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Heterogeneity of DNA Ploidy in Endometrial Carcinoma: Comparison of Different Tissue Samples Obtained during Diagnosis and Treatment

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Abstract. *Aim: Comparison of DNA ploidy status of different tumour tissue samples (fresh/frozen vs. paraffin-embedded; curettage vs. hysterectomy samples) obtained during diagnosis and treatment of patients with endometrial carcinoma. Patients and Methods: DNA ploidy status and conventional prognostic parameters were recorded for 74 patients with endometrial carcinoma prospectively. Results: In 59 (79.7%) patients the DNA status was described as diploid in all analyzed tissue samples. The remaining 15 (20.3%) cases were described as DNA aneuploid in at least one of the corresponding tissue samples. The concordance between DNA ploidy status in fresh vs. paraffin-embedded hysterectomy samples as well as curettage vs. hysterectomy paraffin-embedded samples was high (kappa coefficient κ=0.6348, 95% confidence interval CI=0.3673-0.9023, and p=0.6408, 95% CI=0.3977-0.8838), however, the methods are not interchangeable. Conclusion: The DNA ploidy discordance observed in our study group seems to document intratumoral heterogeneity that should be expected when applying DNA ploidy status in the clinical management of endometrial carcinoma.*

Endometrial carcinoma (ECA) is the most common gynaecologic malignancy in the Western world. Approximately 80% of cases are diagnosed at an early stage, being well to moderately differentiated at the time of diagnosis. Due to the high cure rate, the survival of patients with ECA is generally good. The overall 5-year relative survival rate is 84% (96% for stage I, 68% for stage II and III, 17% for stage IV, respectively (1). The key prognostic parameters are International Federation of Gynecology and Obstetrics (FIGO) stage, grade, histologic subtype, depth of myometrial invasion, vascular invasion and lymph node metastases. However, none of these conventional prognostic factors allows selection of all patients with poor prognosis. Additional parameters related to high risk of recurrence or death from the disease are under investigation.

Immunohistochemical examination of molecular, biological markers and DNA ploidy are in the focus of research for ECA prognostic parameters. DNA aneuploidy is related to more advanced disease at diagnosis and high risk of recurrence, and consequently to significantly lower overall survival of patients with ECA (2-4). Even patients with disease of the same stage and grade have worse survival if DNA aneuploidy is found as compared to DNA diploidy (5-7). In most studies, DNA ploidy has been found to be a stronger prognostic factor than any other tumour-associated parameter, with the possible exception of p53 overexpression (8).

The aim of this study was to compare the DNA ploidy status in different tissue samples obtained during diagnosis and treatment of patients with ECA. We analyzed tissue samples from pre-treatment biopsy (curettage or hysteroscopy) and primary surgery (hysterectomy) with different time of sample collection. Usually it takes from 2 to 4 weeks from pre-treatment biopsy to surgical therapy, the most frequent primary treatment in patients with ECA. Regarding the specimen category, formalin-fixed paraffin-embedded (FFPE) and fresh/frozen samples were analyzed; samples differ substantially in the sampling method

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(microscopic selective sampling in FFPE *vs*. macroscopic observation in fresh/ frozen samples) and storage. We aimed i) to compare the DNA ploidy status of fresh/frozen and FFPE samples obtained from hysterectomy specimens; ii) to compare the DNA ploidy status of curettage and hysterectomy specimens (both FFPE).

Although a fresh tissue sample is generally preferred for analysis of DNA ploidy by flow cytometry, obtaining the material can create both practical and logistic problems and may sometimes interfere with the demands of routine pathological evaluation. Use of FFPE samples allows standard examination of specimens of the uterus and cervix and thus avoids any future medicolegal consequences. Originally, FFPE samples were used to perform retrospective studies but they have also become widely used for prospective evaluation when fresh material is not available. Above all, microscopic examination of parallel sections allows for selective sampling from a tumour-rich area.

