SGML

Virchows Archiv (1994) 424:647–651

ORIGINAL ARTICLE

J. G. Müller · S. Demel · M. P. Wirth · A. Manseck
H. G. W. Frohmüller · H. A. Müller

DNA-ploidy, G2M-fractions and prognosis of stages B and C prostate carcinoma

Received: 17 May 1993 / Accepted: 29 March 1994

Abstract Paraffin embedded tissue of 49 stage C and 27 stage B prostate adenocarcinomas was investigated by flow cytometry. All patients were treated by radical prostatectomy with pelvic lymphadenectomy and followed up for 5–10 years. The tumour was separated from the benign tissue to increase the proportion of tumour cells. Ten stage C and seven stage B carcinomas had to be excluded because of poor fixation. Six of the 39 (15%) stage C and 1/20 (5%) stage B carcinomas were aneuploid. Cell cycle analysis was done with correction for sliced nuclei and background subtraction. The threshold between carcinomas with low and with increased ("tetraploid") G2M-fraction was determined by comparing carcinomas with and without tumour progression. Sixty-seven percent of the patients with non-euploid stage C carcinomas and 11% of those with euploid carcinomas suffered from tumour progression ($P<0.01$). The respective values for the stage B carcinomas were 67% and 6% ($P<0.01$). These results demonstrate the strong prognostic impact of DNA-ploidy and G2M-fractions for each individual patient.

Key words Prostate carcinoma · Radical prostatectomy
Flow cytometry, DNA-ploidy · Prognosis

Introduction

The prognosis of a patient with prostate carcinoma is currently best predicted by the pathological tumour stage. The majority of patients with stage A carcinoma have a normal life expectancy, but most patient with stage D tumours will die from their carcinoma. However, there is a marked heterogeneity in each stage. In stage C carcinoma, 10 years survival rates following radical retropubic prostatectomy and bilateral pelvic lymphadenectomy are reported to reach about 55% [12, 28]. Variables selecting for the remaining 45% of patients are urgently needed as these patients will suffer from disease progression and might benefit from further therapy.

Since the first studies of DNA-ploidy of prostate carcinomas using static cytometry [34] a number of flow-cytometric investigations have documented the significant prognostic value of DNA-ploidy. This was confirmed by using fresh material for flow cytometry [11, 35] or for static cytometry [2, 10, 30]. The additional determination of the G2M-fraction [17, 27, 29] was reported to increase the sensitivity. In all these studies it was shown that the rate of aneuploid or "tetraploid" carcinomas increased with tumour stage, but there are few studies with an accurate stage-related evaluation of ploidy, G2M-fraction and prognosis. Reports from the Mayo Clinic for stage B, C and D1 carcinomas showed that the determination of DNA-ploidy and G2M-fraction are especially useful in predicting individual prognosis [24, 26, 36]. Therefore, we investigated our stage C as well as stage B cases with known follow-up, using 5–10 year old paraffin embedded archival material [37]. The technique of the separation of the neoplastic tissue from the benign surroundings tissue and the dissoziation technique of the tumour tissue was optimized to increase the proportion of tumour cell nuclei and to reduce the amount of the euploid non-tumour cells within the final suspension. The sensitivity and specificity of the flow cytometric parameters for the "prediction" of a recurrence were calculated.

Materials and methods

Forty-nine patients with stage C carcinoma and 27 with stage B carcinoma, operated on between 1 January 1980 and 31 Dezember 1985, were evaluated. Ten cases of stage C and seven stage B had uninterpretable DNA-histograms because of poor fixation. Tumour tissue of 59 patients (39 stage C and 20 stage B) with a mean age of 60.7 years (range 39–73 years) was analysed (Table 1).

