Expression of *erbB-1* and *erbB-2* genes in normal and pathological human endometrium

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Abstract. Overexpression of the erbB-1 (EGFR, epidermal growth factor receptor) and erbB-2 (HER2/neu) proteins contributes to the aggressive behavior of malignant tumors originating from the endometrium. We currently examined whether the trend of these proteins to overexpression is a direct effect of their gene transcriptional activities. Expression of the *erbB-1*/*erbB-2* genes was measured applying the quantitative RT-PCR technique in 25 uterine carcinomas, 12 normal endometria, a carcinosarcoma and a case of botryoid sarcoma of the uterine cervix. We showed that *erbB-1* mRNA was overexpressed in 48% (12/25) and *erbB-2* mRNA was overexpressed in 8% (2/25) of the analysed tumors. The level of expression appeared to be significantly higher in the malignant tumors as compared to the benign ones for *erbB-1* and for *erbB-2* ($p=0.0001$ and $p=0.008$, respectively). A significant correlation between *erbB-1* overexpression and tumor differentiation was found (Spearman rank correlation test, $p<0.001$). Concomitant *erbB-1* and *erbB-2* overexpression was detected only in 1 out of 25 (4%) uterine neoplasms. *erbB-1* was overexpressed in a sarcoma botryoides of the uterine cervix. Our data suggest that erbB-1/erbB-2 overexpression is a direct effect of higher than normal transcriptional activity of the encoding genes in a subset of human endometrial carcinomas.

Introduction

Endometrial cancer is one of the most common female genital tract malignancies in the Western world (1). In the United States, 40,230 new cases and 7,090 deaths were anticipated in 2004, making it the 4th most common cancer in women (2). The incidence of endometrial cancer has also increased in Poland during the last three decades, and 3,796 new cases were diagnosed in 2002 (3). Many risk factors are involved in the etiology of endometrial malignancy, including obesity, late menopause, anovulatory cycles, hypertension, diabetes mellitus and unopposed estrogenic stimulation (4). Family history may also be a risk factor as familial clustering of endometrial cancers has been reported (5). Sandles and co-investigators (5) suggested that two 'genetic models' may be implicated in the development of endometrial adenocarcinoma: the cancer family syndrome (Lynch II or hereditary nonpolyposis colorectal cancer syndrome) and a predisposition for endometrial adenocarcinoma alone.

Several studies have demonstrated the importance of epidermal growth factor (EGF)-related peptides and their receptors (erbB-2/HER-2, erbB-3/HER-3 and erbB-4/HER-4), on the background of sex steroid effects, in normal, hyperplastic and neoplastic human endometrium (6,7). EGF-related growth factors play a regulatory role, particularly influencing the mitogenic activity of human endometrial adenocarcinoma cell lines in serum-free medium (8,9). The phosphatidylinositol cascade appears to be an important signal transduction pathway in cell culture, mediating the growth effects of EGF on endometrial cancer cell line HEC-1-A (10). In the poorly-differentiated endometrial cancer cell line KLE, EGF stimulation of cell growth is exerted through both protein kinase C-dependent and -independent manners (11).

EGFR, HER-2 and EGF or TGFα can be detected in various epithelial tumors originating from the female genital tract (12-16). Expression of mRNA for EGF, HB-EGF and TGFα has been detected in normal human endometrium (15). In endometrial cancer, the same authors detected EGF, HB-EGF and TGFα mRNA expression in 76.5, 66.7 and 68.4% of cases, respectively (15).

Previously, erbB-2 protein overexpression was associated with impaired overall survival of patients affected by endometrial cancer (17,18). To note, uterine papillary serous carcinomas (UPSC), notorious for their aggressive behavior and unfavorable prognosis, revealed erbB-2 overexpression in 80% of cases (19).

However, one can speculate that erbB-1 and erbB-2 protein overexpression results from the differential expression pattern of related genes during endometrial tumorigenesis.

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Abbreviations: EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding epidermal growth factor; TGFα, transforming growth factor α; SD, standard deviation

Key words: erbB-1, *erbB-2*, RT-PCR, endometrial cancer

Therefore, the current study was aimed at investigating *erbB-1* and *erbB-2* gene expression in normal and pathological human endometrium. Moreover, the correlation between gene expression and various clinicopathological variables of cancer was also studied. Uterine carcinosarcoma and a botryoid sarcoma of the uterine cervix were also included.