Most of the retrospective and prospective studies used hysterectomy specimens. Receiving information on DNA ploidy status at the time of the first diagnosis from the curettage (hysteroscopy) specimen could help clinicians. The ability to predict the risk of extrauterine disease and risk of post treatment recurrence before initiation of the therapy would be of great importance. It would allow prompt action and the patient could be referred to an oncogynaecological center, enabling rapid selection of the required extent of the surgery, and adequate application of adjuvant therapy. The DNA ploidy status could identify patients at risk even in cases where other high-risk factors (high-grade, non-endometrioid histopathology, deep myometrial invasion, extrauterine spread) are absent.

Patients and Methods

Patients with ECA treated at the Department of Gynaecology and Obstetrics of the Third Faculty of Medicine of Charles University and The Kralovske Vinohrady University Hospital in Prague, Czech Republic, between January 2009 and September 2010 were enrolled into a prospective study. Patients underwent standard diagnostic procedures, staging examinations, primary surgery, and adjuvant treatment depending on final staging.

Patients. Patients with diagnosis of ECA were enrolled into the study; we strived to obtain different kinds of tumour tissue specimens for DNA analysis in each case (Figure 1). Clinicopathological variables were recorded as follows: age, related risk factors, histological subtype, stage, grade, myometrial invasion, lymphatic node metastasis, type of surgery, adjuvant therapy, and clinical outcome. The surgical procedure included hysterectomy with bilateral salpingo-oophorectomy (BSO). Aortopelvic lymphadenectomy was accomplished to complete surgical staging if unfavourable prognostic features were recorded, such as grade 3, non-endometroid histopathology, or deep myometrial invasion. Adjuvant chemotherapy was applied in cases of advanced disease; pelvic irradiation and/or vaginal brachytherapy was indicated for patients with tumour mass outside the uterine corpus, high-grade or deep myometrial invasion depending on the final histological staging. After completing treatment, patients with ECA were re-appointed each 3 months for the first 3 years, each 6 months for the next 2 years and each 12 months thereafter.

Preparation of nuclei suspension from paraffin-embedded tissue. An area of representative tumour tissue in paraffin-embedded and haematoxylin-eosin stained sections was selected by an experienced pathologist. Three to four 50 μm-thick sections were cut from the corresponding paraffin blocks. The samples were prepared for flow cytometric DNA analysis using the Hedley technique adapted for routine practice (9). Sections were placed into fine nylon mesh bags (loop size 162 μm), deparaffinized twice in xylene (for 20 and 10 minutes at room temperature), rehydrated in a series of ethanolic solutions of decreasing concentration (96%, 90%, 70%, 50% ethanol for 10 minutes, each at room temperature) and finally washed twice in distilled water. The nuclei suspension was prepared by mechanical homogenization and enzyme digestion. Firstly, tissue sections were disintegrated mechanically using a GentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and were then incubated in 1 ml of 0.5% pepsin solution at 37˚C for 30 minutes, with intermittent vortex mixing. Suspensions were centrifuged, resuspended in a solution of RNAse and the fluorochrome marker propidium iodide (BD Biosciences, San Jose, CA, USA), filtered through 50 μm cup Filcon filters (BD Biosciences) and left at 37˚C for 10 minutes.

Preparation of nuclei suspension from fresh/frozen tissue. Fresh samples for flow cytometry were obtained from the fresh hysterectomy specimen by a pathologist after thorough macroscopic observation anticipating it as being representative of tumour tissue. Samples were processed immediately or stored frozen at –80˚C. We performed examination of both fresh and frozen tissue in several pilot cases and found there to be no major differences caused by freezing. Approximately 0.5 cm^2 of fresh or defrosted tissue was minced with scissors in 2 mM EDTA/PBS solution. GentleMACS™ dissociator (Miltenyi Biotec) was used to obtain a single-cell suspension. Suspensions were filtered through 50 μm Filcon cup filters (BD Biosciences), centrifuged and resuspended in a solution of 2 mM EDTA/PBS solution to prevent formation of aggregates. The small part of the examined suspension (50 μl) was processed using Cell Cycle BD kit (BD Biosciences) containing enzymes and nuclear-staining propidium iodide. Internal standard was prepared from female peripheral blood using Cycle Cell BD kit (human lymphocytes) to verify the quantity of DNA of examined samples.

