Flow Cytometric DNA-heterogeneity in Paraffin-embedded Endometrial Cancer

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To evaluate the degree of intratumoral DNA ploidy heterogeneity in endometrial carcinoma, the authors examined curettage specimens from 30 patients with clinical stage I and II endometrial carcinoma. The curettage material was obtained before the onset of treatment. A representative sample from each tumour was chosen and a 50 μm section was cut. The paraffin block of tumor was then divided into 4 equal parts and a 50 μm section was cut from each part. All tumour samples were analysed separately by flow cytometry. DNA ploidy heterogeneity was noted in 5/29 cases (17%). In three cases DNA-aneuploid stem cell lines were found only among the 4-part sections and were not detected when the whole tumor section was analysed.

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During the last decade flow cytometric analysis of the DNA content of endometrial tumors has gained an increasing importance as an independent prognostic variable (1–4). DNA flow cytometric evaluations performed on paraffin-embedded tumor material have shown a good correlation with results obtained on fresh tissue (5, 6). However, the reliability of this method as prognostic tools could be greatly hampered in the case of intra-tumoral DNA-heterogeneity. Heterogeneity concerning morphology, growth rate, antigenicity, radio- and chemotherapy sensitivity, etc. has been demonstrated in many neoplasms, including endometrial carcinoma. In addition, variations in intra-tumoral DNA content and S-phase fraction (SPF) has been found in several types of malignancies, such as cancer of the ovary, breast, stomach, bladder and colon (5, 7–9), as well as in endometrial cancer (1). The aim of the present study was to investigate the degree of intratumoral variation of DNA index and SPF as determined by flow cytometry on paraffin-embedded endometrial cancer material. This was made by comparing DNA histograms obtained from different parts of the endometrial curettage material.

MATERIAL AND METHODS

Formalin-fixed paraffin-embedded curettage specimens from 30 patients with endometrial carcinoma in clinical stage I and II obtained before the onset of treatment were used. The median age of the patients was 65 years and the median observation time 67 months. All specimens were classified and graded by a pathologist and the results are presented in Table 1. A representative block from each tumor was chosen and a 50 μm section (whole-tumour section) was cut from each specimen. The block was then cross divided into four equal parts which were separately re-embedded and a 50 μm section from each quarter part (4-part sections) was cut. The whole- tumor and 4-part section respectively were analysed by flow cytometry on the same day. The tumor material was deparaffinized, rehydrated and trypsinized as described by Schütte et al. (10) and the nuclear suspension was stained by propidium iodide according to Vindeløv et al. (11). A FACscan flow cytometer (Becton Dickinson) equipped with a 15 mW argon laser (488 nm) was used. A minimum of 20 000 events were recorded. The SPF value was calculated by the method described by Baisch et al. (12) assuming cells in S-phase having a rectangular distribution. Thus, the SPF value was calculated by multiplying the number of channels between the $G_{0/1}$ and $G_{2/M}$ peaks by the mean number of cells per channel in the mid-S-phase region. In aneuploid tumors, the diploid $G_{0/1}$ cells contained in the tumor cause an overlap in the aneuploid region and might influence the calculated level of SPF. In order to minimize this problem an area apart from the overlapping region without cell aggregates was selected for calculation of the S-phase.

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Table 1

Histologic type, architectural grade and nuclear grade in 30 endometrial carinoma curettage specimens

Histological type	Number	Architectural grade			Nuclear grade		
		Grade 1	Grade 2	Grade 3	Grade 1	Grade 2	Grade 3
Endometrioid	20	13	1	6	4	11	5
Serous	7	0	4	3	0	0	7
Adenosquamous	2	0	0	2	0	0	2
Mucinous*	1	1	0	0	1	0	0

* excluded due to DNA-histograms of poor quality

The first peak in the histogram was considered to represent DNA-diploid cells (DNA index = 1). Histograms with an additional peak to the right of the $G_{0/1}$-peak were defined as aneuploid (13). The DNA-index for aneuploid tumors was calculated as the quotient of the $G_{0/1}$ aneuploid peak position divided by the $G_{0/1}$ DNA-diploid peak position (channel number). This definition does not allow for DNA-indices less than 1. However, in our experience hypodiploid stem cell lines are very seldom found in endometrial cancer. Internal standards was not used as there are no reliable reference cells when archival material is analyzed. Only histograms from whole-tumour sections with a coefficient of variation (CV) $\leq 8\%$ were accepted.

The interpretation of DNA-histograms was performed in a blinded fashion.

RESULTS

In one case (a mucinous carcinoma) the DNA histograms were of too poor a quality to be acceptable, and in some tumors the 4-part sections contained too little material to create acceptable histograms. However, in all tumors at least two (mean 3.4) 4-part-sections were interpretable. The median CV in histograms from whole-tumor sections was 5.1.

In five tumors the DNA histograms obtained from the whole tumor section showed a different ploidy status compared to one or several of the 4-part sections, thus revealing a DNA heterogeneity rate of 17% (5/29). In three of these cases the aneuploid stem cell line was detected only among the 4-part sections (Table 2).

The remaining 24 tumors displayed no difference in their ploidy status between the various sections. Four of these tumours were homogeneously aneuploid (14%) while the other 20 were homogeneously DNA-diploid (69%) (Tables 2 and 3).

