Tumor Heterogeneity at Protein Level as an Independent Prognostic Factor in Endometrial Cancer

Anna Supernat*, Sylwia Łapińska-Szumczyk†, Hanna Majewska‡, Jacek Gulczyński§, Wojciech Biernat‡, Dariusz Wydra† and Anna J. Żaczek*

*Department of Medical Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, 80-211 Gdańsk, Poland; †Department of Gynaecology, Gynaecological Oncology and Gynaecological Endocrinology, Medical University of Gdańsk, 80-402 Gdańsk, Poland; ‡Department of Pathomorphology, Medical University of Gdańsk, 80-214 Gdańsk, Poland; §Department of Pathology and Neuropathology, Medical University of Gdańsk, 80-211 Gdańsk, Poland

Abstract

Intratumor heterogeneity implies heterogeneous protein function, facilitating tumor adaptation which results in therapeutic failure. We hypothesized that tumor heterogeneity at protein level may influence the course of the disease. As a single biopsy might not represent the full biologic complexity of the tumor, we have analyzed immunohistochemically four different cores obtained from each primary tumor within the cohort of 364 patients with endometrial cancer (EC). The following proteins were examined: estrogen receptor 1 (ESR1), progesterone receptor, epidermal growth factor receptor, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, receptor tyrosine-protein kinase erbB-3, v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4, phosphatidylinositol-4,5-bisphosphate 3-kinase, phosphorylated v-akt murine thymoma viral oncogene homolog 1, v-myc avian myelocytomatosis viral oncogene homolog, DNA topoisomerase II alpha 170 kDa (TOP2A), cyclin-dependent kinase inhibitor 2A (CDKN2A), tumor protein p53, RAD21 homolog, S. pombe, and runt-related transcription factor 1. Particularly strong correlation was found between TOP2A and CDKN2A heterogeneity and higher stage of the disease (P = .0002 and P = .0003, respectively). Most correlations with clinicopathologic data were observed for ESR1 heterogeneity that correlated with non-endometrioid carcinomas (P=.02), higher stage (P=.005), grade (P=.01), and the presence of metastases (P = .01). Thirty-nine (11.0%) patients were classified as “globally heterogeneous”. Cumulative tumor heterogeneity strongly correlated with the presence of metastases, higher stage, and higher grade of the disease (all P b .05). It also carried negative prognostic value (P=.0008). We show that the degree of heterogeneity in EC might serve as a clinically valid molecular marker.
Introduction
Endometrial cancer (EC) is the most frequent malignancy of the female genital tract in the Western world, with approximately 90,000 new cases registered each year in the European Union [1]. Despite the high prevalence, the understanding of the molecular background of EC with regard to its pathogenesis and disease progression remains insufficient. Data concerning tumor heterogeneity in EC are especially scarce. Recent discoveries have shown that tumor composition is heterogeneous and consists of various cell clones. This intratumor heterogeneity depends on heterogeneous protein function, which can facilitate tumor adaptation, resulting in therapeutic failure through Darwinian selection [2]. Furthermore, intratumor heterogeneity was detected in all types of studied cancers [3,4] and may lead to more aggressive tumor behavior and unfavorable outcome [5,6].

As a single biopsy might not represent the full biologic complexity of the tumor, we used immunohistochemistry (IHC) to analyze four different cores obtained from each primary tumor within the cohort of patients with EC. Tumor heterogeneity might affect the response to treatment. Thus, the study included the expression analysis of the proteins often related to target therapies. The following proteins were examined: estrogen receptor 1 (ESR1), progesterone receptor (PGR), epidermal growth factor receptor (ERBB1), v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (ERBB2, also known as HER2), receptor tyrosine-protein kinase erbB-3 (ERBB3), v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (ERBB4), phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA), phosphorylated v-akt murine thymoma viral oncogene homolog 1 (pAKT1), v-myc avian myelocytomatosis viral oncogene homolog (MYC), DNA topoisomerase II alpha, 170 kDa (TOP2A), cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16), tumor protein p53 (TP53), RAD21 homolog, S. pombe, and runt-related transcription factor 1 (RUNX1). We hypothesized that the existence of cellular heterogeneity within the tumor, identified in four different cores (analyzed by IHC) belonging to the same patient, may influence the course of the disease and affect patients’ survival.

