The tumour volume and histological tumour grade (6, 12, 26, 34) have been shown to correlate with tumour stage and prognosis (28, 30). However, the correlation to tumour stage has been established only in lesions harbouring a low or high grade malignancy and, above all, in more advanced lesions. The characterization of prognostically important features in localized moderately differentiated tumours needs to be improved.

The assessment of DNA-ploidy in prostatic cancer has been shown to provide prognostic information (3, 19, 23, 24, 31, 35, 40). However, a substantial difficulty associated with this technique is the reported heterogeneity in the DNA-ploidy of prostatic cancer (13, 19).

The aims of this study were to compare the DNA patterns of prostatic intraepithelial neoplasia (PIN) and invasive cancer (IC) in preoperative biopsies with those of multiple postoperative samples taken from total prostatectomy specimens and to assess whether DNA-ploidy aberrations due to a combination of heterogeneity and limited sampling efforts are a possible source of underestimation. The intention was also to correlate DNA ploidy to pT stage, seminal vesicle invasion, histological grade and tumour volume in a consecutive group of patients who underwent total prostatectomy.

MATERIAL AND METHODS

Patients and preoperative diagnostic and surgical procedures

Fifty-four consecutive patients, mean age 63 years (range 55–72) with WHO grade 1–2, clinical stage $T0_d$-2, N0, M0 prostatic cancer, underwent total retropubic prostatectomy. The preoperative diagnosis of the primary tumour was performed by means of standard procedures, i.e. 1–3 fine-needle aspirates in 35 of the cases, 1–3 core biopsies (1.2 × 20 mm, Biopty^R) in 37 cases, a combination of both in 23 cases and by means of transurethral resection (TUR) (incidental carcinomas) in 5 cases. All the histological and cytological specimens were reviewed blindly by two pathologists (CB, MdT). Preoperative staging included an analysis of serum levels of prostate acid phosphatases (PAP) with an upper limit of 150% of normal value, a bone scan and a chest X-ray. Surgical lymph node staging was carried out in all cases. The patients were operated with Walsh technique (39).

DNA ploidy analysis was performed as a single cell analysis of the fine-needle aspirates and by flow cytometry of the material obtained from the core biopsies, as described below. The most aberrant ploidy pattern was considered diagnostic.

Handling of the surgical specimens and morphological techniques

The specimens (prostate including seminal vesicles) were completely covered with Indian ink for the purpose of delineating the surgical margins and fixed in a 10% buffered formalin solution for 4–5 days. They were then step-sectioned at 5 mm intervals, directed perpendicularly to the rectal surface. The apical slice was cut twice in a sagittal projection. Thereafter, 6–11 slices were obtained from each gland. After paraffin embedding and whole section mounting, 4 μm sections stained with Weigert-van Gieson were examined and the extent of the IC and PIN areas outlined. The total volume of IC, the volume of the largest single IC focus and the volume of PIN in each specimen were calculated utilizing computerized planimetry (Epsilon Image work-station, Imtec, Uppsala, Sweden). The areas were assumed to have the same thickness throughout the entire 5 mm of the slices. The calculated volumes of PIN and IC were then added in order to obtain their respective total volumes. The volumes thus obtained were multiplied by a factor of 1.22 (27) in order to compensate for tissue shrinkage during processing. The accuracy of the shrinkage factor was confirmed in 5 randomly chosen samples from our own material. The WHO system was used for grading and the UICC 1978 system for pT-staging (38). A pT_3 stage was defined as that in which cancer cells extended throughout the entire thickness of the capsule.

DNA-measurements

Preoperative biopsies from fine-needle aspirates stained with May-Grünwald-Giemsa were used for the single cell cytophotometric DNA analysis. After destaining in absolute methanol (99.8%) overnight, the cells were refixed in 10% neutral formalin and stained according to the Feulgen procedure (9). The criteria for di-, tetra- and aneuploidy were the same as those defined by Forsslund and Zetterberg (9); i.e. the diploid region with an upper limit of 2.5c; the tetraploid, 4c region; 3.5–4.5c. The modal value was defined as the most frequent c value using a class of 0.5c. Cells outside and between the 2c and 4c region, i.e. the non-2c and non-4c cells were considered aneuploid. Single cell cytometry was performed with an image analysis technique using the Excell DNA Measurement programme on the Epsilon work-station developed by Imtec, Uppsala, Sweden.

