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Reproducibility of flow cytometric assessment of follicular tumours of the thyroid

Th P H VAN THIEL,* J C VAN DER LINDEN,† J P A BAAK,* M M VAN DE SANDT,† C VAN GALEN, P D BEZEMER‡

*From the *Institute of Pathology and ‡Department of Medical Statistics, Free University Hospital, Amsterdam, and †SSDZ, Department of Pathology, Delft, The Netherlands*

SUMMARY The reproducibility of the DNA index of paraffin wax sections from 44 follicular tumours of the thyroid (18 follicular adenomas and 26 follicular carcinomas), which had been assessed by flow cytometry was analysed in two laboratories, using consecutive sections of the same specimens and two different commercially available flow cytometers. Two slightly different cell preparation and staining techniques were used in the two laboratories. Using strictly defined criteria the histograms were classified blind as diploid, peritetraploid, aneuploid, or inadequate and insufficient by two independent investigators. Both the concordance between the two different flow cytometers and the agreement of duplicate assessments within the same flow cytometers were assessed. The mean coefficient of variation of the G_0/G_1 peak of the diploid tumours in the PARTEC flow cytometer was 5·5 (range 2·3–9·8) and in the FACS flow cytometer 5·2 (range 3·7–8·3); this difference was not significant. There was concordance of classification between the two laboratories in 35 of 36 cases. In 25 cases (18 diploid, seven aneuploid) the intralaboratory variation showed a 100% concordance in histogram classification.

It is concluded that flow cytometer DNA index assessment of follicular tumours of the thyroid is reproducible and can be used to evaluate the discriminating and prognostic value of this feature.

There is some evidence to suggest that in thyroid tumours there is a correlation between nuclear DNA content and biological behaviour.[1–4] The DNA index may allow a distinction to be made between clinically indolent tumours and aggressive carcinomas by the degree of ploidy abnormality.[5] Diploid malignant tumours are generally associated with a better prognosis than aneuploid ones.[6 7] Nevertheless, the diagnostic and prognostic value of DNA cytometric measurements is still controversial. Malignant thyroid tumours with a DNA content in the diploid range have been observed, and, in fact, diploid malignant tumours can be as invasive and metastatic as aneuploid ones.[6 7] On the other hand, aneuploidy has been reported in benign thyroid lesions[8] and to explain this it has been suggested that adenomas with aneuploidy might be in malignant transformation.[8] Another explanation for these conflicting data could be that the flow cytometric assessments of nuclear DNA content are not always perfectly reproducible, either as a result of differences in sampling,[5] cell preparations, equipment used, or histogram classifications.

The lack of methodological studies and the possible discrepancies in results[6] required a closer analysis of the reproducibility of flow cytometric measurements of DNA content for tumours of the thyroid.

Material and methods

Tissue specimens from 18 follicular adenomas and 26 follicular carcinomas were analysed by two observers on two different flow cytometers. Neither the technicians who performed the flow cytometer measurements nor the pathologists classifying the DNA histograms were aware of the histopathological diagnosis and the results of flow cytometric measurements in the other laboratory. Furthermore, from 25 patients two samples of the same tumour section were processed and analysed independently on the FACS flow cytometer. The results were statistically analysed and compared.

All 44 patients had undergone surgery for thyroid tumours between 1975 and 1985. Tissues were samples

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and fixed in 4–5% buffered formalin because many quantitative microscopic features remain stable at that concentration.[9,10] Histopathological diagnoses were made on sections from paraffin wax blocks that contained the worst differentiated areas of the tumour. The sections were stained with haematoxylin and eosin. Consecutive thick (50 or 30 μm) and 5 μm sections were cut. Flow cytometric analyses were done on the thick sections and the 5 μm stained slides served to control the tumour cell content of the thick sections.

The flow cytometric samples were analysed in two laboratories using different (mercury lamp) flow cytometers (PAS-II flow cytometer, PARTEC Instruments, Arlesheim, Switzerland, and FACS-analyser, Becton Dickinson, Heidelberg, West Germany). In one laboratory RNAse treatment was used to ensure that the propidium iodide (50 mg/ml) staining was specific for DNA; the other laboratory used DAPI because of the colour specificity of the DNA staining.

In the first laboratory (Institute of Pathology, Free University, Amsterdam) the nuclei suspensions were made from 50 μm sections according to a method described by Hedley *et al.*[1] The sections were centrifuged in 10 ml tubes and dewaxed in 6 ml xylene for 15 minutes at room temperature. Rehydration was performed in an ethanol sequence of 100%, 96%, 70% and 50% (10 minutes for each step). Inbetween centrifugation and decantation of the supernatant were done. The cells were then washed in 5 ml phosphate buffered saline (PBS), pH 7·4. Three ml of 0·05% protease (Sigma Chemical Company, Saint Louis, ISA; P-5255 10 U/mg, type 7) was added and the tubes were incubated for 30 minutes at 37°C, with intermittent vortex mixing. The reaction was stopped with 6 ml ice-cold PBS and washed again. Mechanical dispersion was performed with a capillary pipette; filtration of the sample occurred through a 50 μm nylon gauze. Finally, 2 ml of 0·02% 4', 6 diamidino-2-phenyl-indole dihydrochlorider (DAPI, Sigma, D-1388, 100 mg) was added to the nuclei (residue) and further dispersion into a single nuclei suspension was obtained with a spinal needle (Becton Dickinson, Heidelberg, West Germany; 20 G 3·5). All samples were analysed within three hours of DAPI staining on the PARTEC flow cytometer.