Patients and Methods

Patients and Tissues
The study included 364 formalin-fixed paraffin-embedded (FFPE) primary tumor samples retrospectively collected from a cohort of EC patients who were operated in the Department of Gynaecology, Gynaecological Oncology and Gynaecological Endocrinology, Medical University of Gdańsk (Gdańsk, Poland) between 2000 and 2010. Each patient was primarily treated by surgery, with the possible option of radiotherapy and/or chemotherapy administration. The inclusion criteria were operable EC (stage IVB patients underwent cytoreductive surgery) confirmed by histologic examination and a signed consent form. The study was accepted by the Independent Ethics Committee of the Medical University of Gdańsk (NKEBN/269/2009, date: 14 September 2009). Procedures involving human subjects were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

The tumor samples included all stages of endometrial carcinoma, from stage IA to IVB, as distinguished by the International Federation of Gynecology and Obstetrics (FIGO) in 2009 [7]. We analyzed all primary carcinomas of the uterine corpus, separating them into endometrioid and non-endometrioid tumors. The latter included serous, clear cell, mucinous, mixed, squamous cell, and undifferentiated carcinomas [8]. Metastases included lymph node and distant metastases. The patients’ characteristics are summarized in Table 1. The median age was 63 (range, 26-89 years). Patients with a body mass index higher than 30 were classified as obese [9]. A survival analysis was performed for 362 (99.5%) patients. After a median follow-up of 72.5 months (range, 0-158), 107 (29.4%) patients had died. The last follow-up data were collected in September 2013. The study was performed in accordance with the REcommendations for Tumor MARKer Prognostic Studies (REMARK) criteria [10].

IH on Tissue Microarrays
Samples were collected by surgical excision before any systemic treatment and were fixed in 10% (vol/vol) neutral buffered formalin for up to 24 hours, dehydrated in 70% ethanol, and embedded in paraffin. FFPE tissue blocks were stored at room temperature for up to 14 years. The percentage of tumor cells in each FFPE specimen was evaluated by hematoxylin and eosin staining reviewed by a certified pathologist. Tissue microarrays (TMAs) were constructed from FFPE surgical resection tumor specimens and control samples. Four 1.5-mm-diameter cores from each tumor were obtained from the most representative areas (well-preserved fragments of invasive carcinoma, without necrosis, autolysis, and squamous metaplasia) using a tissue-arraying instrument (MTA-I; Beecher Instruments, Sun Prairie, WI), and then reembedded in microarray blocks.

Table 1. EC Patients’ Characteristics (N = 364)

| Variable                                      | Number of Cases (%) |
|-----------------------------------------------|---------------------|
| Menopausal status                             |                     |
| Premenopausal                                 | 27 (7.4%)           |
| Perimenopausal                                | 22 (6.0%)           |
| Postmenopausal                                | 314 (86.3%)         |
| Missing data                                  | 1 (0.3%)            |
| Age                                           |                     |
| ≤50 years                                     | 39 (10.7%)          |
| >50 years                                     | 325 (89.3%)         |
| Obesity                                       |                     |
| Absent                                        | 173 (47.5%)         |
| Present                                       | 190 (52.2%)         |
| Missing data                                  | 1 (0.3%)            |
| Histology                                     |                     |
| Endometrioid                                  | 332 (91.2%)         |
| Non-endometrioid                              | 27 (7.4%)           |
| Missing data                                  | 5 (1.4%)            |
| Stage (FIGO)                                  |                     |
| IA-IB                                         | 246 (67.6%)         |
| II                                            | 53 (14.6%)          |
| IIB-IBC                                       | 44 (12.1%)          |
| IVA-IVB                                       | 16 (4.4%)           |
| Missing data                                  | 5 (1.4%)            |
| Grade                                         |                     |
| I                                             | 173 (47.5%)         |
| II                                            | 134 (36.8%)         |
| III                                           | 47 (12.9%)          |
| Missing data                                  | 10 (2.7%)           |
| Cervical invasion                             |                     |
| Absent                                        | 268 (73.6%)         |
| Present                                       | 91 (25.0%)          |
| Missing data                                  | 5 (1.4%)            |
| Mysometrial infiltration                      |                     |
| ≤1/2                                          | 168 (46.2%)         |
| >1/2                                          | 191 (52.3%)         |
| Missing data                                  | 5 (1.4%)            |
| Metastases                                    |                     |
| Absent                                        | 321 (88.2%)         |
| Present                                       | 34 (9.3%)           |
| Missing data                                  | 9 (2.5%)            |
Punches of normal tissues were added to each array to introduce built-in internal controls to the system. Consecutive 4-μm-thick TMA sections were cut and placed on charged polylysine-coated slides (Superfrost Plus; BDH, Braunschweig, Germany) for subsequent IHC analysis.