J. G. Müller (✉) · S. Demel · H. A. Müller
Institute of Pathology, University of Würzburg,
Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany

M. P. Wirth · A. Manseck · H. G. W. Frohmüller
Department of Urology, University of Würzburg,
Würzburg, Germany

Table 1 The flow cytometric results of 38 of the 39 stage C and of the 20 stage B prostate carcinomas in relation to pathologic tumor stage and outcome as compared to the controls. One patient with stage C disease was lost to follow up and therefore not included in this table

Stage	Outcome	Number of cases	Euploid	G2M>10% ("tetraploid")	Aneuploid
C	No recurrence	29	27	0	2
	Recurrence	9	3	3	3
B	No recurrence	17	17	0	0
	Recurrence	3	1	1	1
Controls		36	36	0	0

By serial whole mount sections of the in-toto formalin fixed prostate specimens the tumour stage was determined using the TNM system of 1978. Grading was done according to Müller [25]. Metastases at the time of surgery were ruled out by routine histological investigation of the pelvic and parailiac lymph nodes and by a preoperative bone scan and chest X-ray. The area with the tumour was separated from the adhering non-neoplastic tissues; thus the proportion of the euploid non-tumour cells which could mask a small aneuploid tumour cell population was kept as low as possible.

The original method of Hedley [13] was modified: 50 µm sections [32] were deparaffinized (three changes of 3–5 ml xylol) and rehydrated to phosphate buffered saline. The efficiency of trypsin digestion (2–3 ml, pH 1.5, 37° C; 3200 units/mg; Sigma) was controlled in selected cases by reembedding and histological investigation of the residual tissue, which was retained after filtration through a 30 µm nylon mesh. Nearly complete removal of the tumour cells from the tissue collagen without increase in nuclear debris was obtained with three-times strong vortex mixing and pipetting during and after a 90 min enzymatic digestion. The resulting cell suspension was washed twice and then stained with DAPI (4,6-diamidino-2 phenylnidole; 4 mg/ml; Sigma). The quality of the preparation of nuclei was controlled by cytological investigation of the DAPI-stained solutions, showing single laying well preserved nuclei. It was shown by comparative evaluation with the original histological sections, that about a half to two-thirds of all measured nuclei were tumour cell nuclei.

The flow-cytometer (EPICS V, Coulter) was adjusted to a CV <2% using beads. The laser was run at 150–200 mW and filtered to excite at 368 nm. Twenty thousand events per case were processed. The mean coefficient of variation of the GOG1-peak, measured at half-maximal hight according to Dean [5], was 7.9% for the whole collective. For cell cycle analysis, Multicycle software (version 1.6 A by Rabinovitsch PS) using the polynomal S-phase algorithm [5] was used on a separate microcomputer. This pro-