Materials and methods

Patients and material. The studied material comprised endometrial carcinomas from 25 patients who received surgical management at the Second Department of Gynecology, Lublin University School of Medicine, Lublin, Poland between 2003 and 2005. The mean patient age was 61 years (range, 50-80). None of the subjects had received hormonal therapy, radiation therapy or chemotherapy before surgery. As a control, 12 normal endometrial tissues (2 from the proliferative and 2 from the secretory phase of the menstrual cycle, as well as 8 atrophic endometria) were collected from women who underwent surgery unrelated to endometrial pathology (uterine leiomyomas, early-stage cervical cancer or ovarian cysts). Uterine carcinosarcoma and a case of botryoid sarcoma of the uterine cervix (rhabdomyosarcoma embryonale) were also included in the study.

The clinicopathological features of the endometrial cancer patients are summarized in Table I. Briefly, gross examination revealed that 20 of the 25 (80%) tumors were of stage I according to FIGO classification (20), and most (19 out of 25, 76%) were of the endometrioid type at presentation (21).

The Independent Ethics Committee of the Lublin University School of Medicine, Lublin, Poland, approved the experiments, and informed written consent was obtained from all subjects.

At surgery, after the uterus had been removed and cut, a portion of the tissue was immediately fixed in buffered formalin (pH 7.4) for routine pathological assessment. The remaining portion was immediately scraped into a sterile eppendorf tube, frozen in liquid nitrogen and stored in a deep-freezer (-80˚C) until assayed. During this procedure, the tissue obtained from the uterine cavity was not cross-contaminated by the material from the cervical canal.

RNA extraction and RT-PCR. Total RNA was isolated according to the method of Chomczynski and Sacchi (22) and quantified spectrophotometrically at 260 nm. High quality RNA was defined by a ratio 260 nm/280 nm of 1.8-2.0. RT-PCR was performed applying the RNA PCR kit, version 2.1 (Takara Shuzo Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions.

erbB-1, *erbB-2* and *glyceraldehyde phosphate dehydrogenase* (*GAPDH*) as a control, were independently amplified according to Thøgersen and co-workers (23). The specific primer sequences are shown in Table II. PCR products were separated on 1.5% TBE-agarose gel at 100 V for 30 min and visualised with ethidium bromide staining. For qualitative and quantitative analysis of ethidium bromide-stained gels, a video densitometer (Biotec-Fischer, Germany) and software Gel-Pro® analyzer 3.0 (Media Cybernetics, USA) were applied.

Table I. Clinicopathological variables of endometrial cancer patients.

Variable	Number	Percent (%)
Age of patients (years)		
<60	11	44
≥60	14	56
Surgical stage		
I	20	80
II	1	4
III	2	8
IV	2	8
Histologic type		
Endometrioid adenocarcinoma	19	76
Adenosquamous carcinoma	5	20
Clear-cell carcinoma	1	4
Histologic grade		
G1	7	28
G2	11	44
G3	7	28
Myometrial invasion		
<1/2	12	48
>1/2	13	52
Coexistence of hyperplastic and neoplastic endometrium		
yes	5	20
no	20	80
Lymph node metastasis		
yes	4	16
no	14	56
n. e.	7	28

n.e., not evaluated.

The ratio between the PCR-amplified gene and its amplified control was obtained for each analysed sample. In the PCR reactions, all ratios were normalised to the appropriate control ratio. All PCR and RT-PCR reactions were repeated 3 times for each sample, and the ratios were separately quantified.

To exclude contamination of genomic DNA as a source for amplified products, each reaction was also carried out without reverse transcriptase.

The integrated optical density (IOD) of the bands, in a digitalised picture, was separately measured as described previously (24). The relative expression of the *erbB-1* or *erbB-2* genes was determined as the IOD ratio of the analysed gene over the IOD of *GAPDH*. Overexpression of the gene was revealed when *erbB-1*/*GAPDH* and *erbB-2*/*GAPDH* IOD ratios were higher than 1.2 and 1.33, respectively (above the mean IOD value for the control group + 3 SD values).

Statistical analysis. None of the parameters recorded in the tumor material passed tests for being normally distributed

Table II. Specific primers for quantitative RT-PCR.

Gene		Primer	Base pairs (bp)
erbB-1	Upstream	5'-GACCCTCCGGGACGG-3'	350
	Downstream	5'-GGCATAGGAATTTTCGTAGTACATAT-3'	
erbB-2	Upstream	5'-AACTGCACCCACTCCTGTGT-3'	340
	Downstream	5'-CAGGGATCCAGATGCCCTTG-3'	
GAPDH	Upstream	5'-GCCACATCGCTCAGACACCA-3'	483
	Downstream	5'-GATGACCCTTTTGGCTCCCC-3'	