In the histological sections, the number of specimens taken for DNA analysis was determined by the estimated IC and PIN volumes and their distribution within the gland. Thus, an average of 1 sample per

Table I. *Comparison of pre- and postoperative DNA ploidy*

		Ploidy in preoperative biopsies		
		D	ND	Total
Ploidy in prostatectomy specimens	D	24	4	28
	ND	8	13	21
	Total	32	17	49

Sensitivity 62%, specificity 86%, positive predictive value 76%, negative predictive value 75%.

Table II. *Capsular penetration related to DNA ploidy in prostatectomy specimens*

pT stage	Diploid	Non-diploid	Total
< pT 3	13	1	14
⩾ pT 3	17	23	40
Total	30	24	54

cc of cancer was taken. Samples were obtained from the main tumour, from separate lesions on the ipsi- and contralateral side of the gland and from PIN areas.

Thus, an average of 8 (1–15) samples were obtained from the marked IC and PIN areas in the paraffin blocks and prepared for flow-cytometric DNA ploidy analysis. One case, from which only one single sample was taken, had the smallest tumour volume in the whole series (0, 11 cc).

The above-mentioned samples and those taken from core needle biopsies were processed for DNA flow cytometric analysis with a technique modified from that described by Hedley and co-workers (15, 20). Sections with a thickness of 50 μm were deparaffinated in xylene, rehydrated and subjected to protease digestion (Sigma, St. Louis, MO, USA). The nuclear suspension was stained with propidium iodide. The cellular DNA content was measured in a Leitz MPV flow cytometer. In each sample, an average of 10^4 nuclei were analyzed. The limit for the coefficient of variation (CV) in the ploidy peaks was set at 10% and samples with higher values were excluded. A total of 308 samples from IC foci in total prostatectomy specimens were considered evaluable and 24 were excluded. Excluded samples were evenly distributed among the preoperative tumour grades and volumes. The DNA pattern was considered non-diploid if the 4c peak exceeded 10% of the total number of processed nuclei or if a peak appeared at other points than 2c and 4c.

Statistics

Statistical analysis was carried out on a MacIntosh computer with the JMP software programme. The Student's unpaired *t*-test was used to calculate confidence intervals and the chi^2-test to test for differences in distribution between the groups. *p*-values of less than 0.05 were considered statistically significant.

RESULTS

Validity of preoperative biopsies (Table I)

Preoperative biopsies revealing non-diploid DNA patterns had a sensitivity of 62% and a specificity of 86%, when compared with multiple sampling from the prostatectomy specimens. In other words, a limited preoperative biopsy entails a risk of underestimating the chromosomal aberration in the prostatic malignancy. A comparison of the DNA contents in fine-needle aspirates with those of core biopsies will be described in a future communication.

DNA-heterogeneity in the total prostatectomy specimens

Non-diploid DNA samples were found in 24 cases of 54 (44%). In all of these 24 cases, diploid cancer was also present. The largest single tumour focus contained both tetraploid and diploid DNA. In the glands with tetraploid DNA patterns, an average ratio of 2 diploid samples to 1 tetraploid was found. Thirty-four of 44 (77%) DNA samples from capsular penetration areas were diploid (Table 4). Twenty-three of 24 (96%) tumours with tetraploid areas penetrated the capsule compared with 17 of the 30 (57%) diploid tumours ($p < 0.0001$) (Table II). If only grade 2 tumours were taken into consideration, all 21 non-diploid tumours penetrated the capsule whereas this was the case

Table III. *G2 tumours (WHO). DNA ploidy compared with capsular penetration (n = 40)*