Protein expression was examined by IHC on TMA blocks using the following antibodies: ESR1 – clone SP1 (Roche, Basel, Switzerland), PGR – clone 1E2 (Roche), ERBB1 – clone EGFR113 (Novocastra, Wetzlar, Germany), ERBB2 – clone 4B5 (Roche), ERBB3 – clone DAK-H3-IC (DAKO, Glostrup, Denmark), ERBB4 – clone HFR1 (Abcam, Cambridge, United Kingdom), PIK3CA – clone C73F8 (Cell Signaling Technology, Danvers, MA), pAKT1 – clone D9E (Cell Signaling Technology), MYC – clone 9E11 (Novocastra), TOP2A – clone Ki-S1 (DAKO), CDKN2A – clone JC8 (Santa Cruz Biotechnology, Dallas, TX), TP53 – clone BP-53-11 (Roche), RAD21 – polyclonal antibody (Abcam), and RUNX1 – clone DW71 (Santa Cruz Biotechnology). The staining has been performed in accordance with the manufacturers' guidelines; details are presented as Supplementary Materials (Table W1).

Protein expression evaluation was performed by two pathologists (H.M. and J.G.) blinded to clinical data. ESR1 and PGR evaluation of the nuclear staining was performed on the basis of Allred score [11]. ERBB2 receptor status was determined on the basis of the criteria of HercepTest (DAKO) according to the manufacturer's guidelines, as previously described [12,13]. The interpretation criteria for the remaining proteins were based on the intensity of the staining and the percentage of cells showing positive reaction (0-100%), which gave the final staining score, as the result of either sum or multiplication, dependent on reported criteria for a particular protein [14–20]. Data published on The Human Protein Atlas were also taken into account (http://www.proteinatlas.org/, last accessed: 16 June 2014). Cutoff point determination of expression positivity, based on result distribution, was performed with the use of Cutoff Finder Web Application [21]. Cutoff point determination of the tumor heterogeneity, understood as different staining intensities between the cores belonging to the same patient, was performed individually for each protein as the proteins differed in staining characteristics. Details are presented as Supplementary Materials (Table W2). For tumor heterogeneity evaluation, staining determination of at least three cores was required. As an example, ESR1 and TOP2A tumor heterogeneity is presented in the four cores taken from the same primary tumor sample (Figures W1 and W2). Additionally, cumulative heterogeneity was determined for each patient, based on nine proteins that correlated with clinicopathologic characteristics and/or survival (ESR1, PGR, PIK3CA, pAKT1, MYC, TOP2A, CDKN2A, RAD21, and RUNX1). For each patient, a score between 0 and 9 was obtained (1 point for each protein classified as heterogeneous, according to the criteria described in Table W2). On the basis of the result distribution, primary tumors with a score of at least 3 were classified as “globally” heterogeneous.

