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Original Article

Immunohistochemical characterisation of molecular subtypes in endometrial cancer

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Abstract: Four molecular subtypes have lately been established in endometrial cancer basing on estrogen receptor (ER), progesterone receptor (PR) and HER2 status: ER+/PR+/HER2+, ER+/PR+/HER2-, ER-/PR-/HER2+ and ER-/PR-/HER2-. The subtypes have shown diversity in terms of prognosis, clinicopathological and molecular characteristics, with ER+/PR+/HER2- and ER-/PR-/HER2+ group exhibiting exceptionally benign and aggressive behavior, respectively. We have further characterized the subtypes in the context of pathways known to drive endometrial carcinogenesis: phosphatidylinositol 3-kinase (PI3K)-AKT pathway (ERBB/PI3K pathway), TP53 system, and the mismatch repair (MMR) mechanism. Analysis of tumor heterogeneity was also included. ER+/PR+/HER2+ was characterized by active ERBB/PI3K pathway occurring in 58% of cases. Subtype ER-/PR-/HER2+ was characterized by the most frequent TP53 mutations (83% of cases). Triple negative phenotype utterly lacked active ERBB/PI3K pathway. Analyzed major pathways rarely correlated with clinicopathologial data but mutated TP53 and retained MMR did correlate with shorter overall survival (both $P<0.01$). The presence of tumor heterogeneity was most frequent in ER-/PR-/HER2+ subtype (53% of all cases). The presented results further emphasize that the molecular subtype distinction, along with MMR and TP53 status, could be a useful diagnostic tool in guiding individualized therapy.

Keywords: Molecular subtypes, ERBB/PI3K pathway, Mismatch Repair system, TP53, endometrial cancer

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]. We have recently established molecular subtype classification based on hormone receptor status (Estrogen Receptor-ER, Progesterone Receptor-PR, Human Epidermal growth factor Receptor 2-HER2) analyzed by immunohistochemistry (IHC). Four molecular subtypes have been distinguished: ER-positive and/or PR-positive, HER2-positive (ER+/PR+/HER2+); ER-positive and/or PR-positive, HER2-negative (ER+/PR+/HER2-); ER-negative and PR-negative, HER2-positive (ER-/PR-/HER2+); ER-negative and PR-negative, HER2-negative (ER-/PR-/HER2-). The subtypes have shown diversity in terms of prognosis, clinicopathological and molecular characteristics, with ER+/PR+/HER2- and ER-/PR-/HER2+ group exhibiting exceptionally benign and aggressive behaviour, respectively [2]. As the presented classification could find clinical application in terms of prognosis and treatment personalization, we have decided to further characterize the subtypes in the context of pathways known to drive endometrial carcinogenesis: phosphatidylinositol 3-kinase (PI3K)-AKT pathway (ERBB/PI3K pathway), TP53 system, and the mismatch repair (MMR) mechanism [3], hoping to present a clearer view on the different behavior of tumors belonging to the delineated subtypes.

Protein tumor heterogeneity has been additionally analyzed in the context of the delineated subtypes as we have found out that also this parameter strongly correlates with patients' clinicopathological characteristics and survival [4].

Patients and methods

Patients and tissues

The study included 400 formalin-fixed paraffin-embedded (FFPE) primary tumor samples retrospectively collected from a cohort of consecutive endometrial cancer patients who were operated in the Department of Gynaecology, Gynaecological Oncology and Gynaecological Endocrinology (Medical University of Gdansk) between 2000 and 2010. Samples included in the study were the total sum of eligible cases with available tissue material. Each patient was primarily treated by surgery, with the possible option of radiotherapy and/or chemotherapy administration. The inclusion criteria were operable endometrial cancer (IVB stage patients underwent cytoreductive surgery) confirmed by histological examination and a signed consent form. The study was accepted by the Independent Ethics Committee of the Medical University of Gdansk (NKEBN/269/2009). Procedures involving human subjects were in accordance with the Helsinki Declaration, as revised in 1983.

Immunohistochemistry (IHC) on tissue microarrays (TMA)

The following proteins were examined immunohistochemically: a) phosphatidylinositol 3-kinase (PI3K)-AKT pathway: ERBB1 (epidermal growth factor receptor), HER2 (v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2, also known as HER2), ERBB3 (receptor tyrosine-protein kinase erbB-3), ERBB4 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4), PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase), phosphorylated AKT1-pAKT1 (v-akt murine thymoma viral oncogene homolog 1), stathmin 1 (STMN1), PTEN; b) TP53 system: TP53 (tumor protein p53); c) mismatch repair mechanism: MLH1 (mutL homolog 1), and MSH2 (mutS homolog 2); d) protein tumor heterogeneity: ER, PR, PIK3CA, pAKT1, MYC (v-myc avian myelocytomatosis viral oncogene homolog), TOP2A (DNA topoisomerase II alpha 170 kDa), CDKN2A (cyclin-dependent kinase inhibitor 2A, also known as p16), RAD21 (RAD21 homolog, S. pombe) and RUNX1 (runt-related transcription factor 1).