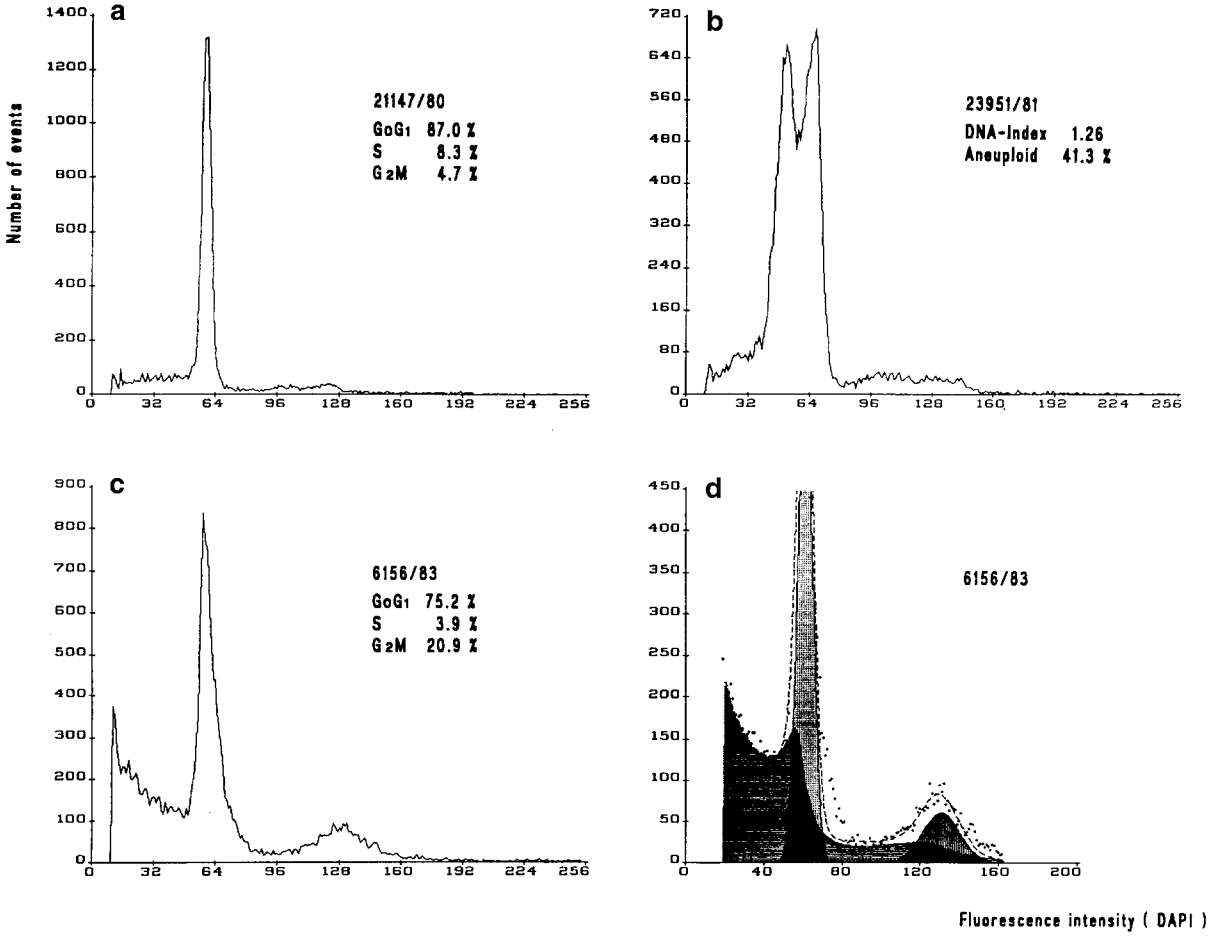
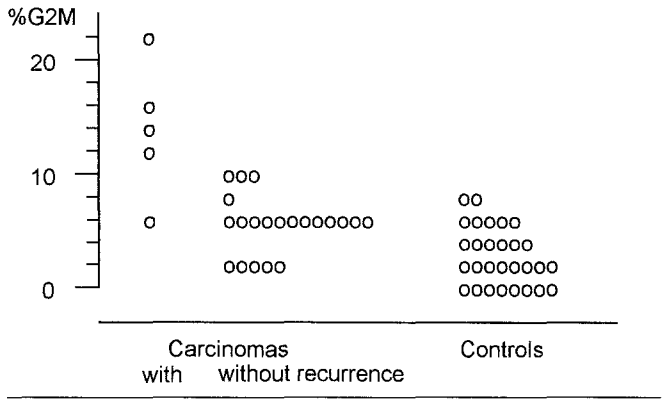


Fig. 1a–d **a** Euploid DNA-histogram (case 21147/80). **b** Aneuploid DNA-histogram, showing the distinct G0G1- and G2M-peaks (case 23951/81). **c** DNA-histogram with an increased G2M-fraction, amounting 20.9% (case 6156/83). **d** Selected area of the histogram shown in **c**, with the computed curves for debris, G0G1-, S-phase and G2M-fraction

Table 2 The G2M-fraction of all non-aneuploid carcinomas with recurrence and without recurrence and of all controls evaluable for cell cycle analysis (N=29)



vides the opportunity for background subtraction with correction for sliced nuclei (see Fig. 1d).

Histograms were classified as euploid, with increased G2M-fraction ("tetraploid") or aneuploid (see Fig. 1a–d). Because no absolute DNA quantification is possible in paraffin embedded tissues, we followed the convention of considering the first peak of the histogram to be the euploid peak [13]. DNA aneuploidy was considered only in cases with at least one additional peak or a clearly bimodal G2M peak [14, 15]. Only cases with a DNA-index >1.10 were considered aneuploid.