Flow cytometry. Flow cytometric DNA analysis was performed using a four-color FACS Calibur instrument (BD Biosciences). Thirty thousand events per sample were scanned using low flow rates to maximize resolution. The quality of histogram was described using the coefficient of variation (CV), which generally should not exceed 8%; however, the CV of the tumour population may be higher due to the presence of multiple subpopulations (10). Debris and aggregates that can cause interference in histogram analysis were monitored.

Data analysis. Flow-cytometric data were expressed as DNA histograms using the Flow-Jo software (Tree Star Inc., Ashland, OR, USA). The DNA ploidy was given by the DNA index. For FFPE

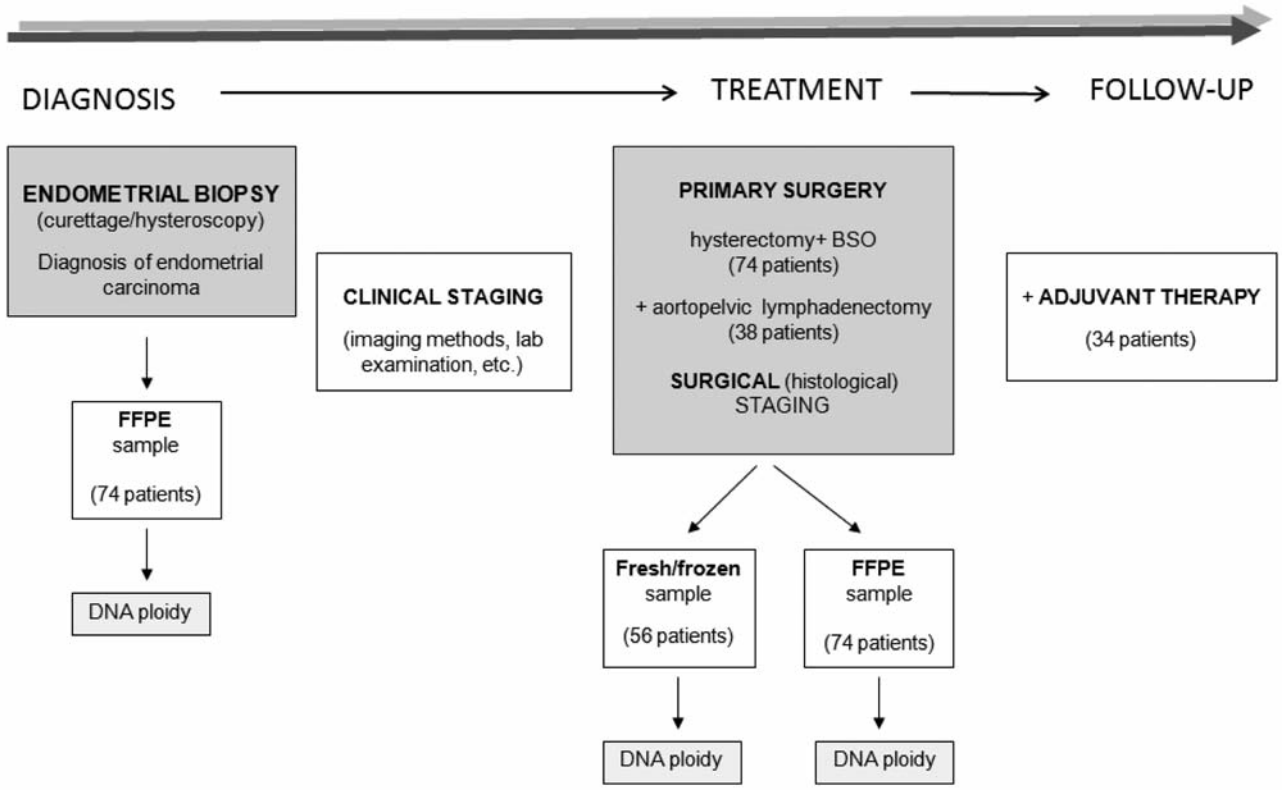

Figure 1. *Acquiring tissue samples for DNA analysis. FFPE: Formalin-fixed paraffin-embedded; BSO: bilateral salpingo-oophorectomy.*

samples, no internal standard of cellular DNA content was used as crosslinking between DNA and other molecules, caused by initial formaldehyde fixation of the tissue sample, unpredictably influences the DNA staining and renders comparison with internal or external standard unreliable (11). By convention, the first G_0/G_1 peak with the lowest DNA content in the histogram is considered diploid and the DNA index is calculated by comparing other G_0/G_1 peaks to its modal position. It can be assumed that the presence of a single G_0/G_1 peak indicates a DNA diploid or very near diploid tumour, whereas multiple G_0/G_1 peaks are only found in DNA aneuploidy. From substantial experience with fresh and fixed tissue, it is known that a peak representing diploid cells is present in all instances, and aneuploidy can be identified by the presence of multiple G_0/G_1 peaks. Thus, with the exception of hypodiploidy, which occurs in fewer than 2% of most solid tumours, aneuploid clones can be distinguished by their higher DNA content compared to diploid cells (9). If only a single peak was present, the specimen had a DNA index of 1.00. If two peaks were present, the DNA index was calculated by dividing the peak channel of the second peak (nondiploid G0/G1 cell population) by the peak channel of the first peak (reference peak). Histograms with a G_2/M population greater than 15% were classified as indicating aneuploidy.

