Intratumoral variations in DNA ploidy and s-phase fraction in human breast cancer

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To study intratumoral DNA ploidy heterogeneity and S-phase fraction (SPF) variability, we prospectively collected five different samples from 48 breast carcinomas and each sample was analysed separately by flow cytometry. Aneuploidy rate was 89.6% after analysis of four or five samples. DNA ploidy heterogeneity, i.e., different samples classified as either DNA euploid or DNA aneuploid in the same tumor was seen in 17%, and DNA index heterogeneity, i.e., tumor populations with different DNA indices (DIs) seen in different samples was 44%. A statistical model defining SPF heterogeneity is proposed. SPF heterogeneity as defined by us was 71%, and as expected the SPF heterogeneity rate increased significantly with increasing number of analysed samples. Four or more samples are needed to detect the most deviant (highest) SPF values. An unrecognized intratumor heterogeneity of DNA ploidy and SPF may partly explain the conflicting results reported in the literature on the above prognostic indicators.

Keywords: Breast cancer, DNA ploidy, heterogeneity, s-phase fraction, SPF

1. Introduction

A large number of studies on DNA ploidy with correlations to tumor stage and different histopathologic, cytometric and prognostic parameters have been performed in human breast cancer. In the majority of these studies only one tumor sample was analysed, and no attention was paid to possible intratumor DNA heterogeneity. The first studies on DNA heterogeneity found it to be rare, but in more recent articles higher rates have been reported, especially when four or more samples have been examined [4,6,8,12,17,19]. The prognostic power of DNA ploidy has varied considerably between different studies, a fact which to some extent may be explained by methodological problems and differences in interpretation of the DNA curves, but also to an unrecognized intratumor heterogeneity. Proliferation studies have gained large interest and SPF as determined by flow cytometry are used in clinical protocols to identify risk patients for adjuvant treatment [21]. The variation in SPF values within a tumour and its influence on prognosis has not been sufficiently evaluated. Indeed, no consensus on the definition of SPF heterogeneity has been reached.

In the present study we have analysed intratumor DNA ploidy and SPF variations in breast carcinomas of sufficient size to permit analysis from five different parts. A statistical model for evaluation of SPF heterogeneity is presented.

2. Material and methods

2.1. Patients

In a prospective study on prognostic indicators in breast cancer, specimens for histopathology, estrogen receptor, progesterone receptor, DNA ploidy and SPF analyses were collected. In 48 patients with primary non-metastatic breast cancer and a tumor size permitting further sampling of tumor tissue, a central slice was cut and divided into five pieces; one central and four quadrant pieces. Thus, five different parts from each tumor were separately analysed for DNA ploidy and SPF. Tumor characteristics are presented in Table 1.

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Table 1
Tumor characteristics of 48 breast carcinomas

	n
Tumor size (mm)	
0–20	1
20–50	42
50–200	5
Histologic grading	
highly differentiated ductal carcinoma	3
intermediately differentiated ductal carcinoma	19
poorly differentiated ductal carcinoma	25
Axillary lymph nodes	
negative	16
positive	27
not examined	5
Tumor stage	
stage I	2
stage II	41
stage III	5

2.2. Staging and histologic grading

For stage, the TNM classification of the International Union Against Cancer was used, based on histopathologic data [14]. The original histologic specimens were regraded blindly and all malignant types were graded as well differentiated, intermediately differentiated or poorly differentiated. The grading was based on the percentage of tubule formation, the degree of nuclear pleomorfism and the number of mitoses in a defined field area [10]. The original pathologists' reports were used for data on tumor size and axillary lymph node metastases.