Samples were collected by surgical excision prior to any systemic treatment and processed as reported before [4]. Protein expression was examined by immunohistochemistry on TMA blocks using the following antibodies: ER-clone SP1 (Roche, Switzerland), PR-clone 1E2 (Roche), ERBB1-clone EGFR113 (Novocastra, Germany), HER2-clone 4B5 (Roche), ERBB3-clone DAK-H3-IC (DAKO, Denmark), ERBB4-clone HFR1 (Abcam, United Kingdom), PIK3CA-clone C73F8 (Cell Signalling, Massachusetts, USA), pAKT1-clone D9E (Cell Signalling), STMN1-clone EPR1574 (Abcam), PTEN-clone 6H2.1 (DAKO, Denmark), TP53 (antibody directed against the mutant TP53 phosphoprotein)-clone BP-53-11 (Roche), MLH1-clone M1 (Roche), MSH2-clone G219-1129 (Roche), MYC-clone 9E11 (Novocastra), TOP2A-clone Ki-S1 (DAKO), CDKN2A-clone JC8 (Santa Cruz Biotechnology, Texas), RAD21-polyclonal antibody (Abcam) and RUNX1-clone DW71 (Santa Cruz Biotechnology). The staining has been performed in accordance with manufacturers' guidelines, details are presented as supplementary material (Table S1; Figure S1).

Protein expression evaluation was performed by two pathologists (HM and JG) blinded to clinical data. ER and PR evaluation of the nuclear staining was performed based on Allred score [5]. HER2 receptor status was determined based on the criteria of HercepTest™ (DAKO) according to the manufacturer's guidelines, as previously described [2, 4]. The interpretation criteria for the remaining proteins were based on the intensity of the staining and/or the percentage of cells showing positive reaction (0-100%), as reported in the literature [6-10]. For proteins where both parameters could have been assessed the final staining score (histological score, H-score) was obtained as the result of multiplication [11]. Cut off point determination of expression positivity of all the proteins studied was performed with the use of Cutoff Finder Web Application [12], basing on the results' distribution. The same cut off determination criteria were assumed for ERBB/PI3K pathway activation status (1 point for each protein classified as positive) where all the 8 proteins were scored together with a new cut off point determined (the only exception was made

Table 1. Clinicopathological data (N=400)

Variable	Number of cases (%)
Menopausal status	
Premenopausal	28 (7.0%)
Perimenopausal	26 (6.5%)
Postmenopausal	345 (86.3%)
Missing data	1 (0.3%)
Age	
≤50 years	42 (10.5%)
>50 years	358 (89.5%)
Obesity	
Absent	197 (49.3%)
Present	202 (50.5%)
Missing data	1 (0.3%)
Diabetes	
Absent	300 (75.0%)
Present	100 (25.0%)
Hypertension	
Absent	140 (35.0%)
Present	260 (65.0%)
Histology	
Type I	307 (76.8%)
Type II	40 (10.0%)
Grade 3	40 (10.0%)
Missing data	13 (3.3%)
Stage (FIGO*)	
IA-IB	277 (69.3%)
II	55 (13.8%)
IIIA-IIIC	47 (11.8%)
IVA-IVB	16 (4.0%)
Missing data	5 (1.3%)
Cervical invasion	
Absent	300 (75.0%)
Present	95 (23.8%)
Missing data	5 (1.3%)
Myometrial infiltration	
≤1/2	198 (49.5%)
>1/2	197 (49.3%)
Missing data	5 (1.3%)
Metastases	
Absent	334 (83.5%)
Present	63 (15.8%)
Missing data	3 (0.8%)

with regard to PTEN: as this protein is the suppressor, not the activator of ERBB/PI3K pathway, 1 point was scored when PTEN status was negative). MMR status was considered functional when the expression of at least one protein (MLH1 or MSH2) was assumed positive.

In addition to the studied pathways we have also included the analysis of tumor heterogeneity in the distinguished molecular subtypes. Protein tumor heterogeneity has been assessed as previously described [4]. In brief, cumulative heterogeneity was determined for each patient, based on 9 proteins which correlated with clinicopathological characteristics and/or survival (ER, PR, PIK3CA, pAKT1, MYC, TOP2A, CDKN2A, RAD21, RUNX1). For each patient a score between 0 and 9 was obtained (1 point for each protein classified as heterogeneous). Based on the results distribution, primary tumors with the score of at least 3 were classified as "globally" heterogenous. Cumulative tumor heterogeneity strongly correlated with the presence of metastases, higher stage, and higher grade of the disease (all *p* values below 0.05). It also carried negative prognostic value (P=0.0008).

Details of immunohistochemical evaluation of all the aforementioned proteins are presented as supplementary material (Table S2).

Statistical analysis

STATISTICA software (Statsoft Co, USA, version 10, Oklahoma) was used for all calculations. The tests that were performed and their applications were as follows: testing normality of the data set-Shapiro-Wilk test; comparison of pathway activation with molecular subtypes-crosstabs statistics with Pearson's Chi-square test; correlations between pathway activation and clinicopathological data-crosstabs statistics with Pearson's Chi-square test; comparison of global tumor heterogeneity with molecular subtypes-crosstabs statistics with Pearson's Chi-square test. The Kaplan-Meier estimator was employed for survival analysis, and the generated curves were compared with the F Cox test. The endpoint for the study was overall survival (OS). OS was defined as the time from sample collection to death of any cause or censoring. Censoring was defined as loss of follow-up or alive at the end of follow-up. Statistical significance was assumed when P≤0.05.

