations were performed under a microscope with chiffre-labeled slides. Slides were evaluated by two independent operators (K.S. and M.S.), who obtained identical results for the classification as "high" versus "low" HER3 expression. Confocal microscopy[18] and immunostaining with Cy3-labelled antibodies[19] was performed as described. Briefly, the slides were deparaffinized, cooked twice in citrate buffer, blocked for 1 hour in 3% BSA, incubated with the first antibody (c-erbB-3, rabbit polyclonal antibody; Santa Cruz Biotechnology; dilution: 1:50) for 1 hour at room temperature, washed, and incubated for 1 hour at room temperature with the second antibody (donkey antirabbit-Cy3; Dianova; 1:100). Nuclear staining was performed with DAPI. The cell lines OVCAR-3, SK-OV-3, IGROV-1, and MCF7 were cultured as described,[11,20] fixed with 4% paraformaldehyde at room temperature for 1 hour, washed in phosphate-buffered saline, incubated with 3% BSA/saponin (0.1 mg/mL) for 1 hour at room temperature, washed and further incubated with the first (c-erbB-3, rabbit polyclonal antibody) and second (donkey antirabbit-Cy3; Dianova; 1:100) antibodies as described above. HER2 was immunohistochemically detected using a commercially

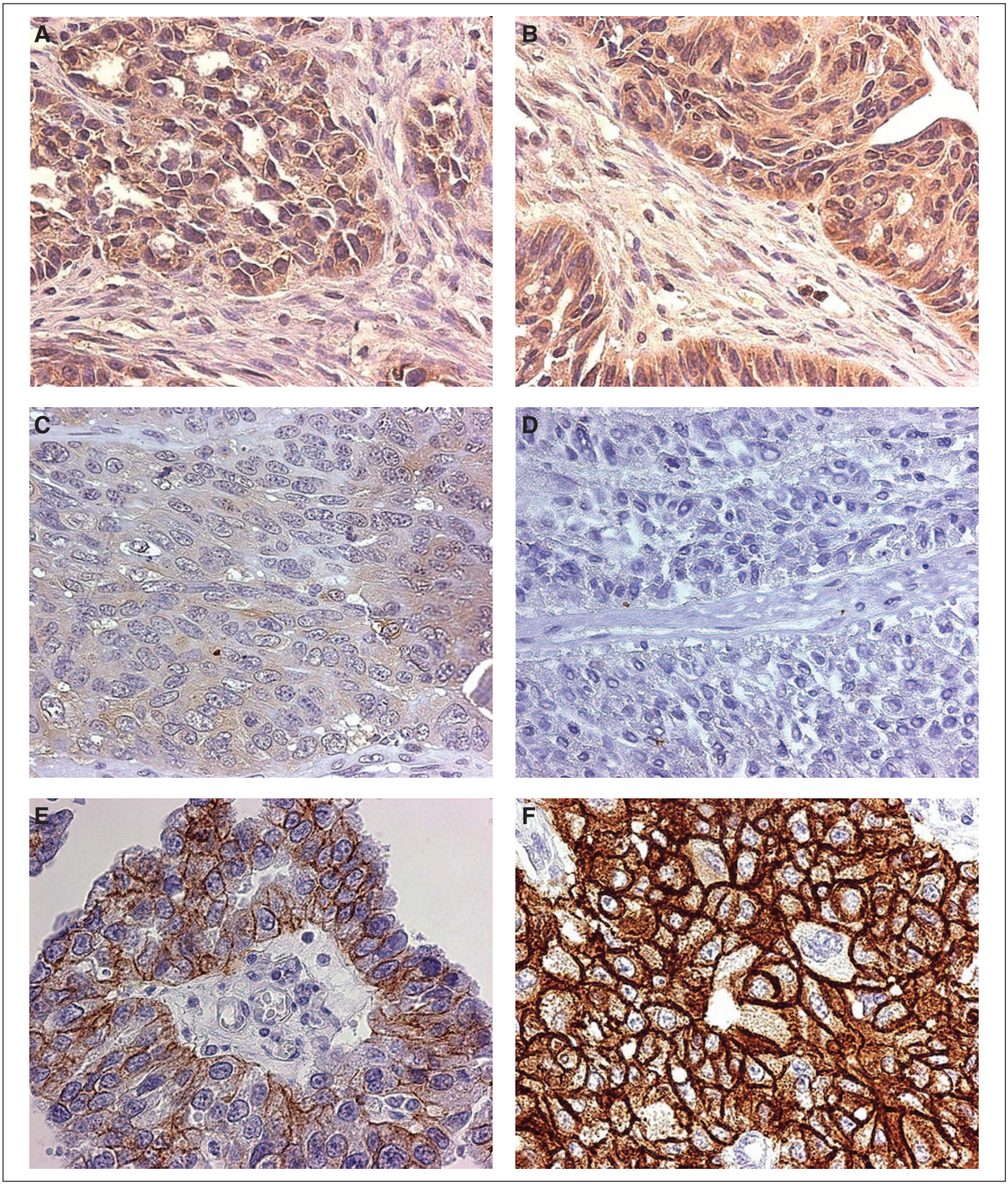


**Fig 1.** Representative examples of epithelial ovarian carcinomas after immunostaining with antibodies specific for HER3 (A-D) and HER2 (E, F). A and B, Strong expression of HER3 (Rajkumar score > 8); C, weak HER3 expression (Rajkumar score < 8); D, negative staining for HER3; E and F, strong membrane staining for HER2. Magnification, 100×.

available kit (HercepTest; Dako, Hamburg, Germany) according to the manufacturer's instructions.

### Statistical Analysis

Kaplan-Meier curves were plotted to assess overall survival. Different survival curves were compared using the log-rank test. The proportional hazard modeling was used to examine whether HER3 was an independent prognostic factor. The HER3 Rajkumar-scores were dichotomized at the median ($< 8\ v \geq 8$). As we were well aware of the danger of "*P* value optimization" by varying cut-points, we have defined the median as the cut point for the analysis [6,7,21,22] prior to analyzing any survival data. The proportional hazards assumption was checked by viewing Cox-Snell residuals, which showed a good fit of the final Cox regression model. The $\chi^2$ test was applied to test a possible association between HER3 expression and FIGO stage, histological grade and type, residual disease, and age. Statistical analysis was performed using SPSS version 10.0 (SPSS Inc, Chicago, IL) software.

## RESULTS

### HER3 Immunostaining

HER3 expression was evaluated in ovarian cancer tissue of 116 patients, resulting in a median Rajkumar score of 8. The patients were dichotomized at the median (score $< 8\ v \geq 8$) resulting in the HER3-high (n = 62; 53.4%) and HER3-low (n = 54, 46.6%; Appendix Fig A1, online only) groups. Typical results of tumors with high (Fig 1A and 1B) and low (Fig 1C and 1D) HER3 expression are shown in Figure 1. The predominant pattern of HER3 staining in ovarian carcinomas was cytoplasmic with only weak membrane staining (Appendix Fig A2, online only, parts A and B). This pattern was similar in the ovarian cancer cell lines OVCAR-3, SK-OV-3, and IGROV-1 (Fig A2, parts C through F). The degree of HER3 membrane staining in these ovarian cancer cell lines was smaller compared with the breast cancer cell line MCF7 (Appendix Fig A3, online only). Because the analyzed paraffin blocks were collected throughout a period of 9 years, we compared blocks older and younger than the median (12 years) to detect a possible loss or masking of the HER3 antigen over time. However, no decrease in HER3 expression due to long storage was found ($\chi^2 = 0.71$; $P = .71$).

### Prognostic Significance of HER3 Expression

HER3 expression (high *v* low expression) in tumor tissue was associated with survival of patients with primary ovarian cancer when the univariate proportional hazards model was applied (Table 2). The relative risk (RR) was 1.894 with a 95% CI of 1.226 to 2.925 ($P = .004$). As in previous studies, the established clinical prognostic factors FIGO stage, histological grade, residual disease and age were clearly associated with survival (Table 2). In contrast, only a trend was observed for histologic type.