In some histograms, however, the DNA-diploid peak displayed an assymetric shoulder which, even though it did not fulfill the criteria for aneuploidy, suggested the presence of an additional non-diploid cell population. There were 4 tumors with such a histogram from the whole-tumor section. In 2 of these a DNA-aneuploid histogram was indeed found among the 4-part sections (50%). These 4-part sections often displayed a CV > 8 (Table 2). On the other hand, among the 19 tumors with a DNA-diploid pattern without a shoulder in the whole-tumor section, only one DNA-aneuploid histogram was found among the 4-part sections from one tumor (5%). The two tumors with DNA-heterogeneity displaying an aneuploid whole-tumor section and DNA-diploidy among the 4-part sections also revealed DNA-diploid 4-part sections with shoulders (Table 4).

A significantly higher mean SPF was noted among the DNA-aneuploid histograms compared with the DNA-diploid ones (mean SPF of diploid tumors vs. mean SPF of aneuploid tumors, $p < 0.0001$).

Seven patients died of their disease during a median observation time of 67 months (15–121 months). Five of

Table 2

DNA-index (DI) and S-phase fraction (SPF) in the different samples of the five tumors that revealed DNA-heterogeneity

		DI	CV	SPF
Tumour 1	Whole part section	1.0	8.0	14%
	section 2	1.0	7.3	16%
	section 3	1.0	5.6	4%
	section 4	1.91	7.4	19%
	section 5	1.86	7.3	25%
Tumour 2	Whole part section	1.0	7.8	11%
	section 2	1.0	9.6	12%
	section 3	1.0	8.5	8%
	section 4	1.11	10.2	22%
	section 5	1.0	9.4	14%
Tumour 3	Whole part section	1.29	7.9	2%
	section 2	1.16	11.0	2%
	section 3	1.0	7.7	2%
	section 4	–	–	–
	section 5	–	–	–
Tumour 4	Whole part section	1.76	7.5	25%
	section 2	1.0	6.1	7%
	section 3	1.0	6.3	10%
	section 4	–	–	–
	section 5	–	–	–
Tumour 5	Whole part section	1.0	8.0	9%
	section 2	1.0	9.2	9%
	section 3	1.16	10.4	8%
	section 4	1.0	6.9	8%
	section 5	–	–	–

Table 3

Ploidy status of the 29 evaluable endometrial carcinomas. Five samples of each tumor were analysed (whole tumour section plus four-parts sections)

Histologic type/grade	Homogeneously DNA-diploid	Homogeneously DNA-aneuploid	Different ploidy in one or several sections
Endometroid grade 1	10	0	3
Endometroid grade 2	1	0	3
Endometroid grade 3	5	1	0
Serous	2	3	2
Adenosquamous	2	0	0
	20	4	5

these suffered from tumors with DNA-aneuploid whole-tumor sections. No cancer death was observed among the 19 patients with DNA-diploid whole part sections without shoulders. On the other hand, 2 of the 4 patients with shoulders in their DNA-diploid histograms from the whole-part section died of disease.

DISCUSSION

The mean SPF of the DNA-aneuploid tumor samples were significantly higher compared with the DNA-diploid ones but in several cases an aneuploid DNA index was accompanied by a low S-phase (<10%). Due to the small number of patients and the short follow-up time it was not possible to make any prognostic conclusions regarding the S-phase.

The present study revealed a DNA-ploidy heterogeneity in 5/29 tumors (17%). If only the whole-tumor sections had been analysed, as is usual in the daily routine work, about 10% of the aneuploid stem cell lines would have gone undetected. This figure is higher than the one reported by Ikeda et al. (1) who detected ploidy heterogeneity in 3/76 paraffin-embedded endometrial carcinomas using 3–4 tissue sections. However, their material contained a higher proportion of low grade carcinomas than in the present study and included adenocarcinomas (endometrial type) without specification of the histologic subtypes.

After examining the whole-part sections, only 6 DNA-aneuploid tumors were detected. In 3 of the 5 tumours with DNA-heterogeneity, an aneuploid cell population was found only among the 4-part-sections. Thus, by dividing the block of tumor into 4 parts and analyzing these separately, we disclosed 3 additional DNA-aneuploid cell populations and thus increased the detection rate of aneuploid cell populations by 50%.

Table 4

Ploidy status in whole-tumour sections vs. four-part sections

	Whole-tumor sections	No. of tumours with aneuploid four-part sections	No. of four-part sections examined
DNA-diploid without shoulders	19	1	73
DNA-diploid with shoulders	4	2	13
DNA-aneuploid	6	6	16

In our material there were several histograms with a shoulder. Although these histograms did not fulfill the criteria for DNA-aneuploidy, a small aneuploid population hidden within the shoulder may well have existed. This type of histogram is difficult to interpret especially in formalin-fixed tumor material, due in part to the slight increase in CV which decreases the possibility to separate adjacent peaks. The presence of shoulders in the DNA-diploid region may reflect a variation in the access of the dye, due to the different structure of the chromatin in malignant and non-neoplastic cells, or it may be due to the presence of a small aneuploid population. The latter hypothesis is, in our opinion, supported by the fact that out of 19 tumors with DNA-diploid whole-tumor sections without shoulders only one aneuploid 4-part section was found, whereas among four tumors with a DNA-diploid whole-tumor section with shoulders the 4-part sections disclosed aneuploidy in two of these tumors. Furthermore, the two cancer deaths among the patients with DNA-diploid whole-tumour sections occurred among the four patients with shoulder histograms, whereas no cancer death was observed among the 19 patients with diploid whole tumour sections without shoulders. The ominous prognosis of an aneuploid DNA histogram is further substansiated by the fact that 5 out of the 6 patients with an aneuploid whole-tumour-section died of their disease.

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