Statistical analysis. Statistical analysis was performed using the SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). The statistical results comparing categorical parameters in subgroups were calculated using a Chi-square test or Fisher's exact test if the expected size in any cell of the contingency table was less than 5. *p*-values were considered statistically significant at the 0.05 level. For agreement between two method, Cohen's kappa (unweighted kappa) was calculated. Kappa is the probability of agreement adjusted for the probability of agreement at random. As reference, Landis and Koch (12) put forth the labels for the strength of agreement based on kappa: poor (<0.00), slight (0.00-0.20), fair (0.21-0.40), moderate (0.41-0.60), substantial (0.61-0.80) and almost perfect (0.81-1.00).

Results

We analyzed samples from a total of 74 patients with histologically proven invasive ECA. In all 74 cases, there were both pre-treatment biopsy (curettage) and primary surgical (hysterectomy) FFPE samples available. Both fresh and paraffin-embedded tissues from 56 patients with histologically proven invasive ECA were available for further analysis. The clinicopathological characteristics of the study group were analyzed using the entry data of all 74 patients. The average age at diagnosis was 66.3±11.0 years (range 39-88 years). The mean body mass index (BMI) was 31.2±7.7 (range 19-57). A total of 17.8% of patients were treated for diabetes; 59.5% were treated for arterial hypertension. From the whole group of 74 patients, 14.8% (n=11) had non-

endometrioid histopathology (serous, clear-cell, etc.); there were 18.9% (n=14) of grade 1, 40.5% (n=30) of grade 2, and 40.5% (n=30) of grade 3 tumours, respectively, in the study group. Most of the patients were diagnosed with early-stage disease: 58 (78.4%) patients were FIGO stage I; 2 patients (2.7%) FIGO stage II; and 14 patients (18.9%) stage III or IV, respectively. Deep myometrial invasion was present in 35.1% of cases. Lymphadenectomy was performed in 51.4% (n=38) cases (patients with high-risk prognostic factors); from this subgroup, positive lymph nodes were confirmed in 18.4% (n=7); from these, 3 cases were DNA aneuploid (see Table I).