2.3. Laboratory methods

Tumor specimens were kept frozen at -80°C until analysis. An imprint was made to assure that adequate malignant tissue was used for analysis. Cell preparation and DNA staining was performed as described by Vindeløv et al. [24]. Chicken and trout red blood cells were used as internal standards. The samples were run in an FACScan flow cytometer (Becton-Dickinson, Sunnyvale, CA, USA). The data were analyzed by the Cellfit software using the RFIT model (Becton-Dickinson). As a rule 10,000 nuclei were examined. In the histograms the peaks from the trout and chicken red blood cells were reference peaks for calculation of DNA indices (DIs) for the tumor cell peaks. The histograms were classified as euploid when only one significant G0/G1 peak was found, and as aneuploid when more than one G0/G1 peak was found. The use of reference peaks enabled us to perform a more exact classification of aneuploid samples as hypoploid clones could also be detected. When the G2/M peak was of significant size (>15%) and the $1.95 < \mathrm{DI} < 2.05$ the sample was judged as tetraploid. Hypoploid tumors had a defined tumour peak with $\mathrm{DI} < 1.0$ and multiploid tumors had more than one aneuploid population. In this study we grouped hypoploid, hyperploid and multiploid tumors in the aneuploid group if not stated otherwise. The term tumor heterogeneity has been used with different definitions. In this report we used the term DNA ploidy heterogeneity to define tumors containing both euploid and aneuploid samples. The term DNA index heterogeneity was used to define tumors with different tumor populations in different samples irrespective of the DNA ploidy classification.

SPF was calculated from the FCM histograms by a computer program (RFIT) using the mathematical method described by Baisch et al. [5]. Correction for background was performed. In diploid and homogeneously aneuploid tumors SPF analysis was successful in the majority of samples (85%). Small aneuploid tumour peaks, multiple peaks, hypoploid peaks (with a SPF impossible to distingish from the SPF from other tumor populations) and background debris made the calculations of SPF unreliable or impossible in 33% of all specimens. In each tumor, for SPF heterogeneity calculation, the SPF values from the tumor population detected most frequently was used and if more than one population with an equal number of SPF determinations was detected, the SPF values from the population with the highest DI was used.

2.4. Statistics

To define the heterogeneity of SPF in analyzed tumor specimens the accuracy of the laboratory analysis for different levels of SPF was determined. In 11 different tumors (not included in this study) with levels of SPF varying between 1.6% and 23% five analyses from the *same* preparation of tumor nuclei were performed by flow cytometry. The relationship between the SPF mean and the SPF standard deviation was found to be linear. The 95th-percentile for the range (maximum SPF − minimum SPF) with 2, 3, 4 or 5 observations from a normal distribution following the observed linear relationship was calculated. The percentiles were estimated using simulation with 10,000 runs at every SPF level between 1 to 40% (Fig. 1). Definition of SPF

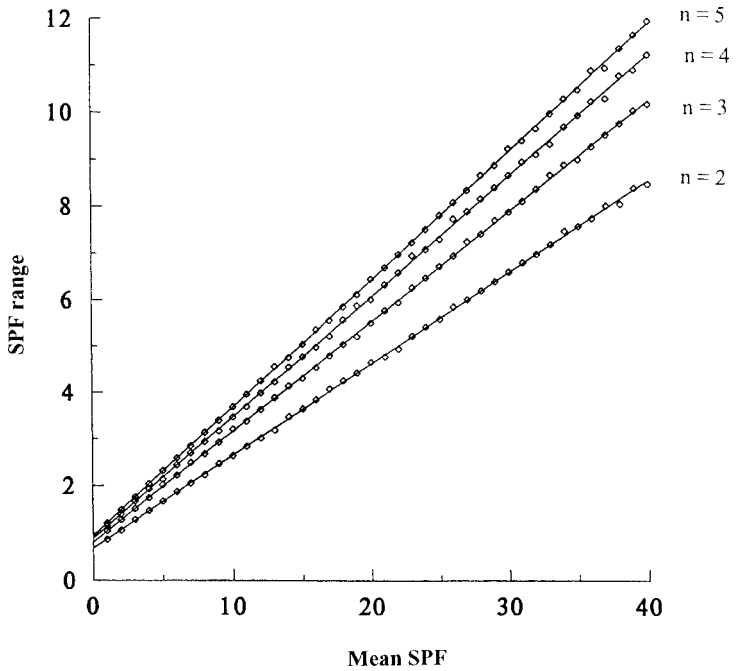

Fig. 1. The statistically calculated 95th percentile curves for linear relationship between the SPF range and mean SPF for each tumor with 2, 3, 4 or 5 observations.

heterogeneity in this study was a SPF range for the different specimens from one tumor exceeding the calculated 95th percentile for SPF range at the mean level of SPF for that specific tumor. The chi-square test was used for contingency tables. A difference was regarded significant if the p-value was less than 0.05.