Tumour stage	Diploid	Non-diploid	Total
< pT 3	5	0	5
⩾ pT 3	14	21	35
Total	19	21	40

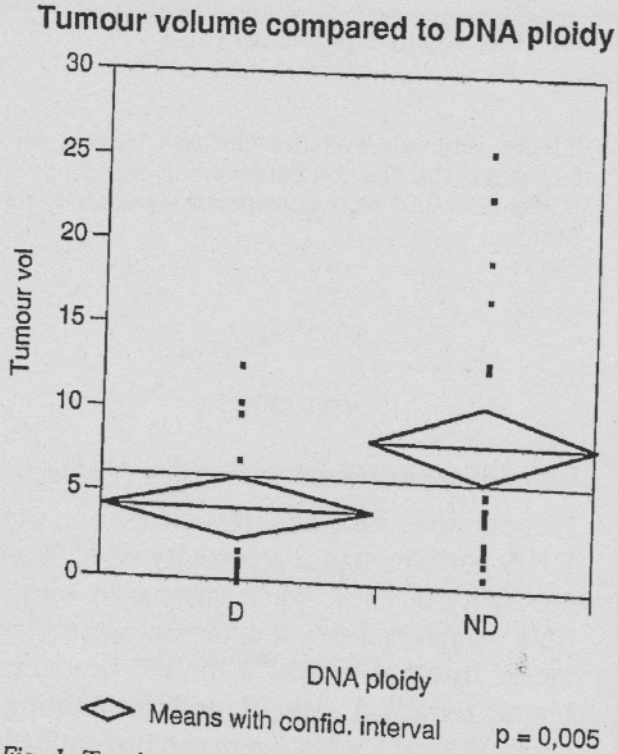

Fig. 1. Total tumour volume (the sum of all foci in multifocal cases) related to DNA ploidy.

in 14 of 19 diploid tumours (74%) (Table III) ($p < 0.0001$). Nine of the 12 tumours (75%) invading the seminal vesicles were non-diploid. Tetraploid tumours had a significantly larger volume (average 9.95 cc) than diploid tumours (average 4.79 cc) ($p < 0.005$) (Fig. 1). All 8 tumours exceeding a volume of 12 cc:s penetrated the capsule and 7 (87%) had non-diploid components. Two of 4 (50%) tumours measuring 8–12 cc:s were non-diploid. The 10 smallest tumours, 1.5 cc or less, were diploid in all but one case (90%) and 7 (70%) of them were entirely intracapsular (Fig. 2*a* and *b*). All of the PIN samples displayed completely diploid DNA patterns (36). Eleven tumours were grade 1, forty were grade 2 and three were grade 3 according to WHO grade criteria. The non-diploid tumours had a statistically significant higher grade than the diploid ones ($p = 0.0001$) (Table V).

DISCUSSION

Methods

Two methods have been used for the preoperative assessment of DNA ploidy; single cell cytometry of fine-needle aspirates and flow cytometry of core biopsies. In the operative specimens, only flow cytometry was performed. In cases where single cell cytometry was used to assess fine-needle aspirates, a few cases displayed aneuploidy. However, tetraploid cell lines were the most aberrant to be found in the operative specimens and/or in the core biopsies taken from cases where both biopsy types had been available. This discrepancy may be explained by the fact that the few aneuploid cells present are difficult to observe among all the normal or diploid malignant cells when the flow cytometric method is used (29). There exists a possibility that the high CV values may mask near-diploid or near-tetraploid aneuploid cells (8, 29). Because of this all the samples have been classified into diploid and non-

Table IV. *DNA-ploidy from different locations in the specimens*

DNA ploidy	Diploid	Non-diploid	Total
Sample site			
Largest tumour focus	169	51	220
Other synchronous tumour foci	42	2	44
Capsular penetration	34	10	44
Total	245	63	308