Thirty-four patients had adjuvant therapy after primary surgical treatment. Five patients were diagnosed with concurrent tumour duplicity (breast carcinoma, cervical carcinoma, rectosigmoid carcinoma, renal carcinoma, borderline ovarian tumour). The median follow-up was 17.7 (range 1-32) months; two patients died of disease; one patient died of other causes; one patient was alive with recurrent disease at the time of the last check-up; four patients were lost to follow-up.

The DNA index was evaluated in all cases. Repeated evaluation was performed in several cases because of low quality histograms until results of good quality were obtained. DNA aneuploidy was described in at least one of the corresponding examined samples in 15 (20.3%) patients. In the remaining 59 (79.7%) patients, DNA diploid status was demonstrated in all examined tissue specimens (FFPE curettage, FFPE hysterectomy, fresh/frozen hysterectomy). We analyzed the relationship between DNA ploidy status and conventional prognostic factors of clinical outcome (Table I). If the DNA status was aneuploid in at least one of the corresponding examined samples from an individual patient, the case was considered to be DNA aneuploid. There was a statistically significant correlation between DNA ploidy status and FIGO stage (p=0.0007); between DNA ploidy status and histological type (p<0.0001); and between DNA ploidy status and the presence of lymph node metastases (p=0.0297), documenting that DNA aneuploidy is related to more advanced, high-risk disease at the time of diagnosis.

With regard to the short follow-up interval, as yet, we have not analyzed the prognostic value of observed parameters (progression-free survival, overall survival). Nevertheless, both the two patients who died of their disease already had advanced stage, grade 3 non-endometrioid tumour with DNA aneuploidy (Table II).

Comparison of DNA ploidy status: Fresh/frozen vs. FFPE hysterectomy samples. DNA ploidy status in both types of samples is shown in Table III. In the group of 56 patients in which fresh/frozen and FFPE samples from hysterectomy were compared, DNA ploidy status was identical in 50 cases and discordant in six cases. There were 43 cases (76.8%) described as being DNA diploid in both types of tissue samples. The remaining 13 cases were described as being DNA aneuploid in at least one of the corresponding tissue samples (fresh or FFPE). The agreement between DNA ploidy status of the two types of specimens (fresh *vs*. FFPE hysterectomy samples) was high (unweighted kappa coefficient=0.6348, 95% confidence interval CI=0.3673-0.9023). However, the analysis shows the two methods cannot be interchanged because DNA ploidy status was assessed as aneuploid in seven patients by both assessments (fresh/frozen and FFPE); in another six patients, only one result was aneuploid but the second was diploid. The fresh/frozen specimen was aneuploid and the FFPE specimen was diploid in three patients; the fresh/frozen specimen was diploid and the FFPE specimen was aneuploid in another three patients. Therefore if aneuploidy is defined as positivity of both tests (fresh/frozen and FFPE) then we detected seven positive cases; if aneuploidy is defined as positivity of either of the two tests, then we detected 13 positive cases. We cannot confirm that the two methods are interchangeable.

The mean coefficient of variation was 4.01% for the fresh/frozen and 7.6% for the FFPE samples, respectively.

Table I. *DNA ploidy status and clinicopathological variables*.

Variable	Total no.	DNA diploid, in all available samples	DNA aneuploid, in at least one sample	*p*-Value
FIGO stage				
I	58	51	7	
II	2	1	1	0.0007
III	11	7	4	
IV	3	0	3	
Histology				
Endometrioid	63	56	7	<0.0001
Non-endometrioid	11	3	8	
Age				
≤50 years	6	6	0	0.2432
>50 years	68	53	15	(NS)
Deep myometrial invasion				
–	57	47	10	0.2854
+	17	12	5	(NS)
LN metastases				
–	31	28	3	0.0297
+	7	4	3	
Grade				
1	14	11	3	
2	30	27	3	0.1551
3	30	21	9	(NS)

FIGO: International Federation of Gynecology and Obstetrics; LN: lymph node.