3. Results

Tumor characteristics are presented in Table 1. In 41 tumors five specimens and in seven tumors four specimens were successfully analysed for DNA ploidy. Thus, 97% (233) of the analysed specimens could be classified as euploid, hypoploid, tetraploid, hyperploid or multiploid. Table 2 shows the DNA ploidy classification of all analysed samples. FCM histograms from one homogenously aneuploid and one multiploid cancer are shown in Fig. 2. Forty tumors were classified as aneuploid (83%) after analysis of the first specimen, and after analysis of the second specimen aneuploid tumor populations were detected in another three tumors with the resulting aneuploidy rate of 89.6%. Analysis of further specimens did not change the aneuploidy rate. Figure 3 demonstrates the increasing aneuploidy rate after analysis of 1–5 samples. Only in five of the 48 tumors all analysed specimens were euploid (10.4%). Hyperploid tumor specimens were found in 25 (52%), multiploid specimens in 14 (29%), homogeneuosly hypoploid specimens in two (4%) and homogeneously tetraploid specimens in two (4%) of the 48 breast cancers.

In eight breast cancers the FCM analyses resulted in both euploid and aneuploid FCM histograms in different specimens from the tumor slice resulting in a DNA ploidy heterogeneity rate of 17%. In those eight tumors the classification as DNA euploid or DNA aneuploid after analysis of one sample would depend on which sample that would have been selected for FCM analysis. The DNA ploidy heterogeneity rate increased from 13% after analysis of two samples to 17% after analysis of 4–5 samples. Further, in 13 aneuploid tumors (27%) the analyses of several samples resulted in the discovery of more than one aneuploid tumor population. Thus, examination of four or five specimens revealed a DNA index heterogeneity rate of 44% ($8 + 13/48$). The DNA index heterogeneity rate increased from 29% to 44% after analysis of 2–5 samples. Figure 3 demonstrates the increasing DNA ploidy heterogeneity rate and DNA index heterogeneity rate after analysis of 1–5 samples.

Two hundred seventy-six tumor populations were detected. Adequate SPF determinations were obtained in 186 populations, i.e., 67%, with a mean SPF of

Table 2
Results of DNA ploidy analysis of all specimens for 48 tumors. (E = euploid, Hp = hyperploid, H = hypoploid, T = tetraploid, M = multiploid)

Tumour	Specimen 1	Specimen 2	Specimen 3	Specimen 4	Specimen 5	DNA index heterogeneity
1	M	M	Hp	Hp		+
2	M	M	Hp	M		+
3	Hp	Hp	Hp	Hp	Hp	
4	M	M	M	Hp	M	+
5	E	E	E	E	E	
6	E	H	E	E	E	+
7	M	M	M	M		
8	Hp	Hp	Hp	Hp	Hp	
9	E	E	E	E	E	
10	Hp	Hp	Hp	Hp	Hp	
11	Hp	Hp	Hp	Hp	Hp	
12	Hp	Hp	Hp	Hp	Hp	
13	Hp	Hp	Hp	Hp	Hp	
14	Hp	Hp	Hp	Hp	Hp	
15	E	E	E	E		
16	Hp	Hp	Hp	Hp	Hp	
17	Hp	Hp	Hp	Hp		
18	Hp	Hp	Hp	Hp	Hp	
19	Hp	Hp	Hp	Hp	Hp	
20	M	Hp	Hp	Hp	Hp	+
21	E	T	E	T	T	+
22	Hp	Hp	Hp	Hp	M	+
23	Hp	M	M	Hp	M	+
24	Hp	Hp	Hp	Hp	Hp	
25	M	M	M	M	M	
26	Hp	Hp	Hp	E	Hp	+
27	Hp	Hp	Hp	Hp	Hp	
28	E	E	E	E	E	
29	M	M	M	M	Hp	+
30	Hp	M	Hp	Hp	Hp	+
31	E	Hp	Hp	Hp	Hp	+
32	Hp	Hp	Hp	Hp	Hp	
33	Hp	Hp	Hp	Hp	Hp	
34	Hp	M	Hp	T	T	+
35	Hp	Hp	Hp	Hp	Hp	
36	Hp	Hp	Hp	Hp	Hp	
37	H	Hp	Hp	H	H	+
38	Hp	E	E	E	E	+
39	Hp	E	Hp	Hp	Hp	+
40	M	Hp	Hp	M	Hp	+
41	T	T	E	T	T	+
42	M	Hp	Hp	Hp	Hp	+
43	Hp	E	Hp	Hp	E	+
44	Hp	Hp	Hp	Hp	Hp	
45	Hp	H	M	Hp	H	+
46	Hp	Hp	Hp	Hp	Hp	
47	Hp	Hp	Hp	Hp	Hp	
48	E	E	E	E	E	
Aneuploidy rate	83%	90%	90%	90%	90%	
DNA ploidy heterogeneity rate		13%	15%	17%	17%	
DNA index heterogeneity rate		29%	36%	40%	44%	