The determination of the cut-off point for the cases with an increased G2M-fraction (the "tetraploid" cases of the literature) was done by comparing the G2M-fractions of carcinomas with and without recurrence. In our collective a G2M-fraction >10% was considered indicative for those carcinomas with recurrence (Table 2). As a control 36 cases of prostatic tissue without carcinoma were investigated. Follow up was done by physical examination, bone scan, chest X-ray and determination of serum levels of prostate phosphatase and prostate specific antigen as reported earlier [37].

Results

Six of the 39 stage C carcinomas were aneuploid (15%). One of these 6 was lost for follow up (Table 1). All 3 patients with stage C carcinoma, who died of tumour progression (3 years, 5.5 years and 6 years after prostactomy), had aneuploid carcinomas. Two further patients with aneuploid stage C carcinomas had no recurrence, being well now 8 years following prostatectomy.

Comparing the G2M-fraction of carcinomas with and without recurrence resulted in a cut-off level of 10% (Table 2). The 3 stage C patients with an increased G2M-fraction suffered from tumour progression 3, 4 and 4 years after prostatectomy. Of the 9 patients with stage C carcinoma who suffered from tumour progression, 3 had aneuploid carcinomas, 3 had an increased G2M-fraction and 3 had an euploid carcinoma. These last 3 carcinomas had flow cytograms insufficient for cell cycle analysis.

Among the 20 stage B carcinoms, there was 1 aneuploid carcinoma. This patient died from recurrence 3 years following prostatectomy. The one patient with an increased G2M-fraction died 8 years following operation with generalized metastases. One further patient with stage B carcinoma suffered from tumour progression 9 years after prostatectomy, having had an euploid carcinoma with a G2M-fraction of 4.4%.

Among the 36 controls, there were no aneuploid cells and no cases with an increased G2M-fraction. The mean G2M-fraction of the controls (3.5±2.1%) was significantly lower than that of all carcinomas (6.3±0.2%, $P<0.05$ in the Student t-test). The mean G2M-fraction of stage C carcinomas (7.4±0.4) was higher, but not significantly different from that of the stage B carcinomas (4.7±0.3). Non-aneuploid carcinomas with recurrence had a significantly higher G2M-fraction (12.7±1.3%) as compared to carcinomas without recurrence (6.1±0.2; $P<0.01$ in the U-test of Mann and Whitney; Table 2). All 12 well differentiated carcinomas were euploid. Among the 32 moderately differentiated carcinomas 1 had an increased G2M-fraction and 3 were aneuploid, and among the 15 poorly differentiated tumours 3 had an increased G2M-fraction and 5 were aneuploid.

Discussion

A strong correlation between ploidy and prognosis was found. Seventy-five percent (8/12) of the patients with a non-euploid, but only 8% (4/48) of those with euploid carcinomas suffered from recurrence ($P<0.01$). These results confirm data reporting a strong correlation between ploidy and prognosis in prostate carcinomas [7, 9, 18, 20, 22, 27, 33, 35]. For example Lee reported that 15% of their euploid but 91% of their non-euploid tumours lead to a recurrence within 60 months ($P<0.001$). This correlation may in part be due to a strong correlation between the pathological tumour stage and ploidy, with most of the stage B carcinomas (96% in the Montgomery's series [24]), but only 87% [36]–68% [33] of stage D1 and only 45% of stage D2 [23] carcinomas being euploid. Frankfurt using native materials described 100% of his stage B carcinomas to be euploid, but in the stage C there were only 53% and in stage D only 32% euploid. This correlation was also found in our material with 90% (18/20) of stage B and 79% (30/38) of stage C carcinomas being euploid. This makes a stage-related evaluation of the prognostic impact of flow cytometry necessary.