Results

Studied cohort and the flow of samples

The tumor samples included all stages of endometrial carcinoma, from stage IA to IVB, as distinguished by FIGO in 2009 (International

Table 2. Flow of the samples within the cohort of 400 patients where molecular subtype was determined (N=400)

Protein expression level assessed	Number of samples	Number of positive samples (%)	System activation assessed	Number of samples	Number of positive samples (%)
PTEN	399	279 (69.9%)	ERBB/PI3K pathway	396	106 (26.8%)
ERBB1	399	166 (41.6%)			
HER2	400	147 (36.8%)			
ERBB3	400	141 (35.3%)			
ERBB4	400	395 (98.8%)			
PI3K	400	7 (1.8%)			
pAKT1	397	325 (81.9%)			
STMN1	400	156 (39.0%)			
TP53	400	160 (40.0%)	TP53 system	400	160 (40.0%)
MLH1	399	13 (3.3%)	Mismatch Repair system	399	93 (23.3%)
MSH2	400	90 (22.5%)			

Table 3. Pathway activation in the context of molecular subtypes

Molecular subtype	Number of positive samples (%)		
	Activation of ERBB/PI3K pathway	Mismatch Repair system functioning	TP53 mutated
PR+/ER+/HER2+	73/127 (57.5%)	43/128 (33.6%)	78/129 (60.5%)
PR+/ER+/HER2-	29/222 (13.1%)	37/224 (16.5%)	50/224 (22.3%)
PR-/ER-/HER2+	4/18 (22.2%)	6/18 (33.3%)	15/18 (83.3%)
PR-/ER-/HER2-	0/29 (0%)	7/29 (24.1%)	17/29 (58.6%)
P value (chi square)	<0.00001	0.002	<0.000001

Federation of Gynecology and Obstetrics) [13]. We analysed all primary carcinomas of the uterine corpus, separating them into type I (endometrioid) and type II tumors (non-endometrioid). The latter included serous, clear cell, mucinous, undifferentiated and mixed adenocarcinomas [14]. As the position of grade 3 endometrioid EC is not clear, and some authors regard them as intermediate group between low-grade endometrioid and serous EC in molecular terms [15], we analyzed it separately. Metastases included lymph node and distant metastases. The patients' characteristics are summarised in **Table 1**. The median age was 64 (range 26-89 years). Patients with a body mass index higher than 30 were classified as obese [16]. A survival analysis was performed for 397 (99.3%) patients, 3 patients were lost to the follow-up. After a median follow-up of 72 months (range: 0-158), 113 (28.5%) patients had died. The last follow-up data were collected in September 2013. The study was performed in accordance with the REMARK criteria [17].

Constructed TMA blocks collectively included 400 patients. Information concerning the expression level of all three receptors simultaneously (ER, PR, HER2) was available for 400 (100.0%) cases. Within the group of 400 samples, 396 (99.0%), 399 (99.8%) and 400 (100.0%) had ERBB/PI3K pathway status, Mismatch Repair system status and TP53 system status assessed, respectively, as presented in **Table 2**.

Pathway activation in the context of molecular subtypes

ER+/PR+/HER2+ was characterized by overexpression of ERBB/PI3K-dependent molecules that occurred in 58% of cases. The least frequent rate of TP53 mutations was identified in ER+/PR+/HER2- subtype (22%), while ER-/PR-/HER2+ subtype showed the highest rate of this alteration (83% of cases); in addition, it often had active MMR system (33% of cases). Triple negative phenotype lacked active ERBB/PI3K pathway utterly (**Table 3**).

Pathway activation in the context of clinicopathological data

The major pathways analysed rarely correlated with clinicopathologial data. Associations were observed for TP53 and MMR. Mutated TP53

Table 4. Pathway activation in the context of clinicopathological data

Variable	Activation of ERBB/PI3K pathway		Mismatch Repair system functioning		TP53 mutated	
	Number of positive samples (%)	*p* value	Number of positive samples (%)	*p* value	Number of positive samples (%)	*p* value
Menopausal status				0.80		
Premenopausal	8/27 (29.6%)	0.73	6/28 (21.4%)		10/28 (35.7%)	0.64
Peri- and postmenopausal	98/368 (26.6%)		87/370 (23.5%)		149/371 (40.2%)	
Age						
≤50 years	11/41 (26.8%)	0.99	7/42 (16.7%)	0.28	12/42 (28.6%)	0.11
>50 years	95/355 (26.8%)		86/357 (24.1%)		148/358 (41.3%)	
Obesity						
Absent	57/195 (29.2%)	0.29	57/197 (28.9%)	0.009	83/197 (42.1%)	0.41
Present	49/200 (24.5%)		36/ 201 (17.9%)		77/202 (38.1%)	
Diabetes						
Absent	81/297 (27.3%)	0.69	65/299 (21.7%)	0.20	116/300 (38.7%)	0.35
Present	25/99 (25.3%)		28/100 (28.0%)		44/100 (44.0%)	
Hypertension						
Absent	41/138 (28.7%)	0.33	33/140 (23.6%)	0.93	64/140 (45.7%)	0.09
Present	65/258 (25.2%)		60/259 (23.2%)		96/260 (36.9%)	
Histology						
Type I	81/301 (26.9%)	0.60	62/303 (20.5%)	0.002	100/304 (32.9%)	<0.000001
Type II	10/40 (25.0%)		18/40 (45.0%)		29/40 (72.5%)	
Grade 3	13/38 (34.2%)		8/38 (21.1%)		23/38 (60.5%)	
Stage (FIGO*)						
IA, IB, II	86/328 (26.2%)	0.70	73/331 (22.1%)	0.10	123/332 (37.1%)	0.01
IIIA, IIB, IIIC, IVA, IVB	18/63 (28.6%)		20/63 (31.8%)		34/63 (54.0%)	
Cervical invasion						
Absent	83/297 (28.0%)	0.28	68/299 (22.7%)	0.72	125/300 (41.7%)	0.17
Present	21/94 (22.3%)		25/95 (26.3%)		32/95 (33.7%)	
Myometrial infiltration						
≤1/2	49/194 (25.3%)	0.55	45/197 (22.8%)	0.47	71/198 (35.9%)	0.11
>1/2	55/197 (27.9%)		48/197 (24.4%)		86/197 (43.7%)	
Metastases						
Absent	87/326 (26.7%)	0.94	78/354 (22.0%)	0.02	127/330 (38.5%)	0.12
Present	16/61 (26.3%)		14/36 (38.9%)		30/61 (49.2%)	