Table II. *Characteristics of patients with endometrial carcinoma who died of the disease.*

Patient	Age at the time of diagnosis (years)	FIGO stage	Tumour grade	Histological subtype	DNA ploidy status (DNA index)	Deep myometrial invasion	Months to death from the disease progression
1	84	IVB	G3	Non-endometrioid	Aneuploid (1.77, 1.00, 1.99)	–	6
2	74	IVB	G3	Non-endometrioid	Aneuploid (2.05, 2.1, 1.97)	+	7

Table III. *DNA ploidy results in fresh/frozen vs. paraffin-embedded hysterectomy samples.*

	Fresh/frozen hysterectomy samples (%)	Paraffin-embedded hysterectomy samples (%)	
		DNA diploid	DNA aneuploid
DNA diploid	46 (82.14)	43 (76.79)	3 (5.36)
DNA aneuploid	10 (17.86)	3 (5.36)	7 (12.5)
Total	56 (100)	46 (82.14)	10 (17.86)

Unweighted kappa coefficient=0.6348.

Table IV. *DNA ploidy results in curettage vs. paraffin-embedded hysterectomy samples.*

	FFPE curretage samples (%)	FFPE hysterectomy samples (%)	
		DNA diploid	DNA aneuploid
DNA diploid	61 (82.4)	59 (79.7)	2 (2.7)
DNA aneuploid	13 (17.6)	5 (6.8)	8 (10.8)
Total	74 (100)	64 (86.5)	10 (13.5)

Unweighted kappa coefficient=0.6408.

Curettage vs. hysterectomy specimens. DNA ploidy status of curettage FFPE samples and corresponding hysterectomy FFPE samples are shown in Table IV. In the group of 74 patients, DNA ploidy status was identical for both types of specimens in 67 cases and differed in seven cases. The agreement between DNA ploidy status of the two types of specimen (curretage *vs.* hysterectomy samples) was high (unweighted kappa coefficient=0.6408, 95% CI=0.3977-0.8838). The mean coefficient of variation was 7.79% for the curettage FFPE samples and 7.58% for the hysterectomy FFPE samples, respectively. However, the analysis shows that the two methods cannot be interchanged because DNA ploidy status was discordant. The curettage FFPE specimen was aneuploid and the hysterectomy FFPE specimen was diploid in five patients; the curettage FFPE specimen was diploid and the hysterectomy FFPE specimen was aneuploid in two patients. Therefore, if aneuploidy is defined as positivity of both tested specimens (curettage and hysterectomy FFPE specimen) then we detected eight positive cases; if aneuploidy is defined as positivity of either of the two tested specimens, then we detected 15 positive cases. We conclude that the two methods are not interchangeable.

Discussion

Studies of DNA ploidy and other molecular biomarkers in patients with ECA were introduced to detect unfavourable factors which help to identify patients at high risk of recurrence and death from their disease. When compared to conventional prognostic factors, DNA ploidy was the strongest variable to predict clinical outcome of ECA, independent of stage or histological subtype (6, 13). These observations were demonstrated on both FFPE and fresh/frozen tissue samples using flow cytometry or image cytometry (14). Some oncological centres have already applied DNA ploidy as routine testing for patients with ECA to specify better the biological character of the tumour and have incorporated it as an integrated part of the treatment algorithm (15, 16).