### Statistical Analysis

STATISTICA software (version 10; StatSoft Co, Tulsa, OK) was used for all calculations. The tests that were used and their applications were given as follows: testing normality of the data set — Shapiro-Wilk test; comparison of the tumor heterogeneity with clinicopathologic data of the patients – crosstabs statistics with Pearson Chi-square test; correlations between the heterogeneity of the studied proteins – crosstabs statistics with Pearson Chi-square test. The Kaplan-Meier estimator was employed for survival analysis, and the generated curves were compared with the log-rank test. The endpoint for the study was overall survival (OS). OS was defined as the time from sample collection to death or censoring. Censoring was defined as loss of follow-up or alive at the end of follow-up. Statistical significance was assumed when \( P \leq .05 \). Cox proportional hazards regression analysis was used to identify the independent predictors of OS. Univariate predictors that are significant with a value of \( P \leq .10 \) were entered into a step-wise multivariate model to identify those with independent prognostic information.

### Results

#### Flow of Samples

For tumor heterogeneity evaluation, staining determination of at least three cores was required. Within the group of 364 patients, tumor heterogeneity was assessed for 310 to 355 (85.2-97.5%) cases, depending on the staining success of a given protein (Table W2). Global heterogeneity was assessed for 355 patients, as cases with less than five assessed proteins were not considered in the context of global heterogeneity due to the lack of significant proportion of data. Graphical representation of tumor heterogeneity within this group is presented in Figure 1.

#### Correlation of Tumor Heterogeneity with Clinical and Pathologic Data

Tumor heterogeneity of the studied proteins was compared with tumor histology, grade, and stage as well as the presence of metastases (Table 2). Parameters such as menopausal status, age, obesity, or myometrial infiltration were not included in the table as these analyses yielded statistically insignificant results. Particularly strong correlation was found between TOP2A and CDKN2A heterogeneity and higher stage of the disease (\( P = .0002 \) and \( P = .0003 \), respectively). Most correlations with clinicopathologic data were observed for ESR1 heterogeneity that correlated with non-endometrioid tumors (\( P = .02 \)), higher stage (\( P = .005 \)), grade (\( P = .01 \)), and the presence of metastases (\( P = .00001 \)). No correlations were found
between the studied parameters (histology, stage, grade, metastases) and the tumor heterogeneity of ERBB1, ERBB2, ERBB3, ERBB4, pAKT1, and TP53, thus these proteins were included in Table W3 only.

**Correlations between the Heterogeneity of the Studied Proteins**

Tumor heterogeneity of the studied proteins was compared with each other. Strong correlation was found between ESR1 and PGR heterogeneity ($r = 0.30, P = .000002$), ESR1 and RAD21 heterogeneity ($r = 0.23, P = .0003$), and pAKT1 and ERBB1 heterogeneity ($r = 0.24, P = .0002$).

**Survival Analysis**

Protein heterogeneity of MYC, TOP2A, ESR1, and RAD21 correlated with shortened OS. The same trend was observed for ERBB4, RUNX1, and CDKN2A. No prognostic impact was detected for ERBB1, ERBB2, ERBB3, PIK3CA, pAKT1, PGR, and ERBB3 expression as well as high ERBB2, TOP2A, and TP53 expression correlated with shorter OS (Table W4).

**Cumulative Tumor Heterogeneity**

As hierarchical clustering of the results brought no satisfying results (data not shown), we scored the heterogeneity of the proteins, which have yielded statistically significant correlations with either clinicopathologic data and/or survival. The proteins included in the cumulative tumor heterogeneity assessment were given as follows: ESR1, PGR, PIK3CA, pAKT1, MYC, TOP2A, CDKN2A, RAD21, and RUNX1. Thirty-nine (11.0%) patients were classified as “globally heterogeneous”, with a score of at least 3. One hundred forty-three (40.3%) patients were entirely homogenous, with a score of 0. Cumulative tumor heterogeneity was compared with clinicopathologic data and OS (Table 4 and Figure 2). It correlated with higher stage, higher grade, non-endometrioid histology, and the presence of metastases as well as shorter OS (all $P < .05$). Due to multiple correlations among the studied parameters, only global tumor heterogeneity, not the heterogeneity of separate proteins, was included into the multivariate analysis. Cumulative heterogeneity remained an independent prognostic factor, along with the stage and tumor histology (Table 5).