*FIGO-International Federation of Gynecology and Obstetrics.

correlated with more aggressive tumor characteristics: histology type II (P<0.00001), stage III and IV (P=0.01), grade 3 (P<0.000001). MMR system was mostly active in non-obese patients (P=0.009). It correlated with the presence of metastases (P=0.02), type II EC (P=0.0006) and grade 3 (P=0.002) (**Table 4**).

Survival analysis

Mutated TP53 and retained MMR system correlated with shorter overall survival. ERBB/PI3K pathway had no prognostic significance (**Figure 1**).

Molecular subtypes in the context of tumor heterogeneity

The presence of tumor heterogeneity correlated strongly with ER-/PR-/HER2+ subtype. The same phenomenon was rare in ER+/PR+/HER2- subtype, as presented in **Table 5**.

Discussion

The distinction of molecular subtypes based on ER, PR and HER2 status has introduced valuable information about underlying tumor biology of endometrial cancer. Performed analysis

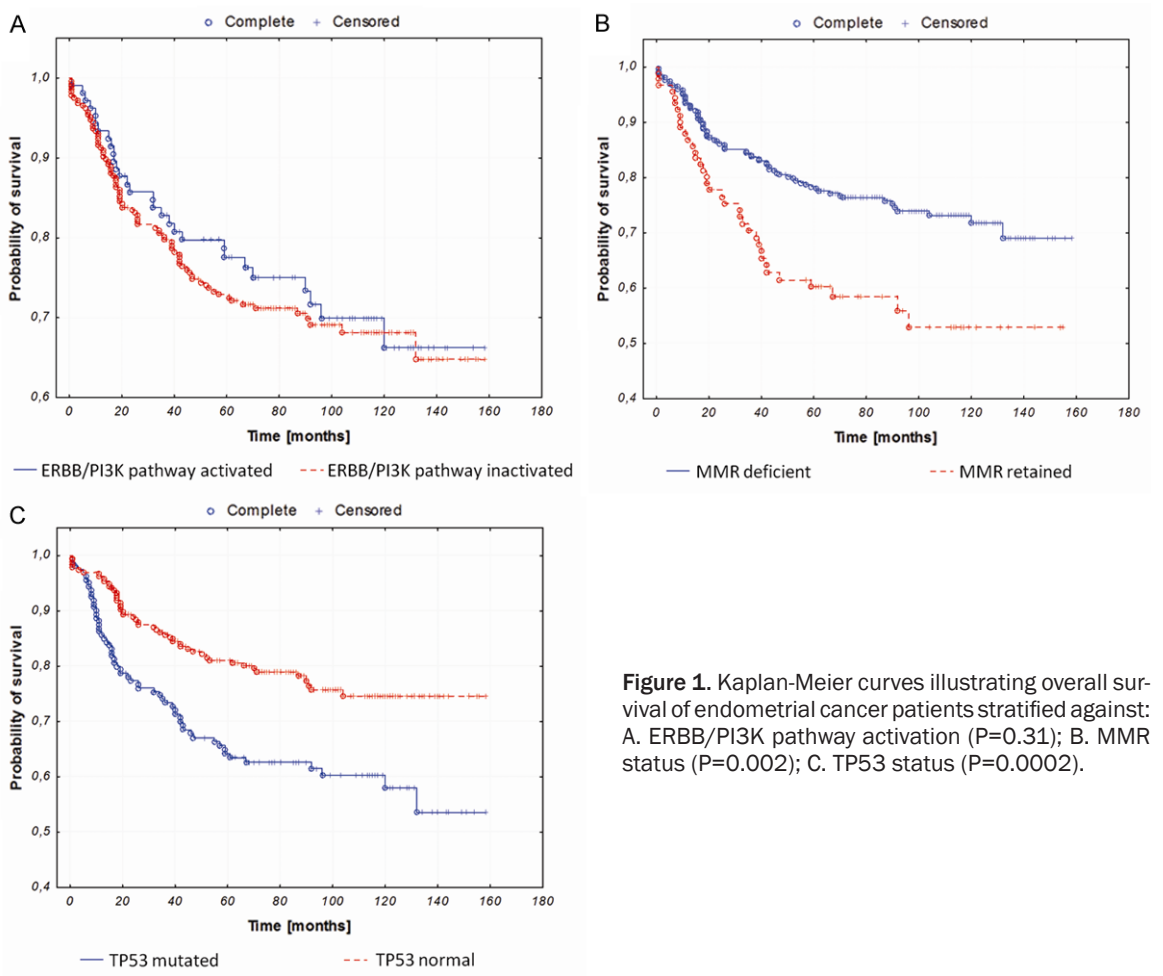

Figure 1. Kaplan-Meier curves illustrating overall survival of endometrial cancer patients stratified against: A. ERBB/PI3K pathway activation (P=0.31); B. MMR status (P=0.002); C. TP53 status (P=0.0002).