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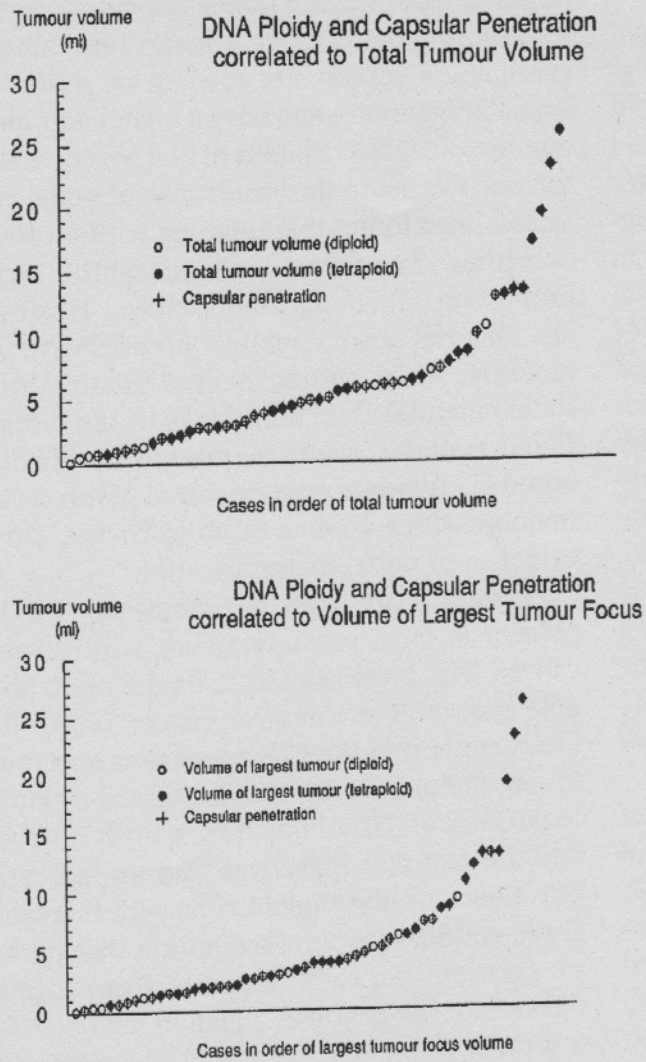

Fig. 2. (*a*) On the horizontal axis are shown individual cases in order of increasing total tumour volume. Filled boxes represent tetraploid tumours. Cases with capsular penetration are marked with a cross. (*b*) Tumour volumes of the largest single tumour focus in order of increasing volume.

Table V. *Tumour grade compared to DNA ploidy* ($n = 54$)

Grade	Diploid	Non-diploid
1	11	
2	19	21
3		3

$p < 0.0001$.

diploid, although, strictly speaking it could be considered more correct to classify them into near-diploid and near-tetraploid. Flow cytometry, when compared with single cell cytometry, may require multiple samples in order to reveal scanty non-diploid components (8). In the future, Hedley techniques may have to be further refined and both flow and static cytometry combined in order may increase the specificity of DNA measurements.

When assessing fine-needle aspirates for the purposes of grading one of the parameters classified is the tendency to dissociate, which is more pronounced with higher malignancy grades. One may assume as the tumour becomes more poorly differentiated, more aneuploid cells can be sampled by means of fine-needle aspiration. However, this assumption requires further study. The technique at present used for flow cytometry, i.e. paraffin embedded specimens is likely to be ineffectual in the detection of non-diploid cells, mainly because of high coefficients of variation. This limitation concerns both the core biopsy samples and the total prostatectomy specimens. This does not mean that the statements about both the DNA heterogeneity in the whole specimens and tendency towards an underestimation of aberrant DNA ploidy patterns in preoperative biopsies are invalid.