In the other laboratory (Department of Pathology, SSDZ, Delft) two 30 μm sections were deparaffinised twice in xylene for two hours, rehydrated in a sequence of 100%, 95%, 70% and 50% of ethanol for 10 minutes each. The material was washed twice in PBS, resuspended in 1 ml 0·5% pepsin in 0·9% saline (pH 1·5 with 2N hydrochloric acid), incubated for one hour at 37°C with intermittent vigourous vortexing. The nuclein were then filtered through a nylon mesh (40 μm), centrifuged for five minutes at 400 × g and the pellet resuspended twice in PBS and centrifuged for five minutes at 400 × g. The cells were stained with propidium iodine (50 μg/ml), and RNAse (0·25 mg/ml) was added. After 10 minutes of incubation at 37°C 100 μl 0·9% saline was added and the samples vortexed. The material was measured within three hours on the FACS analyser.

Unaware of the initial histological diagnosis, two independent investigators (TT and JL) classified the histograms using the following criteria. According to Hedley *et al*[11] and Tribukait[12] the assumption was made that the left (first) peak belonged to diploid cells (G_0/G_1). These cells were either diploid tumour cells, normal thyroid cells, or fibroblasts. Therefore, a diploid tumour showed only one G_0/G_1 peak in the expected range. Peritetraploid tumours had a G_2/M peak more than 10% of the height of the G_0/G_1 peak and showed a clear tail at the right of the G_2/M peak. Aneuploid tumours, however, showed an additional peak. Tumours were classified as multiploid when there was more than one aneuploid peak.

The coefficient of variation (CV) was defined as the ratio of the half width (2 × standard deviation) at 61% of the height of the G_0/G_1 peak to the value of the G_0/G_1 peak on the abscissa. The CV of the G_1 peak in all the measured samples ranged from 2·3 to 13·3% (mean 6·1).

Statistical analyses included single data description and frequency distribution.[13] Agreement was assessed with the weighted κ statistic.

Results

The cv of the G_0/G_1 peak of the diploid tumours was 5·5 (mean) with a range of 2·3–9·8 on the PARTEC and 5·2 (mean) with a range of 3·7–8·3 on the FACS flow cytometer. This difference was not significant. In the interlaboratory study of 44 follicular tumours of the thyroid, eight histograms of the same tumours were inadequate (CV of >10) or insufficient for evaluation in both laboratories. Of the remaining 36, an agreement in classification was present in 33 patients of which 25 were diploid, three were peritetraploid, and five were aneuploid (according to the classification of the first laboratory). Three cases were classified differently. Of these histograms, in two cases discrepancy was based at review on errors in interpretation of the histograms of the FACS analyser. One FACS histogram was erroneously classified as diploid but was in fact peritetraploid (fig 1), and one other "diploid" FACS histogram was in fact an aneuploid tumour (fig 2). One, classified as peritetrapoloid on the PARTEC (DNA = 1·99; CV = 8·9), was aneuploid on the FACS (DNA = 1·75; CV = 4·6) (fig 3).