Regarding prognostic factors for ECA, we are able to determine two of them from the pre-treatment endometrial biopsy: tumour grade and histological subtype; the others (FIGO stage, myometrial invasion, extrauterine spread) can be established precisely after surgical treatment only. But there are significant limitations described for the accuracy of tumour grade and histopathologic subtype examination obtained from endometrial sampling techniques (14). In the study of Leitao *et al.* (17) preoperative endometrial sampling diagnosis of FIGO grade 1 and final hysterectomy pathology did show correlation in 85% of cases. FIGO grade was upgraded in almost 15% of 482 cases. High-risk final pathology (grade 2 and 3, non-endometrioid pathology, deep myometrial invasion) was seen in 18.5% in preoperative grade 1 cases. Frumowitz *et al.* (18) describes that 23% of grade 1 and 2 curettage samples were upgraded in the final histology (group of 153 patients). It shows that decision-making at the time of diagnosis is often based on inaccurate and incomplete information, and positivity of additional

Table V. *DNA aneuploid cases – comparison of available paraffin-embedded (FFPE) and fresh/frozen tissue samples and clinicopathological variables. Concordant cases 1-7; discordant cases 8-15.*

Case	DNA ploidy status (DNA index)			Histological subtype	Grade	FIGO stage	Lymph node status	Deep myometrial invasion
	FFPE sample		Fresh/frozen sample Hysterectomy					
	Curettage	Hysterectomy						
1	A (1.18)	A (1.23)	A (1.20)	N	G2	II	NX	–
2	A (1.81)	A (1.87)	A (1.83)	N	G3	IA	N0	–
3	A (1.58)	A (1.53)	A (1.43)	N	G3	IIIB	NX	+
4	A (2.05)	A (2.10)	A (1.97)	N	G3	IVB	NX	+
5	A (1.32)	A (1.41)	A (1.35)	N	G3	IA	NX	–
6	A (1.38)	A (1.42)	A (1.12)	E	G3	IIIC1	N1	+
7	A (1.21)	A (1.38)	A (1.25)	E	G1	IA	N0	–
8	A (2.08)	D (1.00)	D (1.00)	N	G3	IVB	NX	–
9	A (1.52)	D (1.00)	A (1.48)	N	G3	IIIC1	N1	–
10	A (1.28)	D (1.00)	D (1.00)	E	G2	IA	NX	–
11	A (1.55)	A (1.59)	D (1.00)	E	G3	IIIC1	N1	+
12	D (1.00)	A (1.28)	D (1.00)	E	G1	IB	NX	+
13	A (1.17)	D (1.00)	A (1.19)	E	G2	IB	N0	+
14	A (1.77)	D (1.00)	A (1.99)	N	G3	IVB	NX	–
15	D (1.00)	A (1.25)	D (1.00)	E	G1	IA	NX	–

A: DNA aneuploid status; D: DNA diploid status; N: non-endometrioid histological subtype; E: endometrioid histological subtype.

independent prognostic factor can help to apply adequate treatment. We believe that patients could profit from the preoperative evaluation of DNA ploidy, in particular, patients with DNA aneuploid tumours could be intended for complete surgical staging including aortopelvic lymphadenectomy even if other prognostic parameters do not indicate high risk. In contrast, patients with DNA diploid tumours with no other high-risk factors present could be spared radical surgery associated with higher morbidity.

We modified the original methods of flow cytometric DNA analysis from FFPE (9) and fresh/frozen samples (19) and adapted the technique to be optimal for the evaluation of endometrial carcinoma specimens and suitable for routine practice at our Faculty. But obviously there are some obstacles within the process of DNA ploidy evaluation in different tumour tissue samples acquired from one patient (Table V). The question is why there are cases discordant in DNA ploidy status. We repeated the flow-cytometric examination of available tissue samples in discordant cases. We can conclude the results of repeated processing and evaluating of certain tissue samples were similar, however, the discordance in results remained between tissue samples from one endometrial carcinoma patient taken at different times (curettage *vs*. hysterectomy) or by using different sampling methodology (fresh *vs*. FFPE). In our opinion, the discordance may be related to intratumoural heterogeneity. Similar discordance was observed for tumour grading, which can also vary in different parts of a tumour.