**Discussion**

Intratumor heterogeneity is presumed to be the main reason based on a single biopsy personalized treatment failure [2]. It can also disturb

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**Table 2. Patients Classified as Protein Heterogeneous in the Context of Clinicopathologic Data**

| Protein | Number of Heterogeneous Samples | $P$ Value |
|---------|---------------------------------|-----------|
| PIK3CA  |                                |           |
| MYC     |                                |           |
| TOP2A   |                                |           |
| ESR1    |                                |           |
| PGR     |                                |           |
| RUNX1   |                                |           |
| RAD21   |                                |           |
| CDKN2A  |                                |           |
| TP53    |                                |           |

Note: HR, hazard ratio; CI, confidence interval.

**Table 3. Univariate Analysis of Protein Tumor Heterogeneity as Prognostic Factors in EC**

| Status of the Analyzed Protein (Heterogeneous vs Non-heterogeneous) | Univariate Analysis | $P$ Value |
|---------------------------------------------------------------------|---------------------|-----------|
| ERBB1                                                               | 0.51                | 0.16-1.61 | .25       |
| ERBB2                                                               | 1.36                | 0.80-2.32 | .25       |
| ERBB3                                                               | 0.76                | 0.51-1.13 | .17       |
| ERBB4                                                               | 1.42                | 0.96-2.10 | .08       |
| PIK3CA                                                              | 1.11                | 0.70-1.75 | .65       |
| MYC                                                                 | 1.7                 | 1.08-2.70 | .02       |
| pAKT1                                                               | 1.31                | 0.78-2.20 | .31       |
| TOP2A                                                               | 2.11                | 1.24-3.60 | .006      |
| ESR1                                                                | 1.89                | 1.06-3.34 | .03       |
| PGR                                                                 | 1.42                | 0.88-2.30 | .15       |
| RUNX1                                                               | 0.99                | 0.99-4.00 | .05       |
| RAD21                                                               | 2.23                | 1.17-4.23 | .01       |
| CDKN2A                                                              | 1.75                | 0.97-3.16 | .06       |
| TP53                                                                | 1.11                | 0.66-1.86 | .70       |

Note: HR, hazard ratio; CI, confidence interval.

**Table 4. Patients Classified as Globally Heterogeneous in the Context of Clinicopathologic Data**

| Status of the Analyzed Protein (Heterogeneous vs Non-heterogeneous) | Number of Positive Samples | $P$ Value |
|---------------------------------------------------------------------|-----------------------------|-----------|
| Histology (Endometrioid vs Non-endometrioid)                        | 31/323                      | 0.04      |
| Stage (I, II vs III, IV)                                            | 24/291                      | 0.001     |
| Grade (1, 2 vs 3)                                                  | 24/300                      | .00003    |
| Metastases (Absent vs Present)                                     | 25/313                      | 0.0001    |

Note: HR, hazard ratio; CI, confidence interval.
pathologic evaluation of the tumor and thus diagnosis. However, emerging evidence suggests that the heterogeneity degree itself might serve as a clinically valid molecular marker [22,23]. Although genetic landscape of endometrial carcinoma has been extensively studied [24], the protein heterogeneity in EC has received far less attention. Analysis of the four cores collected from different regions of the primary tumor provided evidence of protein heterogeneity in the majority of the studied patients with EC. Thus, a single biopsy indeed reveals only a small fraction of protein expression changes present in an entire tumor. Heterogeneity of tumors has been extensively studied in breast cancer. Breast carcinomas were shown to possess allelic imbalance, karyotypic diversity, and cell subpopulations of diverse therapy sensitivity [25,26]. Furthermore, variable expression of ERBB2, cyclin D1 (CCND1), MYC, and TOP2A has been reported within breast tumors [27,28]. Triple negative breast carcinoma, known from its aggressiveness and unfavorable prognosis, was characterized by explicit intratumor genetic heterogeneity [29]. In non–small cell lung cancer, ERBB1 amplification heterogeneity lowered targeted therapy efficiency [30]. ERBB2 heterogeneity reported in endometrial and gastric cancers was found to be the reason for discordant results with fluorescence in situ hybridization [31,32]. Yoon et al. have demonstrated that heterogeneous amplification of ERBB2 in esophageal adenocarcinoma confers poor prognosis [33]. Studies of esophageal precancers revealed that the degree of clonal diversity was found to increase the probability of progression from esophageal precancer to adenocarcinoma [22]. Minor subpopulations of primary tumors were shown to be responsible for relapse after drug administration [34]. Intratumor heterogeneity of PTEN protein expression corresponded with loss of heterozygosity and shorter OS in glioblastoma [35]. Tumor heterogeneity of Ki-67 protein in prostate cancer correlated with more aggressive tumor characteristics [5]. In this study, we have demonstrated that heterogeneity of individual proteins, namely PIK3CA, MYC, TOP2A, ESR1, PGR, RUNX1, RAD21, and CDKN2A, correlates with more aggressive tumor behavior and, in case of MYC, TOP2A, ESR1, and RAD21, also confers poor prognosis. Interestingly, prognostic significance of the studied proteins depends on whether the heterogeneity or the expression level is being analyzed. Apart from ESR1, PGR, and TOP2A, which were significantly correlated with prognosis in terms of both the heterogeneity and the expression level, there were also proteins that were either informative in the context of tumor heterogeneity (PIK3CA, MYC, CDKN2A, RAD21, and RUNX1) or protein expression level (ERBB2, ERBB3, and TP53). Thus, protein