The results of the 36 samples are summarised in

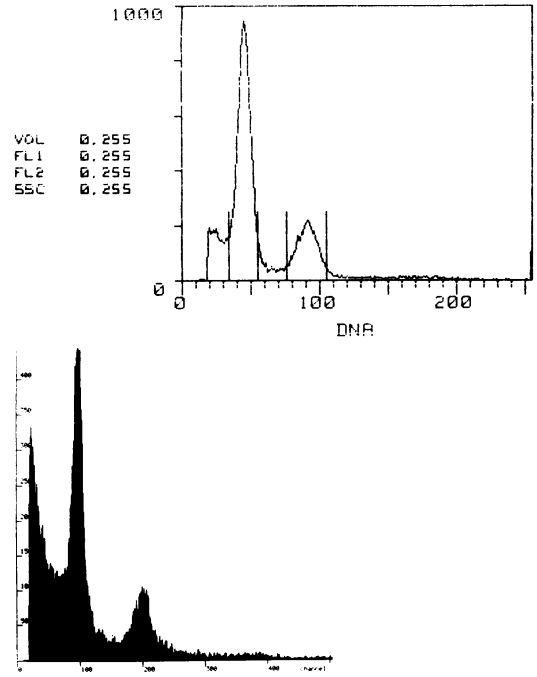

Fig 1 *Peritetraploid histogram erroneously classified as diploid.*

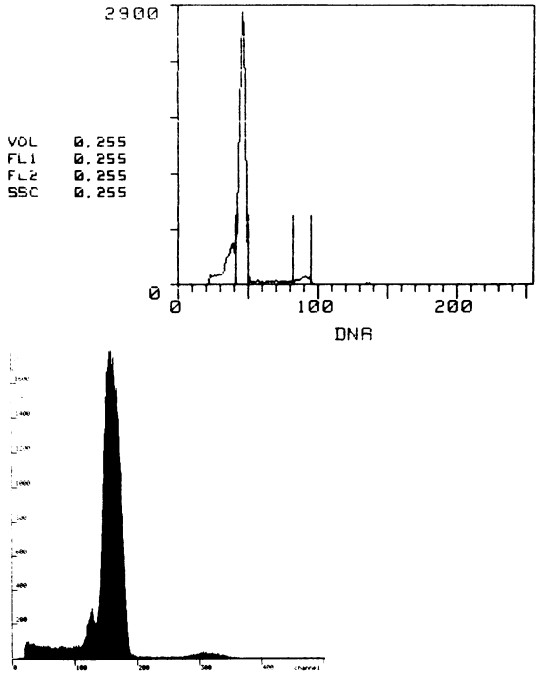

Fig 2 *Aneuploid tumour misclassified as diploid.*

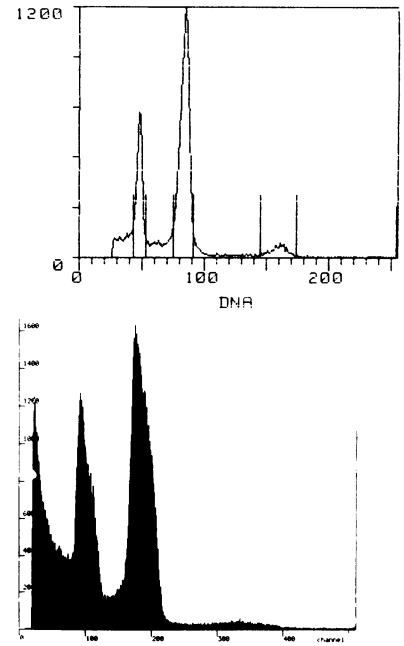

Fig 3 *True difference: aneuploid tumour on FACS (DNA = 1·75; CV = 4·6) and peritetraploid on PARTEC (DNA = 1·99; CV = 8·9).*

table 1 (overall agreement 33/36 = 91%, weighted κ 0·84). Thus both measurements of two different observers on two different instruments were perfectly reproducible. In the intralaboratory reproducibility study 18 of the 25 random samples were regarded as diploid and seven as aneuploid in both measurements. The results are shown in table 2 (classification concordance 100%).

Discussion

Both inter- and intralaboratory results show a strong concordance (inter 91%, intra 100%). Differences included one peritetraploid and one aneuploid tumour on the PARTEC which were classified as diploid due

Table 1 *Interlaboratory variation flow cytometry DNA index on 36 randomised samples of follicular tumours of thyroid using FACS and PARTEC flow cytometers*

	FACS			
PARTEC	*Diploid*	*Tetraploid*	*Aneuploid*	*Total*
Diploid	25			25
Tetraploid	1	3	1	5
Aneuploid	1		5	6
Total	27	3	6	36

Weighted κ statistic 0·84 (range: 0·65–1·00).

Table 2 *Interlaboratory variation DNA index on 25 randomised samples of follicular tumours of thyroid using FACS flow cytometer*

	Measurement 1			
Measurement 2	*Diploid*	*Tetraploid*	*Aneuploid*	*Total*
Diploid	18		0	18
Tetraploid				
Aneuploid	0		7	7
Total	18		7	25

to errors in interpretation of the histograms obtained with the FACS analyser. This failure was partly caused by a minimal difference in the percentage of cells in the G_2M peak. Furthermore, the observers found the histograms produced by the PARTEC flow cytometer easier to interpret. Despite selected and objective criteria used the true interpretation of histograms of the same samples on two different analysers remains a problem. More objective classification rules should be outlined.

The other case remained a "true" discrepancy (aneuploidy on the FACS classified as peritetraploidy on the PARTEC). Sampling differences were the most likely cause. The CV of the G_0/G_1 peaks in the two histograms obtained with both flow cytometers (PARTEC 8·9; FACS 4·6), however, support the need for further refinement of the interpretation "rules".

In conclusion, we feel that the concordances in measurements of DNA content between the two laboratories is acceptably high, although there is a need for more objective criteria for histogram analyses. Furthermore, multicentre studies should be encouraged by the possibility of using different and compatible "methods". Evaluation of the diagnostic and prognostic importance of DNA content in follicular tumours of the thyroids which have been assessed by flow cytometry is currently being undertaken.

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Requests for reprints to: J P A Baak, Professor of Pathology, Institute of Pathology, Free University Hospital, De Boelelaan 1117, 1007 MB Amsterdam, The Netherlands.