Results

This study revealed a heterogenous distribution of DNA-ploidy in well and moderately differentiated localized prostatic carcinoma. Preoperative routine sampling using 1–2 fine-needle and/or core biopsies from the main palpable tumour in order to obtain a cancer diagnosis proved to be inadequate, since the method only detected aberrant DNA-patterns with a sensitivity of 62%. Heterogeneity was observed, not only in the total mass of tumour in each specific case, but also within the largest single focus and also when separate foci were compared. On average, 2 diploid samples to 1 non-diploid were found in glands with one or more non-diploid DNA cell line. These results indicate that, if only one DNA analysis per specimen is undertaken, the chance of finding non-diploid cell lines would not be higher than approximately 30%. This may explain why other investigators (3, 13) have found that

diploid tumours penetrate the capsule as frequently as non-diploid ones.

In our material, tumours with non-diploid foci displayed capsular penetration to a significantly higher degree than the purely diploid ones. In fact, all but one of the non-diploid tumours penetrated the capsule. This indicates that DNA ploidy measurement has good prognostic value. Non-diploid foci were observed in 9/12 (75%) cancers that involved the seminal vesicles. However, interestingly, only 10/44 (22%) of the samples taken from the actual capsular penetration areas were non-diploid. This puzzling finding is hard to explain. In this series, 57% of the purely diploid cancers penetrated the capsule and yet non-diploidy signalled an even more frequent penetration, indicating that there may be a difference in the biological behaviour of different cell lines in a heterogenous tumour. The possibility that the proliferative capacity or some other property present in the diploid components of the two lesions may differ remains to be explored.

Several authors have proven the value of DNA ploidy analysis in the prognostication of prostatic cancer (3, 11, 19, 21, 23, 31, 35, 37, 40). Nativ et al. (24) published a retrospective analysis of 146 cases of totally prostatectomized G1-3 pT_3 tumours and found a good correlation between DNA ploidy and prognosis. Adolfsson et al. (1) found a similar correlation as regards low grade, low stage diseases first treated when symptoms of progression occurred. In a recent material published by Forsslund et al. (10), it has been demonstrated that DNA tetraploidy did not have as great an impact on prognosis as that of true aneuploidy and that there was no significant difference in survival when tetraploid tumours were compared with diploid ones.

Tumour volume has been shown to correlate fairly well to stage in low and high grade tumours. In cancers with intermediate grades, the correlation has not been as impressive (3, 17, 19, 26, 28, 30). It appears from this study that most tumours with a volume exceeding 8 cc:s are non-diploid, whereas most tumours with a volume less than this are diploid. The calculated volume in this study is the sum of the volumes of all the tumour foci, not just that of the largest one. However, even if only the largest tumour focus is taken into consideration the same applies. Thus, it appears that tumours exceeding a certain size tend to be more biologically aggressive and have a higher content of non-diploid DNA. Stamey et al. (34) correlated tumour volume to the occurrence of nodal metastases, and found that tumours with a volume exceeding 3 cc:s had a significantly higher proportion of nodal metastases. However, his material also contains preoperative G3 tumours, which should have a greater metastatic potential at an early stage of the disease. In our material, where no preoperatively diagnosed G3 tumours were operated, even certain tumours with a volume of up to 26 cc:s had no evidence of nodal metastasization.

Prostatic intraepithelial neoplasia (PIN) is present in most prostate glands with invasive cancer. PIN is considered to be the main probable precursor of invasive cancer (4, 5). The DNA content in the PIN specimens was found to be diploid in all the examined samples, regardless of IC ploidy (36). Both this fact and the fact that there was also an increasing prevalence of non-diploid cells with increasing grade and size supports the notion that tumour progression depends on the focal destabilization of the genome, which in turn provides a growth advantage for the clones with more aberrant DNA ploidy patterns.

We conclude that the non-diploid DNA content of prostatic cancer cells is a strong predictor of tumour size and capsular penetration and hence possibly of biological aggressiveness. Therefore, a preoperative measurement of DNA ploidy may be able to yield important information about the malignancy and stage of the prostatic carcinoma, provided measures are taken to optimize the representativity of the samples taken to assess the true properties of the tumour. Our experience of mapping for the purposes of histological diagnosis and grading indicates that the DNA assessment may be rendered more accurate by multiple sampling (Häggman et al., unpublished observations).

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