Table 5. Global tumor heterogeneity in the context of molecular subtypes

Molecular subtype	Number of samples classified as globally heterogenous (%)
ER+/PR+/HER2+	12/117 (10.3%)
ER+/PR+/HER2-	11/193 (5.7%)
ER-/PR-/HER2+	9/17 (52.9%)
ER-/PR-/HER2-	7/26 (27.0%)
P value (chi square)	<0.000001

revealed that the elucidated subgroups differed fundamentally in terms of prognosis, clinicopathological, and molecular characteristics. The greatest distinction was observed between ER-/PR-/HER2+ subtype exhibiting exceptionally aggressive tumor characteristics and ER+/PR+/HER2- subtype being the most benign. ER/PR-/HER2+ subtype was characterized by exceptionally short overall survival, often showing histological features of type II endometrial carcinomas, advanced stage or grade, with frequent signs of cervical invasion, myometrial infiltration, or metastases. The other subtypes have fallen in the middle of this distinction, showing intermediate clinicopathological, prognostic and molecular characteristics, with ER-/PR-/HER2- subtype classified as the second least favourable and ER+/PR+/HER2+ subtype-the second most favourable [2]. Encouraged by the described findings, we have decided to study the elucidated subtypes in the context of pathways known to drive endometrial carcinogenesis: ERBB/PI3K pathway, TP53 system and MMR. This study revealed underlying mechanisms of the tumor formation within different subtypes.

ER+/PR+/HER2+ exhibited the highest rate of ERBB/PI3K pathway activation, which was in compliance with the results obtained in breast cancer where ERBB/PI3K pathway activation signature was found common for luminal B subtype [18]. This subtype was also often charac-

terized by the highest frequency of functional MMR system. Nevertheless, it is difficult to verify this data in the available literature as the data on this subject in EC are nonexistent and the frequency as well as the significance of DNA mismatch repair deficiency in breast cancer (the only surrogate with molecular subtypes distinguished) is unclear [19]. ER+/PR+/HER2- subtype exhibited TP53 mutations only in 22% which was the lowest value for all the studied subtypes. This might partially explain its benignity. Similar frequency of TP53 mutations was observed for luminal A breast cancer. Subtype ER-/PR-/HER2+ was characterized by the most frequent TP53 mutations, which is also common in HER2 amplified breast tumors [20]. Triple negative phenotype utterly lacked active ERBB/PI3K pathway.

Even though ERBB/PI3K activation in EC showed multiple correlations with aggressive tumor behaviour at the gene copy number variation level, as we have reported previously [21], no such association could have been observed in hereby study. Interesting results, however, were obtained in case of MMR system. The DNA mismatch repair is critical for the maintenance of genomic stability and its deficiency is common in many cancers. It might seem that high expression of MMR proteins will be present mostly in low-grade, early stage tumors but in fact retained MMR system correlated with type II histology and grade 3 (P=0.002) and the presence of metastases (P=0.02). Additionally, a trend was observed towards association of MMR system and higher stages of the disease (P=0.10). Similar results, also in endometrial cancer, were reported lately [22], contrary to many reports where deficiency of MMR system showed no associations with clinicopathological features or prognosis [23-25]. Furthermore, retained MMR system was less common in obese patients (P=0.009). Joehlin-Price et al. reported that obesity itself might be less crucial driver of the EC initiation. In their study, obese women had a lower overall MMR deficiency, however statistically significant BMI differences were not observed in all the studied subgroups and were highly dependent on the particular MMR protein analyzed [25]. In our study the accumulation of mutated TP53 correlated with type II EC (P<0.00001) and higher stage of the disease (P=0.01). Other authors also reported similar findings, with TP53 accumulation associated with adverse tumor characteristics [26, 27].

In our study ERBB/PI3K activation showed no prognostic significance. We were also unable to find any prognostic significance of that pathway at the DNA level [21]. Nout et al. managed to present a trend regarding the prognostic significance of high PI3K-AKT pathway activation in EC [28]. Much more informative with regard to OS was MMR status. We have shown that MMR-deficient cases had significantly longer OS, similarly to the data presented by Kato et al. [22]. High expression of mutated TP53 strongly correlated with unfavourable prognosis. The data are in consistence with the results found in the literature [29, 30].

Intratumoral heterogeneity analyzed in the studied specimen was the most frequently observed event in PR-/ER-/HER2+ subtype (P<0.000001). As PR-/ER-/HER2+ subtype carries by far the worst prognosis [2] and tumor heterogeneity has also shown to be of strong adverse prognostic significance [4], it is possible that overexpression of HER2 drives uncontrollable divisions characterized by an extremely high mutation rate, generating genetically diverse subclones within the tumor. To the best of our knowledge, we are the first to present the data concerning intratumoral heterogeneity in the context of molecular subtypes based on ER, PR and HER2 receptor status.

Methodology utilized in hereby study, exploited on a large sample size, showed satisfying reaction performance, specificity, high specimen quality, and thus low sample loss. The protocols used allowed for highly coherent protein expression measurements. Crucial limitation of the study was the lack of treatment response data as both the subtypes and the studied molecular systems carry implications for therapy selection.

EC molecular subtypes based on ER, PR and HER2 status have proven to differ not only in terms of prognosis and clinicopathological data but also in molecular characteristics. The proposed classification, along with molecular characterization, might serve as a clinically valid molecular marker. Continued investigation of the presented data, especially in the aspect of targeted therapies, is necessary, as it has been proven that MMR deficiency can be associated

with resistance to standard therapeutic agents (eg. platinum compounds, methylating agents, fluoropyrimidine agents) [31]. Furthermore, loss of MMR could provide predisposition to adjuvant radiotherapy sensitivity [22]. There are also reports suggesting it might be worthwhile to include effective PI3K/Akt inhibitors in the current treatment regime of endometrial cancer [32]. Moreover, testing of TP53 expression level was suggested to help in the selection of patients eligible for pelvic lymphadenectomy in clinical stage I endometrial carcinoma [33] and tumors overexpressing TP53 have been shown more likely to benefit from anti-IGF-IR therapies [34].