Next, we used the multivariate proportional hazard model to analyze the prognostic impact of HER3 adjusted for well-established clinical prognostic factors: FIGO stage, histological grade and type, residual disease, and age (Table 3). The forward and backward selection process of the Cox regression led to the same model. Among these factors, only HER3 expression, FIGO stage, residual tumor, and age were of prognostic relevance (Table 3). Although FIGO stage and residual tumor are quantitatively dominant prognostic factors, HER3 expression turned out to be a moderate-size independent prognostic factor in our study population. Histologic grade and type were not significant.

The influence of HER3 expression on survival time was visualized by Kaplan-Meier plots. A clear difference was observed between patients with low (n=54) and high (n=62) HER3 expression ($P = .0034$; log-rank test). Median survival time was 3.31 (95% CI: 1.93-4.68) years for patients with low HER3 expression compared to only 1.80 (0.82-2.78) years for patients with high HER3 expression (Fig 2). The influence of HER3 on survival time was also obvious when FIGO stage I+II and FIGO stage III+IV patients were analyzed separately (Appendix Fig A4, online only). As expected the established clinical parameters FIGO stage, histological grade, residual disease and age were clearly associated with survival time (Appendix Fig A5, online only).

### Analysis in Relation to Clinical Parameters and HER2 Expression

HER3 expression was not associated with FIGO stage ($P = .519$), histologic grade ($P = .263$) and type (0.695), residual disease ($P = .095$), and age ($P = .194$; Appendix Table A1, online only). Similarly, no correlation of HER3 expression with parameters previously determined in the same tissue specimens [6,7,21-27] has been observed, namely p53, progesterone and estrogen receptors, ki67, upa, pai-1, cathepsin D, EGF receptor, c-*myc*, mdm-2, aldehyde dehydrogenase, glutathione-*S*-transferases A1 and P1, MGMT, LDH, p21, topoisomerase II$\alpha$, metallothionein, and SPOC1 (data not shown). Since HER3 is a dimerization partner of HER2, it would be interesting to analyze a possible interaction between both parameters. Therefore, we determined HER2 expression in the same tissue specimens using the well-established HercepTest. However, overexpression of HER2 (HercepTest score 2+ or 3+) was observed in only a small fraction of the analyzed tumors (4.9%). This corresponds to the data obtained in previous studies in ovarian cancer, where relatively small numbers patients also exhibited the 2+/3+ staining. [10] The small number of HER2 overexpressing carcinomas did not allow statistical analysis of possible interactions between HER2 and HER3 with respect to

**Table 2.** Association of HER3 Expression With Survival in 116 Patients With Primary Ovarian Cancer Using the Univariate Proportional Hazards Model (Cox analysis)

| Factor | Relative Risk | 95% CI | *P* |
|---|---|---|---|
| HER3 expression (low *v* high) | 1.894 | 1.226 to 2.925 | .004 |
| FIGO-stage (stages I, II *v* III, IV) | 7.912 | 5.797 to 10.799 | < .001 |
| Grading (grade I *v* II, III) | 7.213 | 4.568 to 11.388 | < .001 |
| Histological type (serous *v* nonserous type) | 0.822 | 0.660 to 1.023 | .080 |
| Residual tumor (no residual tumor *v* residual tumor) | 8.241 | 6.277 to 10.819 | < .001 |
| Age at surgery (< 55 *v* ≥ 55 years) | 1.553 | 1.263 to 1.910 | .037 |

**Table 3.** Explanatory Prognostic Factors in 116 Patients With Primary Ovarian Cancer Accepted in the Backward Selection Model (likelihood quotient) of the Cox Regression

| Factor | Relative Risk | 95% CI | *P* |
|---|---|---|---|
| HER3 expression (low *v* high) | 1.711 | 1.095 to 2.674 | .018 |
| International Federation of Gynecology and Obstetrics stage (stages I, II *v* III, IV) | 4.776 | 1.889 to 12.076 | .001 |
| Residual tumor (no residual tumor *v* residual tumor) | 2.688 | 1.399 to 5.167 | .003 |
| Age at surgery (< 55 *v* ≥ 55 years) | 2.062 | 1.166 to 3.648 | .013 |
| Not regarded as explanatory | | | |
| Histological type (serous *v* nonserous type) | | | .548 |
| Grading (grade I *v* II, III) | | | .160 |

survival. Nevertheless, our data show that expression of HER3 is more frequent in ovarian cancer than that of its dimerization partner HER2.