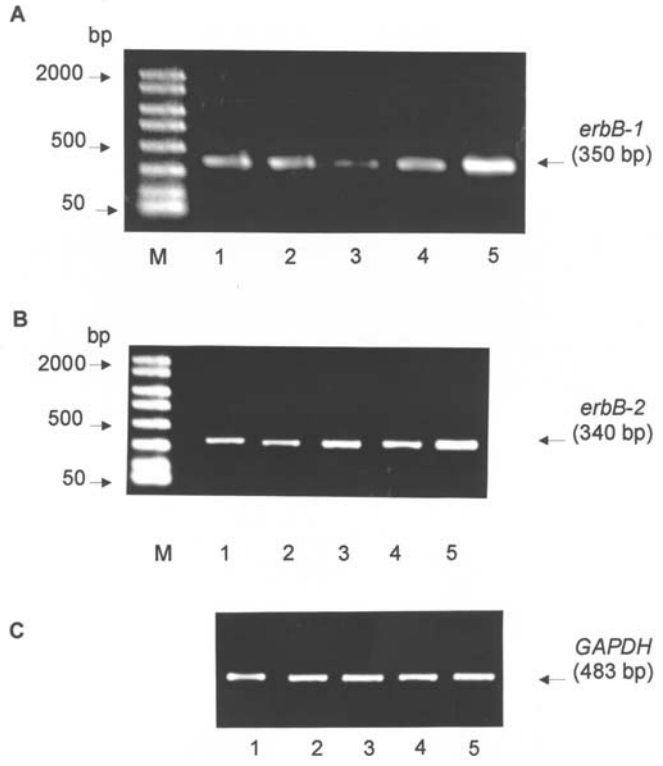

Figure 1. Analysis of *erbB-1* (A) and *erbB-2* (B) mRNA expression in normal (lane 1) and endometrial cancer tissues (lanes 2-6) with *GAPDH* mRNA (C) as a reference gene. M, molecular weight marker (PCR marker, 50-2,000 bp; Sigma-Aldrich, USA).

(Smirnow-Kolmogorov test) and the non-parametrical statistical tests (Mann-Whitney U test, the Fisher exact test or the Spearman rank correlation test) were applied. The data were statistically analysed using STATISTICA for Windows, version 5.1 (Statsoft, Tulsa, OK, USA). $p \leq 0.05$ was considered statistically significant.

Results

Expression of *erbB-1* and *erbB-2* was studied by quantitative RT-PCR analysis with *GAPDH* applied as a reference gene (Fig. 1). There were 12 normal endometrial tissues, and the mean ± SD of the *erbB-1*/*GAPDH* and *erbB-2*/*GAPDH* IOD ratios were 0.96±0.08 (range, 0.85-1.08) and 1.12±0.07 (range, 0.97-1.26), respectively. For tumor samples, analogous IOD ratios (mean ± SD) were calculated as 1.20±0.06 (range, 1.11-1.36) and 1.21±0.09 (range, 0.99-1.38) for *erbB-1* and *erbB-2*, respectively. In both cases, the level of expression appeared to be higher in the malignant cases as compared to the benign ones ($p=0.0001$ and $p=0.008$, respectively).

erbB-1 overexpression was reported in 48% (12/25) of the endometrial carcinomas. A significant correlation between *erbB-1* overexpression and tumor differentiation was found (Spearman rank correlation test, $p<0.001$). There was no correlation between *erbB-1* overexpression and patient age, clinical stage, histological type, depth of myometrial invasion, lymph node metastasis or coexistence of hyperplastic and neoplastic human endometrium.

erbB-2 overexpression was observed in 2 out of 25 (8%) of the uterine malignant tumors. All overexpressed cases were well-differentiated endometrioid-type endometrial carcinomas, stage Ic with deep myometrial infiltration. Concomitant *erbB-1* and *erbB-2* overexpression was detected only in 1 out of 25 (4%) neoplasms.

erbB-1/*GAPDH* and *erbB-2*/*GAPDH* IOD ratios for carcinosarcoma were 1.02 and 1.11, respectively, whereas *erbB-1* was overexpressed in a sarcoma botryoides of the uterine cervix (*erbB-1*/*GAPDH* IOD ratio, 1.24). The *erbB-2*/*GAPDH* IOD ratio for rhabdomyosarcoma embryonale was 1.2.

Discussion

Proto-oncogenes are a group of normal genes that play important roles in the regulation of cell proliferation. Abnormalities in the expression, structure, or activities of proto-oncogene products contribute to the development and maintenance of the cell malignant phenotype. The human *erbB-2* gene product, like the epidermal growth factor receptor, is a transmembrane receptor protein that plays an important role in coordinating the complex erbB signaling network that is responsible for regulating cell growth and differentiation (25,26).