Even with some molecular differences not being striking among the elucidated subtypes and with the lack of correlations between the studied systems and/or clinicopathological data, the presented results further emphasize that the molecular subtype distinction, along with MMR and TP53 status, could become a useful diagnostic tool in guiding individualized therapy, as suggested before [2, 35, 36]. The presented results should be subject to further investigation which could be therewith verified in a clinical setting. Additionally, studies of MMR system in a larger cohort of patients are necessary to explain observed discrepancies in reports concerning endometrial cancer.

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Disclosure of conflict of interest

None.

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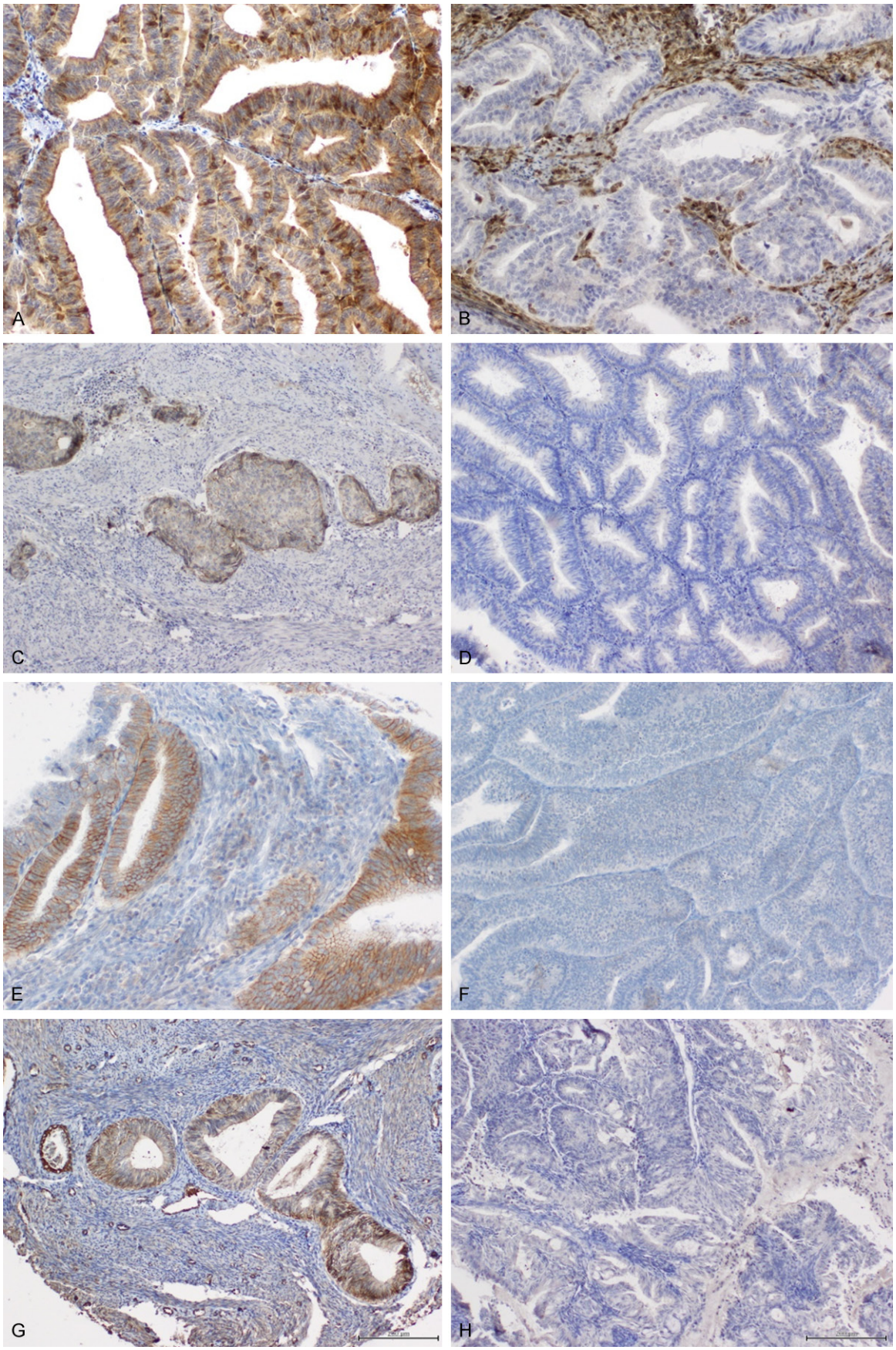
Table S1. Immunohistochemistry staining details