Our observation corresponds to the results documenting intratumoural heterogeneity of solid tumours, including ECA. Intratumoural variation in DNA index was observed in 24% of breast and 21% of ovarian carcinomas in the study of Kallioniemi (20). He suggests examining several samples from each tumour to account for the intratumoural heterogeneity regardless of the type of starting material. In another study comparing fresh and FFPE specimens from 30 solid neoplasms, only 26 of the results were concordant and two more were concordant after assessment of additional deparaffinized specimens, indicating DNA ploidy heterogeneity only (21). Other studies did not find significant differences between the two types of samples and the quality of histograms, in spite of mentioning the clonal heterogeneity of tumours (22). ECA heterogeneity is revealed in studies comparing results from curettage and hysterectomy specimens showing a significant number of cases with divergent DNA ploidy status. In the study of Rosenberg *et al*. (23), ECA curettage material was analyzed (four separate samples from each curettage) and 17% heterogeneity was noted. In contrast, Susini *et al*. (24) prospectively showed the same accuracy comparing preoperative and postoperative biopsies in a group of 50 patients. In the largest study group (463 patients) Baak *et al*. (25) described DNA ploidy as being prognostic in samples from hysterectomy, but not from curettage in FIGO stage I cases. In a recent study, Pradhan *et al*. (26) described that 72% DNA ploidy results were concordant in the curettage

and hysterectomy specimens only and concluded that DNA ploidy from hysterectomy specimens cannot be used interchangeably with analyses of curettage material because of intratumour heterogeneity.

Another question is whether it is more advantageous to use fresh/frozen or FFPE samples. Fresh samples are usually preferable for DNA ploidy examination by flow cytometry because sample preparation is not so time consuming and DNA histograms are generally of better quality compared to results from FFPE samples. However, acquisition and storage of the material can present both practical and logistic problems and may sometimes interfere with the demands of routine pathological evaluation. Preparation of FFPE samples is more technically demanding and more time consuming. The DNA histograms are generally of poorer quality (higher CVs, more debris). Still, there are several advantages on this method. Originally, it allows retrospective analysis of patients whose clinical outcome is already known, therefore showing prognostic significance of DNA ploidy. Additional retrospective studies are needed for these evaluation purposes. However, it is widely used for prospective evaluations in cases when fresh material is not available, or in cases of very small tumours where the lesion needs to be processed entirely for diagnostic purposes. Pretreatment specimens are also usually stored as FFPE samples only, as the diagnosis of ECA is not known at the time of the biopsy. Some authors used fresh/frozen endometrial biopsy samples for DNA ploidy evaluation as they took a further biopsy before the primary surgery (24); but a repeat endometrial biopsy could be unacceptable for some patients. Another advantage of FFPE samples is the microscopic examination of a parallel tissue section by an experienced pathologist allowing for selective sampling of a tumour-rich area (11). Our experience has led us to prefer the use of FFPE samples as they are widely accessible from all patients; we can seek out certain samples retrospectively (*e.g.* repeated curettage before developing ECA); and we can repeat the examination several times. Furthermore, there is a microscopic inspection of parallel tissue sections identifying the carcinomatous area within the analyzed tissue. A new method, laser microdissection, could be used for the selective excision of the tumour-rich part from the FFPE block in the future.

Conclusion

The search for objective and reproducible prognostic factors for patients with ECA continues. DNA ploidy appears to be an important factor predicting clinical outcome in patients with ECA which could bring additional important information on prognosis in a subgroup of otherwise low-risk patients. Based on DNA ploidy status, we can decide on more radical treatment (primary surgery, adjuvant therapy). However, heterogeneity of DNA ploidy within the same tumour has to be taken into account. We do support the opinions of those who emphasize the necessity for examining multiple tissue sections for DNA ploidy (27). Similar to evaluation of the tumour grade that can vary in different areas of tumour tissue, the DNA index may vary in different areas within a tumour. We should, thus, focus and figure on the heterogeneity of DNA ploidy when applying it in the management of patients with ECA.

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