Several studies have focused on the expression of erbB-1 and/or erbB-2 and their biological or clinical involvement in endometrial tumorigenesis (27-31).

In 65 endometrial carcinomas, 71% showed erbB-1 immunoreactivity but also 77% of atrophic endometria and 54% of endometrial adenomatous hyperplasia were positive (28). ErbB-1 immunoreactivity seems to be related to the endometrial cancer histotype, regardless of the tumor grade or extent of myometrial infiltration (29). Additionally, strong

(3+) or diffuse (2+ or 1+) immunostaining for erbB-1 was found in 21/128 (32%) and 83/128 (65%) of endometrial carcinomas, respectively. However, protein expression did not correlate with any known prognostic variables, whereas expression of erbB-2 was reported to correlate with tumor type (p=0.046), especially with clear-cell carcinomas of the endometrium (30). On the contrary, however, erbB-1 expression was correlated with histological grade (p=0.004) and patient age (p=0.04) in human endometrioid-type endometrial carcinomas (31).

Our results indicated that the *erbB-1* and *erbB-2* mRNA expression level was significantly higher in malignant human endometria compared to benign ones. Moreover, a significant correlation between *erbB-1* overexpression and tumor differentiation was also observed (p<0.001). Previously, Pfeiffer and co-workers (15) reported higher mRNA levels of all EGF receptors in human endometrial carcinomas than in normal endometrial controls. However, this increase was only significant for TGFα and amphiregulin (p<0.005). This allows us to speculate that erbB-1/erbB-2 overexpression is a direct effect of higher than normal transcriptional activity of the encoding genes in a subset of human endometrial carcinomas.

It is worth pointing out that erbB-1 and erbB-2 expression may be a useful prognostic factor in endometrial carcinomas (17,18,31-34). erbB-1-positive endometrial carcinoma patients had a statistically shorter length of survival compared with those with erbB-1-negative tumors (p=0.018), but protein status failed to retain prognostic value applying multivariate analysis (31). Moreover, erbB-1 expression was significantly correlated with decreased patient survival not only for endometrioid-type endometrial adenocarcinomas, but also for the serous papillary and clear cell categories (32). Previously, Hetzel *et al* (17) reported that strong erbB-2 immunostaining in endometrial cancer was an independent prognostic variable applying univariate and multivariate analysis (213 out of 247 tumors were at I-II stages of the disease). Kohlberger and co-investigators (18) showed that erbB-2 oncoprotein expression was significantly correlated with impaired overall patient survival (p=0.04). erbB-2 expression, instead of vascular invasion-associated changes and tumor aneuploidy, was found to independently correlate with survival in FIGO stage I endometrioid endometrial carcinomas (33). In a previous study, p53 overexpression predicted the recurrence of endometrial cancer better that HER-2/neu immunostaining, although simultaneous protein overexpression indicated a worse patient outcome (34). However, there are additional reports which fail to confirm these observations (35,36).

The current results seem to be of great importance when referring to the data concerning erbB-1 and erbB-2 protein levels in human malignancies. The relative mitogenic and transforming abilities of erbB proteins and their ligands suggest that cells co-expressing erbB-1/erbB-2 are more effectively transformed than cells expressing erbB-1 alone (37). The observation that erbB-2 can transpotentiate the proliferative effect of epidermal growth factor more than erbB-3 is interpreted in terms of heterodimer formation, particularly because erbB-1/erbB-2 interactions are more prevalent than erbB-1/erbB-3 (38). Currently, concomitant *erbB-1*/*erbB-2* mRNA overexpression was found only in 4% of endometrial carcinomas. Further investigations on a large group of cases should clarify the influence of *erbB-1*/*erbB-2* overexpression on the clinical behavior of human endometrial carcinomas.

Finally, *erbB-1* was overexpressed (*erbB-1*/*GAPDH* IOD ratio, 1.24) in a case of a highly malignant tumor of the uterine cervix, botryoid sarcoma. Due to its rarity, instead of *TP53* deregulation as reported previously (39), *erbB-1* alterations may also have participated in the development and progression of this uncommon human neoplasm originating from the uterine cervix.

Attempts to elucidate the mechanisms of signal transduction via receptors of the erbB family, and especially the sequence of events which occurs during neoplastic transformation, seem to be enormously important from the therapeutic point of view, and lead to the more selective and effective use of inhibitors or antagonists of some transmitters (40). For example, the efficacy of herceptin therapy and the assessment of HER-2/neu have been proposed for chemotherapy-resistant, advanced-stage UPSC (41). The clinical utility of HER-2/neu or EGFR-targeted therapies in biphasic, highly aggressive neoplasms originating from the endometrium (carcinosarcoma) was also suggested (42).

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