## DISCUSSION

The epidermal growth factor receptor family (HER1, HER2, HER3, and HER4) operates several signal transduction pathways, including phosphoinosite-3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK1/2). [16,20,28,29] Both signal transduction pathways mediate proliferation, adhesion, migration and antiapoptotic effect.[16] Therefore, overexpression of epidermal growth factor receptors is often associated with advanced tumor stages and poor clinical outcome.[6]

In the present study we concentrated on HER3 and studied a possible association with survival in 116 patients with primary epithelial ovarian cancer. We observed a strong expression of HER3 in 53.4% of the patients, which is a relatively high fraction. Contradictory results have been obtained in previous immunohistochemical studies about HER3 expression in ovarian cancer.[30,31] Positive immunostaining has been observed in 69% of ovarian carcinomas,[31] whereas only 3% were positive in another study.[31] This discrepancy demonstrates a need for standardization of HER3 staining techniques. DNA microarray analysis has been applied in another study to identify genes differentially expressed between normal ovary tissue and epithelial ovarian cancer.[32] Interestingly, *HER3* was among the genes upregulated in ovarian cancer.

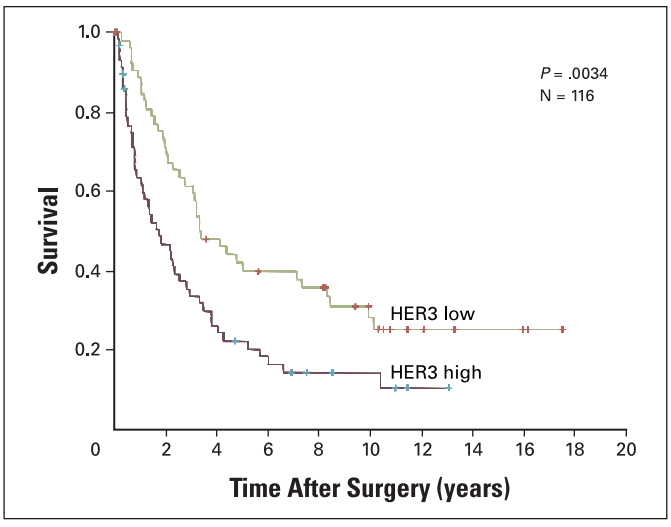


**Fig 2.** Association of HER3 expression with survival time in 116 patients with primary ovarian cancer. Median survival time was 3.41 years for patients with low HER3 expression (HER3 low, n = 54) compared with only 1.80 years for patients with high HER3 expression (HER3 high, n = 62). The difference was significant in the log-rank test (*P* = .0034).

In our study, HER3 expression was clearly associated with survival. In the multivariate proportional hazards model, HER3 (*P* = .018) was associated with survival independent of the established clinical prognostic factors (FIGO stage, histologic grade and type, residual tumor after surgery, and age). HER3 has also been reported to have a negative effect in breast cancer.[33,34] High expression of HER1, HER2, and HER3 is associated with reduced survival among breast cancer patients[33] and with early relapse in patients with estrogen receptor–positive tamoxifen-treated breast cancer.[34]

In previous studies, we analyzed several possible influential parameters on prognosis in the same carcinomas analyzed in the present study, including p53, progesterone and estrogen receptors, ki67, upa, pai-1, cathepsin D, EGF receptor, c-*myc*, aldehyde dehydrogenase, glutathione-*S*-transferases A1 and P1, MGMT, p21 topoisomerase II$\alpha$, glutathione, and metallothionein.[6,7,21-27] However, none of these factors was independent from residual disease or from FIGO stage. This underlines the role of HER3 in our study population.