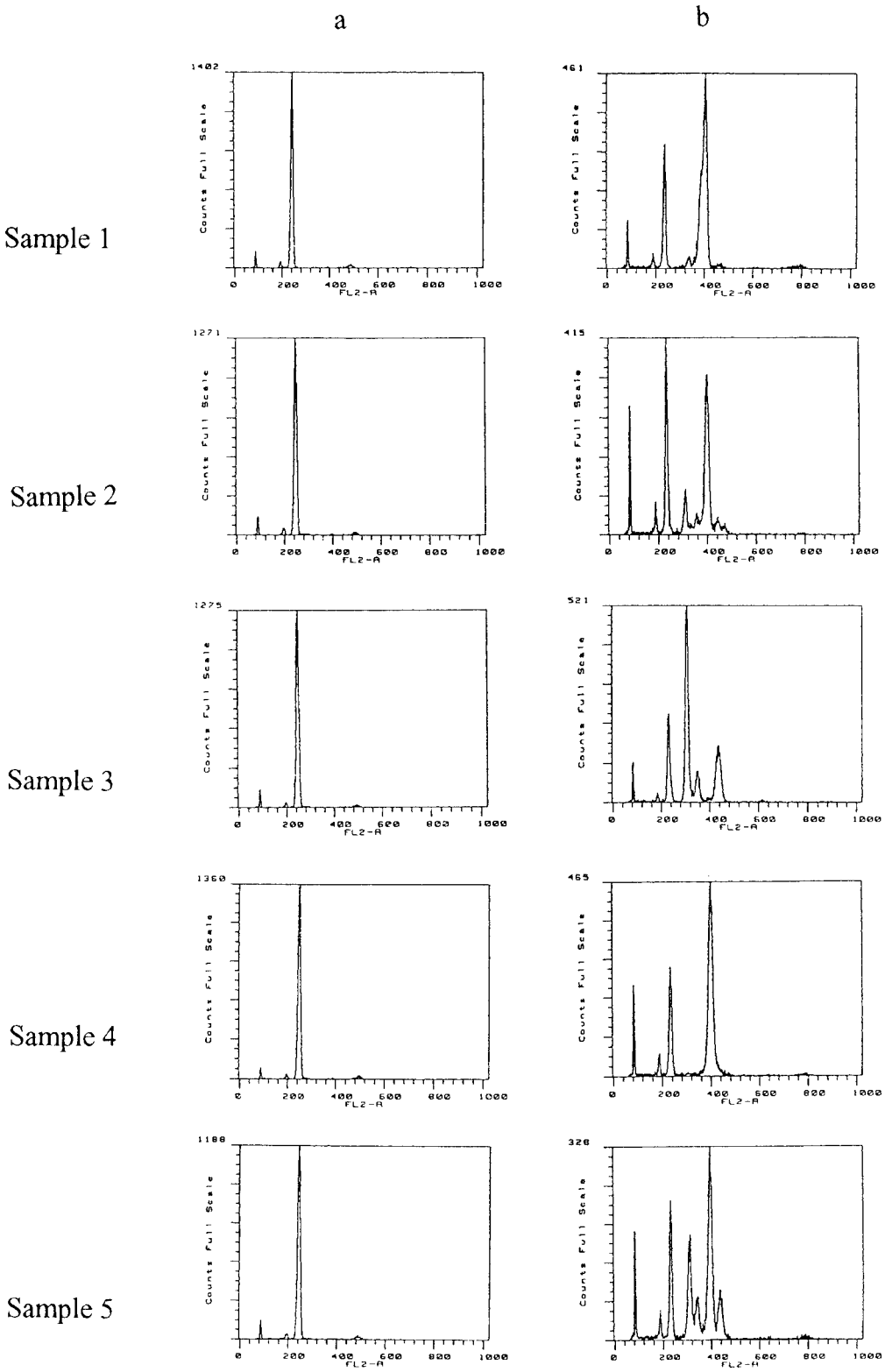

Fig. 2. FCM histograms from one homogenously euploid (a) and one multiploid (b) breast cancer.