The crucial point for each patient is the strong correlation between ploidy and prognosis within the different tumour stages. In our stage C carcinomas 67% (6/9) of all aneuploid or "tetraploid", but only 11% (3/27) of the patients with euploid carcinomas ($P<0.01$) had a recurrence. In our stage B carcinomas 2/3 (67%) of the non-euploid and 1/18 (6%) of the euploid carcinomas recurred ($P<0.01$). This is in agreement with other studies with a stage-related evaluation. Stephenson [33] reported a significantly worse prognosis for non-euploid stage D1 carcinomas (5-year survival 49.5% compared to 87.9% in those with euploid metastases, $P<0.0109$). From the Mayo clinic, three reports concerning stage D1 [36], stage C [26] and stage B carcinomas [24] have further confirmed these results. This demonstrates that DNA

flow cytometry offers strong prognostic parameters even within the prognostic groups defined by the pathological tumour stages. Sixty-seven percent of the patients with stage C carcinomas suffering from tumour progression were detected either by aneuploidy or by an increased G2M-fraction, and the three patients not detected had DNA-histograms inadequate for cell cycle analysis.Therefore, by using well fixed material the sensitivity may be increased further. In stage B carcinomas, there were 3 patients with recurrence, 1 of them being detected by aneuploidy, and the other 2 by an increased G2M-fraction (sensitivity 100%). The ratio of "false positive" cases (aneuploidy or increased G2M-fraction but no recurrence) was 2 out of 38% (5%) in stage C and 0 in stage B. One of these 2 patients was lost for follow up 2 years after prostatectomy. Similar values may be calculated from the data of Nativ [26], with a sensitivity for predicting the recurrence of 75% (41 of their 54 patients suffering from a recurrence had "tetra-" or aneuploid carcinomas), and a specificity of 52% (41 of their 79 patients with "tetra-" or aneuploid carcinomas suffered from a recurrence). Most of the "false positives" in these studies were "tetraploid" cases.

The method of determining the cutoff-point of the G2M-fraction discriminating carcinomas with high and with low risk of future tumour progression is crucial to increase the specificity of the method. Most investigators have made a comparison with carcinoma-free prostatic tissue [26, 29, 36]. There was also a statistically significant lower level of the G2M-fractions in controls than in carcinomas in our study. However comparison with the controls seems inappropriate. Not the differences between benign and malignant are under investigation. To discriminate malignancies with low and with high risk for metastases a direct comparison of the G2M-fractions with the patient outcome was done. In this retrospective study the best sensitivity and specificity for selecting patients with low and high risk of future tumour progression was at the 10% G2M-fraction (Table 2). Because of the low patient number and the non-significant difference in the mean G2M-fractions of the stage B and the stage C carcinomas, for stage B and stage C carcinomas the same cut off-point was used. To avoid an overestimation of the S or G2M phase fractions in the paraffin material a correction for sliced nuclei and a background subtraction is important (Fig. 1d) [5, 16]. Tetraploidy means a tumour cell population with 4n chromosomes in the G0 and G1 phase and 8n chromosomes in the G2M phase [11]. This is a rare finding in prostate carcinoma [24, 26, 36]. Most of the "tetraploid" carcinomas of the literature are those with an "increased" G2M-fraction, so that we prefer the terminus "increased G2M-fraction" to designate these carcinomas.

In the optical evaluation of the flow cytograms, artefacts due to the preparation of the nuclei or due to insufficient fixation have to be considered [4, 8, 15, 33]. In our material 27 consecutive cases had to be excluded because of poor fixation (prolonged storage in an insufficient amount of formalin). No aneuploid peaks were observed in our tumour-free control specimens [1, 6, 21, 22].

Comparative flow cytometric and cytogenetic investigations, reported to show higher rates of aneuploidy by cytogenetic means in various carcinomas [31] are not available for prostate carcinomas [3, 19]. One investigation, using multiple punch biopsies taken from the paraffin embedded material, demonstrated cases with heterogenous ploidy pattern. This method enhances the sensitivity of detection of aneuploidy by increasing the proportion of the aneuploid tumour cell population within the sample [20]. The relatively high rate of aneuploid carcinomas in our study may therefore be due to the selection of tumour tissue out of its non-neoplastic surroundings.

In conclusion, the determination of ploidy and G2M-fraction are important prognostic factors for a patient with prostate carcinoma. In combination with the pathological tumour stage, their sensitivity and specificity for predicting a postoperative tumour recurrence seems sufficiently high to indicate a prospective study. This will require well fixed specimens and a separation of tumour cell nuclei to increase the proportion of tumour cells in the final analysis. A careful optical and mathematical evaluation of the flow cytograms is needed. Without background subtraction the G2M-fraction would be under large bias by the varying amounts of debris. In future standardization of these flow cytometric techniques will be necessary to permit a valid selection of those prostate carcinoma patients who require further therapy following prostatectomy.