Figure 2. Kaplan-Meier curves representing the OS of EC patients stratified against the status of global tumor heterogeneity ($P = .0008$).

Table 5. Univariate and Multivariate Analyses of Clinicopathologic Data and Global Tumor Heterogeneity as Prognostic factors in EC Patients

| Analyzed Parameter | Univariate Analysis | Multivariate Analysis |
|--------------------|---------------------|-----------------------|
|                    | HR  | 95% CI  | $P$ Value | HR  | 95% CI  | $P$ Value |
| Histology (non-endometrioid vs endometrioid) | 4.36 | 2.61-7.29 | .0000001 | 3.68 | 2.16-6.27 | .000002 |
| Stage (III, IV vs I, II) | 1.97 | 1.62-2.39 | .0000001 | 1.58 | 1.20-2.10 | .001 |
| Grade (3 vs 1, 2) | 1.38 | 1.09-1.74 | .008 | Not significant |
| Metastases (present vs absent) | 3.60 | 2.23-5.81 | .0000001 | Not significant |
| Tumor heterogeneity (present vs absent) | 2.33 | 1.44-3.76 | .0005 | 1.71 | 1.05-2.80 | .03 |
heterogeneity and staining intensity might be two distinct phenomena, differently reflecting the course of the disease.

Correlations between protein heterogeneity of ESR1 and PGR, ESR1 and RAD21, and ERBB1 and pAKT1 were especially strong. ESR1 and PGR1 expression was found to correlate strongly in EC [36]. Investigation of ERBB1 and pAKT1 expression revealed strong correlation between those two proteins in head and neck squamous cell carcinoma [37]. Similarly, we have found statistically significant correlations between ESR1 and PGR, ESR1 and RAD21, and ERBB1 and pAKT1 (data not shown). Mentioned proteins are functionally related. Perhaps if their expression is co-dependent, so could the heterogeneity.

Cumulative tumor heterogeneity of selected proteins’ heterogeneity proved to be an independent predictor of survival and showed the strongest correlations with clinicopathologic data. Apparently, simultaneous analysis of a large number of protein markers gives more thorough image of clonal diversity present in the tumor. Therefore, we conclude that the larger the extent of intratumor heterogeneity in EC, the more aggressive the tumor behavior is and thus the worse the prognosis is.

One of the limitations of the study was relatively short follow-up period. Furthermore, due to variable quality and sometimes small amount of collected material, reliable analysis of all four cores per patient was not always possible. Furthermore, due to variable quality and sometimes small amount of collected material, reliable analysis of all four cores per patient was not always possible. Additionally, the correlation between ESR1 and PGR, ESR1 and RAD21, and ERBB1 and pAKT1 was found to be significant. It was observed that the expression of these proteins could be the heterogeneity.

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