Antibody	Clone	Manufacturer	Dilution	Antigen retrival	Incubation	Kit
ESR1	SP1	Roche	ready to use	Benchmark's protocol		ultraView
PGR	1E2	Roche	ready to use	Benchmark's protocol		ultraView
ERBB1	EGFR113	Novocastra	1:20	pH=9 (DAKO S3267), heat induced	90 min, room temperature	Novolink
HER2	4B5	Roche	ready to use	Benchmark's protocol		ultraView
ERBB3	DAK-H3-IC	DAKO	1:50	pH=9 (DAKO S3267), heat induced	90 min, room temperature	Novolink
ERBB4	HFR1	Abcam	1:50	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
PIK3CA	C73F8	Cell Signaling	1:50	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
pAKT1	D9E	Cell Signaling	1:20	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
STMN1	EPR1574	Abcam	1:400	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
PTEN	6H2.1	DAKO	1:200	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
TP53	BP-53-11	Roche	ready to use	Benchmark's protocol		ultraView
MLH1	M1	Roche	ready to use	Benchmark's protocol		ultraView
MSH2	G219-1129	Roche	ready to use	Benchmark's protocol		ultraView
MYC	9E11	Novocastra	1:100	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
TOP2A	Ki-S1	DAKO	1:200	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
CDKN2A	JC8	Santa Cruz	1:200	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
RAD21	Polyclonal	Abcam	1:500	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink
RUNX1	DW71	Santa Cruz	1:50	pH=6.1 (DAKO S1699), heat induced	90 min, room temperature	Novolink

Table S2. Details of immunohistochemical evaluation

Evaluated protein	Staining localisation	Staining intensity	% of stained cells
ER	nucleus	Allred score*	
PR	nucleus	Allred score	
HER2	membrane	HercepTest**	
ERBB1	membrane	HercepTest***	
ERBB3	cytoplasm	assessed	not assessed****
ERBB4	cytoplasm	assessed	not assessed
PIK3CA	cytoplasm	assessed	not assessed
pAKT1	cytoplasm, nucelus	assessed (sum of intensities)	not assessed
STMN1	cytoplasm	assessed	assessed
PTEN	cytoplasm, nucelus	not assessed	assessed
TP53	nucleus	assessed	assessed
MLH1	nucleus	not assessed	assessed
MSH2	nucleus	not assessed	assessed
MYC	cytoplasm	assessed	assessed
TOP2A	nucleus	not assessed	assessed
CDKN2A	cytoplasm, nucleus	assessed	assessed
RAD21	nucleus	assessed	not assessed
RUNX1	cytoplasm	assessed	assessed