References

1. Alanen KA, Joensuu H, Klemi PJ (1989) Autolysis is a potential cause of false aneuploid peaks in flow cytometry DNA histograms. Cytometry 10:417–425
2. Böcking A, Chatelain R, Orthen U, Gien G, Kalckreuth G von, Jocham D, Wohltmann D (1988) DNA-grading of prostatic carcinoma: prognostic validity and reproducibility. Anticancer Res 8:129–135
3. Brothman AR, Lesho LJ, Somers KD, Schellhammer PF, Ladage LE, Merchant DJ (1989) Cytogenetic analysis of four primary prostatic cultures. Cancer Genet Cytogenet 37:241–248
4. Camplejohn RS, Macartney JC (1986) Comments of "effects of section thickness on quality of flow cytometric DNA content determinantion in paraffin-embedded tissues". Cytometry 7:612–615
5. Dean PN (1985) Methods of data analysis in flow cytometry. In: Dilla MA van, Dean PN, Laerum OD, Melamed MR (eds) Flow cytometry: instrumentation and data analysis. Academic Press, pp 195–221
6. Deitch AD, Stand MA, White RW deVere (1989) Deoxyribonucleic acid flow cytometry of benign prostatic disease. J Urol 142:759–762
7. Dejter SW, Cunningham RE, Noguchi PD, Jones RV, Moul JW, McLeaod DG, Lynch JH (1989) Prognostic significance of DNA ploidy in carcinoma of prostate. Urology 33:361–366
8. Feichter GE, Goerttler K (1986) Pitfalls in the preparation of nuclear suspensions from paraffin-embedded tissue for flow cytometry. Cytometry 7:616–619
9. Fordham MVP, Burdge AH, Matthews J, Williams G, Cooke T (1986) Prostatic carcinoma cell DNA content measured by