As HER3 dimerizes with HER2, it would be interesting to study the influence of both factors on prognosis. Therefore, we determined HER2 expression in the same ovarian carcinomas using the well-established HercepTest. However, only 4.9% of the ovarian carcinomas showed a strong overexpression (HercepTest score 2+ or 3+). Similarly, only 11.4% of 2+ or 3+ expression levels (determined by the 4D5 monoclonal antibody directed against HER2) were observed in a previous study in 837 patients with ovarian cancer.[10] Unfortunately, the fraction of HER2 overexpressing carcinomas was too small for statistical analysis of possible interactions between HER2 and HER3. Nevertheless, our data show a higher fraction of patients overexpressing HER3 (53.4%) than HER2 (4.9%), highlighting the relevance of HER3 as a therapeutic target in ovarian cancer.

Recently, an antibody directed against the extracellular domain of HER3 ($\alpha$-HER3$^{ECD}$) has been established.[11] $\alpha$-HER3$^{ECD}$ causes internalization of its target receptor and blocks HER3-mediated signaling. As HER3 overexpression is strongly associated with worse prognosis in ovarian cancer, HER3 blocking therapies seem to be a promising new strategy for ovarian cancer treatment.

However, the predominant pattern of HER3 expression is cytoplasmic, with only weak membrane expression, in contrast to the classic membrane staining observed with HER2. Cytoplasmic staining of HER3 has also been observed in squamous tumors of the head and neck.[35] Therefore, it will be of clinical relevance to test whether antibody targeting of HER3 is a viable strategy, despite of the only weak HER3 membrane expression in ovarian cancer or whether other strategies, such as small molecule inhibitors, will be superior.

In conclusion, HER3 is overexpressed in a fraction of epithelial ovarian carcinomas and may represent a new influential parameter on prognosis, independent from the established clinical parameters.

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### *Acknowledgment*

This article is dedicated to the late Werner J. Kleeman.


### *Appendix*

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

## Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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## GLOSSARY

**ErbB:** Also called the human epithelial growth factor receptor (HER), ErbB belongs to the EGFR receptor family. ErbB1 (EGFR/HER-1), ErbB2 (HER-2), ErbB3 (HER-3), and ErbB4 (HER-4) are the four members that comprise this receptor family.

**FIGO staging:** A tumor staging system established and revised by the International Federation of Gynecology and Obstetrics (FIGO) that takes into account the postoperative histopathologic evaluation of the specimen. The FIGO stage classification has prognostic value.

**HercepTest:** HercepTest is an immunohistochemical assay for HER2 overexpression in carcinomas to determine if patients could potentially benefit from treatment with trastuzumab.

**Histological grade:** Histologic grade provides prognostic information in many tumors, including ovarian cancer. It is based on a combination of cellular features (nuclear, cytological, and architectural). The more nuclear atypia and mitotic figures, the higher the grade.

**Histological type:** The histologic type is determined by microscopic examination of the cell morphology and architecture of tissues. More than 90% of ovarian neoplasms arise from the epithelial surface of the ovary, the rest come from germ cells and stromal cells. The epithelial neoplasms are classified as serous (30% to 70%), mucinous (5% to 20%), endometrioid (10% to 20%), clear cell (3% to 10%), and undifferentiated (1%).

**Multivariate proportional hazards model:** Proportional hazards or COX regression modeling is a general method in medical statistics to analyze the influence of several (patient specific) covariates on time-to-event end points. No assumption is made concerning the form of the underlying time-to-event curve. The only assumption made is that the effect of the covariates on the hazard rate in the study population is multiplicative and does not change over time.

**Trastuzumab:** A humanized anti-ErbB2 monoclonal antibody approved for treating patients whose breast cancers overexpress ErbB2 protein or demonstrate ErbB2 gene amplification, it is currently being tested in combination with other therapies.