*The score of staining intensity (0-3) and the % of the stained cells (divided into 5 intervals - 1: 0-1%, 2: 2-10%, 3: 11-33%, 4: 34-66%, 5: 67-100%). **Score ranging from 0 to 3, with 3 defined as "A strong complete membrane staining observed in more than 10% of the tumor cells". ***modification: cut-off 1% instead of 10%. ****not assessed-homogenous staining or equal staining intensity in all specimen.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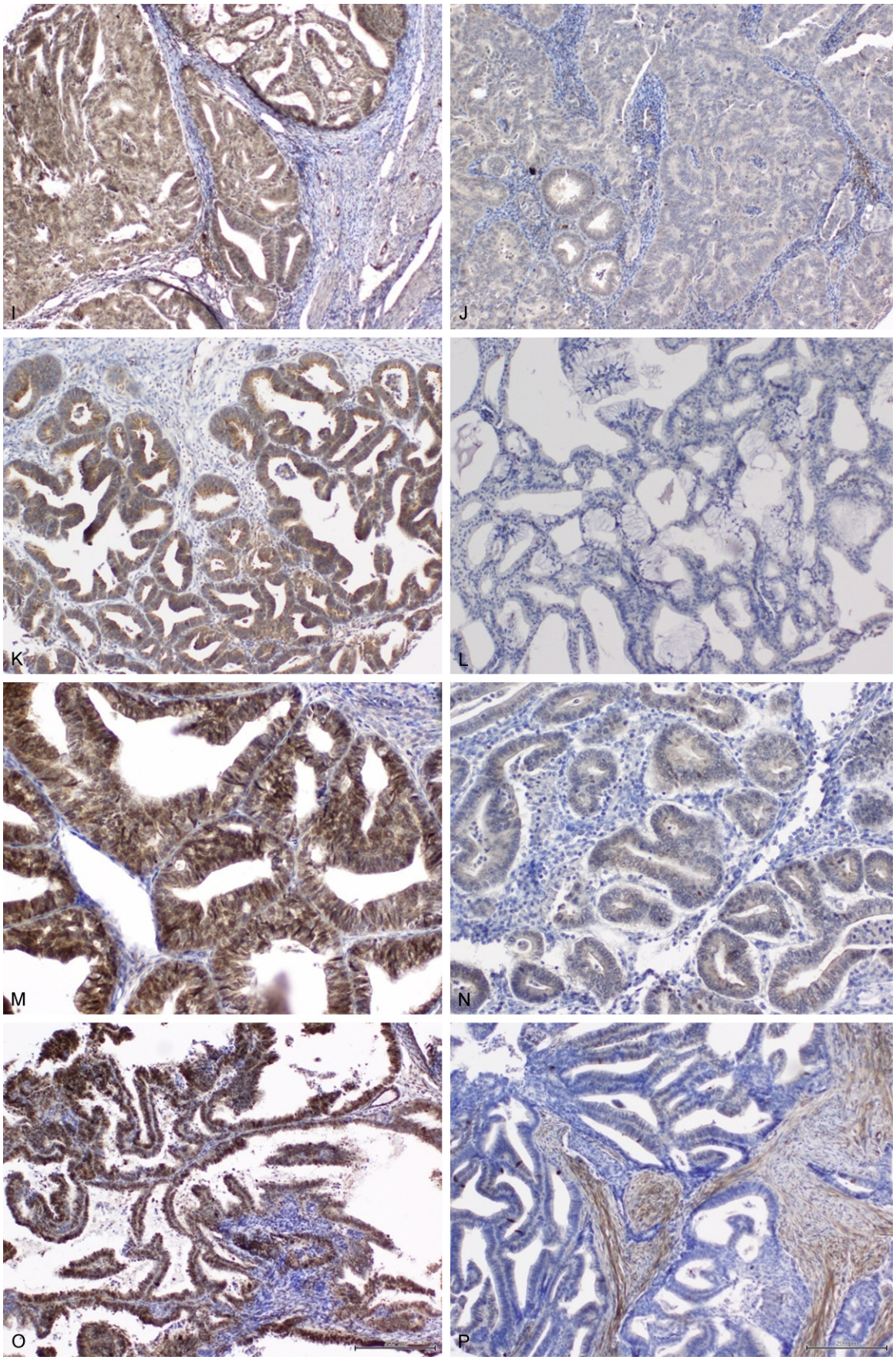
I
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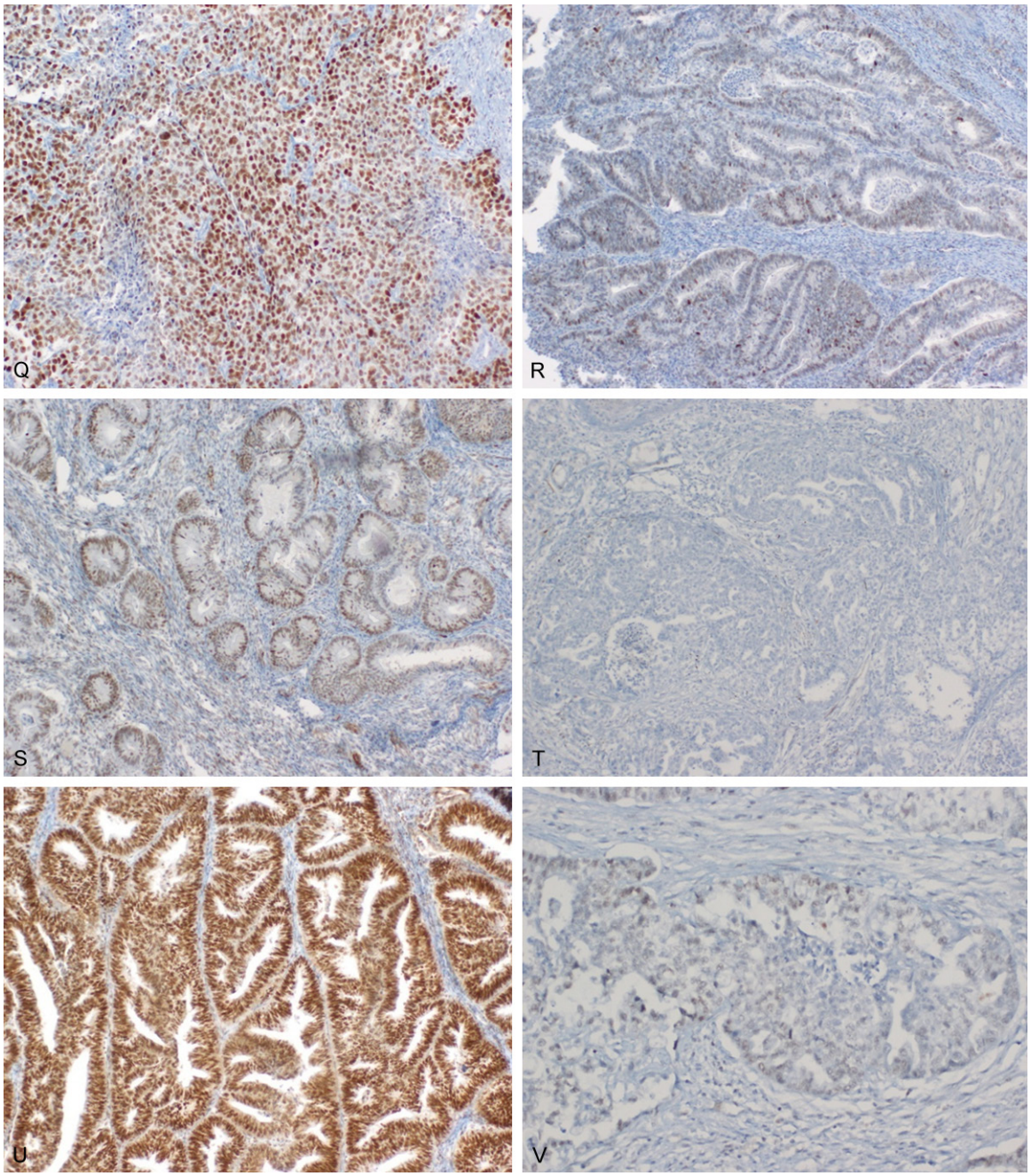

Figure S1. Representative immunohistochemistry images of: A. Positive PTEN staining; B. Negative PTEN staining; C. Positive ERBB1 staining; D. Negative ERBB1 staining; E. positive HER2 staining; F. Negative HER2 staining; G. Positive ERBB3 staining; H. Negative ERBB3 staining; I. Positive ERBB4 staining; J. Negative ERBB4 staining; K. Positive PI3K staining; L. Negative PI3K staining; M. Positive pAKT1 staining; N. Negative pAKT1 staining; O. Positive STMN1 staining; P. Negative STMN1 staining; Q. Positive TP53 staining; R. Negative TP53 staining; S. Positive MLH1 staining; T. Negative MLH1 staining; U. Positive MSH2 staining; V. Negative MSH2 staining.