flow cytometry and its relation to clinical outcome. Br J Surg 73:400–403
10. Forsslund G, Zetterberg A (1990) Ploidy level determinations in high-grade and low-grade malignant variants of prostatic carcinoma. Cancer Res 50:4281–4285
11. Frankfurt OS, Chin JL, Englander LS, Greco WR, Pontes JE, Rustum YM (1985) Relationship between DNA ploidy, glandular differentiation and tumor spread in human prostate cancer. Cancer Res 45:1418–1423
12. Frohmüller H, Theiß M, Wirth MP (1991) Radical prostatectomy for carcinoma of the prostate – long term follow up of 115 patients. Eur J Urol 19:279–283
13. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA (1983) Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333–1335
14. Hiddemann W, Schumann J, Andreeff M, Barlogie B, Herman CJ, Reif RC, Mayall BH, Murphy RF, Sandberg AA (1984) Convention on nomenclature for DNA cytometry. Cytometry 5:445–446
15. Joensuu H, Kallioniemi O-P (1989) Different opinions on classification of DNA histograms produced from paraffin-embedded tissue. Cytometry 10:711–717
16. Kallioniemi OP, Visakorpi T, Holli K, Heikkinen A, Isola J, Koivula T (1991) Improved prognostic impact of S-phase values from paraffin-embedded breast and prostate carcinomas after correcting for nuclear slicing. Cytometry 12:413–421
17. Klein FA, Ratliff JE, White FK (1988) DNA distribution patterns of prostatic tissue obtained at time of transurethral resection. Urology 31:260–265
18. Lee SE, Currin SM, Paulson DF, Walther PJ (1988) Flow cytometric determination of ploidy in prostatic adenocarcinoma: a comparison with seminal vesicle involvement and histopathological grading as a predictor of clinical recurrence. J Urol 140:769–774
19. Limon J, Lundgren R, Elfving P, Heim S, Kristoffersson U, Mandahl N, Mitelman F (1989) Double minutes in two primary adenocarcinomas of the prostate. Cancer Genet Cytogenet 39:191–194
20. Lundberg S, Carstensen J, Rundquist I (1987) DNA flow cytometry and histopathological grading of paraffin-embedded prostate biopsy specimens in a survival study. Cancer Res 47:1973–1977
21. Malinin TI, Hornicek FJ, Block NL, Malinin GI (1988) Aneuploidy of glandular epithelial cells in histological normal prostate glands. Experientia 44:247–249
22. McIntire TL, Murphy WM, Coon JS, Chandler RW, Schartz D, Conway S, Weinstein RS (1988) The prognostic value of DNA ploidy combined with histologic substaging for incidental carcinoma of the prostate gland. Am J Clin Pathol 89:370–373
23. Miller J, Horsfall DJ, Marshall VR, Rao DM, Leong SY (1991) The prognostic value of deoxyribonucleic acid flow cytometric analysis in stage D2 prostatic carcinoma. J Urol 145:1192-6
24. Montgomery BT, Nativ O, Blute ML, Farrow GM, Myers RP, Zincke H, Therneau TM, Lieber MM (1990) Stage B prostate adenocarcinoma. Flow cytometric nuclear DNA ploidy analysis. Arch Surg 125:327–331
25. Müller H-A, Altenähr E, Böcking A, Dhom G, Faul P, Göttinger H, Helpap B, Hohbach Ch, Kastendieck H, Leistenschneider W (1980) Über Klassifikation und Grading des Prostatakarzinoms. Verh Dtsch Ges Pathol 64:609–611
26. Nativ O, Winkler HZ, Raz Y, Therneau TM, Farrow GM, Myers RP, Zincke H, Lieber MM (1989) Stage C prostatic adenocarcinoma: flow cytometric nuclear DNA ploidy analysis. Mayo Clin Proc 64:911–919
27. Neill WA, Norval M, Habib FK (1989) Nuclear DNA analysis of prostate tissues: correlation with stage and grade of tumour. Urol Int 44:141–146
28. Schellhammer PF (1988) Radical prostatectomy. Patterns of local failure and survival in 67 patients. Urology 31.191–197
29. Schultz RE, Varello MA, Tsou KC, Wein AJ, Murphy JJ (1985) Simultaneous flow cytometric deoxyribonucleic acid and acid phosphatase analysis of benign and malignant lesions of the prostate. J Urol 134:1133–1136
30. Seppelt U, Sprenger E (1984) Nuclear DNA cytophotometry in prostate carcinoma. Cytometry 5:258–262
31. Shackney SE, Burholt DR, Pollice AA, Smith CA, Pugh RP, Hartsock RJ (1990) Discrepancies between flow cytometric and cytogenetic studies in the detection of aneuploidy in human solid tumors. Cytometry 11:94–104
32. Stephenson RA, Gay H, Fair WR, Melamed MR (1986) Effect of section thickness on quality of flow cytometric DNA content determinations in paraffin-embedded tissues. Cytometry 7:41–43
33. Stephenson RA, James BC, Gay H, Fair WR, Whitemore WF, Melamed MR (1987) Flow cytometry of prostate cancer: relationship of DNA content to survival. Cancer Res 47: 2504–2507
34. Tavares AS, Costa J, De Carvalho A, Reis M (1966) Tumour ploidy and prognosis in carcinomas of the bladder and prostate. Br J Cancer 20:438–441
35. Tribukait B, Ronstrom L, Espositi P (1983) Quantitative and qualitative aspects of flow DNA measurements related to the cytologic grade in prostatic carcinoma. Anal Quant Cytol Histol 5:107–111
36. Winkler HZ, Rainwater LM, Myers RP, Farrow GM, Therneau TM, Zincke H, Lieber MM (1988) Stage D1 prostatic adenocarcinoma: significance of nuclear DNA ploidy patterns studied by flow cytometry. Mayo Clin Proc 63:103–112
37. Wirth MP, Müller HA, Manseck A, Müller J, Frohmüller HGW (1991) Value of nuclear DNA ploidy patterns in patients with prostate cancer after radical prostatectomy. Eur Urol 20:248–252