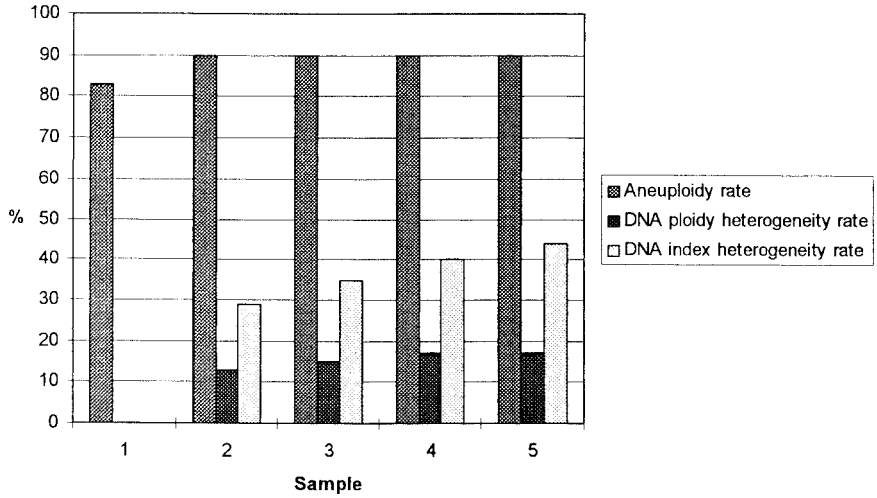

Fig. 3. Cumulative aneuploidy rate, DNA ploidy heterogeneity rate and DNA index heterogeneity rate after analysis of 1–5 samples.

9.6%, median of 8.4% and a range of 0.3–37.1%. Coefficient of variation ranged between 1.1 and 8.8% with a mean of 3.3 ± 1.4%. For hypoploid, euploid, tetraploid and hyperploid tumor populations SPF analysis was successful in 55%, 76%, 64% and 61%, respectively. Mean SPF values for the different ploidy groups were 11%, 5%, 6% and 11%. Adequate SPF determinations for the euploid or most frequent aneuploid tumor population were obtained for 41 of the 48 (85%) analysed tumors. Only SPF values for the same tumor population were compared between samples. Among those 41 tumors SPF analysis was performed in five specimens for 18, in four specimens for 12, in three specimens for three and in two specimens for eight tumors. SPF heterogeneity rate as defined by us was 71% (29/41). The SPF heterogeneity rate tended to increase with increasing number of analysed samples; in tumors with two and three analysed specimens the SPF heterogeneity rate was 38% and 67% compared to 83% and 78% in tumors with four and five analysed samples respectively. The heterogeneity rate was significantly higher when four or five samples were collected compared to only two or three ($p = 0.03$, Chi-square test).

4. Discussion

In this report mainly stage II breast cancers were analysed for DNA ploidy and SPF, since these tumors were large enough to permit analyses from five separate pieces. Since aneuploidy rate is largely independent of stage an aneuploidy rate of 89.6% was surprisingly high. In previous studies using FCM, aneuploidy rates have varied between 60% and 92%, but mostly between 60–70% [1,11,13,18,19,25]. The high aneuploidy rate could be due to technical improvement of the FCM technique giving histograms of high resolution permitting small aneuploid tumor peaks to be detected. The fact that several specimens were analysed from each tumor also contributed to the detection of more non-euploid tumor peaks. A summary of results from different studies regarding aneuploidy rate, DNA ploidy heterogeneity rate and DNA index heterogeneity rate discovered after FCM analysis of several samples is presented in Table 3. Teixeira et al. in a study of seven carcinomas by cytogenetic analysis found multiple clones in four of the tumors (57%) and interestingly the clones were unevenly distributed within the tumor mass [22]. The results from the present study with a DNA index heterogeneity of 44% and a DNA ploidy heterogeneity of 17% after analysis of 4–5 samples are well in line with previously published results.

It is obvious that analysis of multiple samples from different parts of a tumor increases the detection of non-diploid tumor populations, and thus DNA ploidy heterogeneity rate [2,3,8,9,12,17]. FCM seems to be a more sensitive tool than cytophotometry in this respect [2]. In a study of Shackney et al. different solid tumors were examined and the authors showed that 65% of the tumors had numerical chromosomal abnormalities found by karyotyping, but not detected by FCM [20]. By extensive sampling and different methods to analyse the tumor populations in breast carcinoma tissue several populations and chromosomal abnormalities may be detected. According to Beerman et al. [7] studying breast carcinomas four samples were

Table 3
Aneuploidy rate, DNA ploidy heterogeneity rate and DNA index heterogeneity rate as reported by different authors

Author	Tumors n	Samples n	Aneuploidy rate %	DNA ploidy heterogeneity rate %	DNA index heterogeneity rate %
Askensten [2]	21	3–5	67	24	–
Bergers [6]	17	6	–	53	–
Bonsing [8]	18	1–11	89	–	67
Fernö [12]	79	2	–	10	–
	13	4	69	23	–
Prey [17]	8	5–11	63	13	25
Schvimer [19]	28	3	82	14	43
Tirindelli Danesi [23]	102	not given	78	–	28
Present study	48	4–5	90	17	44

needed to permit detection of all DNA stemlines with a probability of about 90%, and according to Ljungberg et al. [16] studying renal cell carcinoma at least five samples taken at random were needed to by more than 90% probability detect an aneuploid or polyploid tumor sample. The prognostic implication of such data have not been sufficiently evaluated. The power of DNA ploidy as a prognostic indicator has been influenced by different techniques for analysis, different methods of classification and also incorrect classification as the aneuploidy rate is underestimated if only one sample is analysed.

In an earlier prospective investigation from our hospital FCM on frozen tumor tissue from breast cancer specimens in an unselected patient material showed the median SPF to be 6.6% and the mean SPF to be 7.2% (range 0.3–30.1%, $n = 126$, unpublished data). The higher values in the present study may be explained by our studying cancers of more advanced stages and using multiple samples. The problem with different SPF values in different specimens from of a tumor has been discussed by some authors [6,12,15]. There is no agreement on how to define SPF heterogeneity. We have described a statistical model based on the laboratory variation in assessment of SPF values in several samples related to the size of SPF. By our definition of SPF heterogeneity a rate of 71% was found. Kallioniemi et al. defined a difference of greater than 24% ($\pm 2 \times$ CV) between different SPF values as a true intratumor variation and this was found in 36% of breast cancers after FCM analysis of paraffin-embedded tumors [15]. Bergers et al. found wide ranges for SPF in breast cancers after analysis of up to six samples and especially among aneuploid tumors (0.0–62.7%), [6]. Fernö et al. divided SPF into three categories (0–7.0%, 7.0–11.9% and 12–100%) and after analysis of two or four samples 25% and 45% respectively showed values in different categories [12]. They also demonstrated that there was a stepwise increase in recurrence rate among patients with increasing SPF category and that patients whose SPF categories varied, from low or intermediate in one part of the tumor to high in another, seemed to have a poor prognosis. Further studies are needed to determine whether the maximal SPF value detected is the one with the greatest prognostic power.

In conclusion, the analysis of several samples from a tumor revealed high DNA aneuploidy, DNA ploidy heterogeneity and DNA index heterogeneity rates. A DNA aneuploidy rate of near 90% and a high DNA ploidy heterogeneity rate decrease the possible prognostic value of DNA ploidy analysis. SPF values showed large variations between different samples and SPF heterogeneity by our definition was 71%. The influence of the intratumoral variation of SPF on prognostication is not known, but a random sample cannot be expected to give true prognostic information. Multiple sampling seems to be necessary to detect tumor populations with non-euploid DNA pattern and to detect the population with the highest S-phase fraction. According to our study four or more samples are needed to detect the most deviant SPF values. To truely evaluate the prognostic strength of a prognostic predictor, it is essential that it represents the whole tumor and not only a part of it. In future research intratumoral variation must be thoroughly analysed before any prognostic factor is used for clinical decisions.

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