The Value Of Mitotic Counting In Prostate Carcinomas

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Abstract-Mitosis counting remains one of the most valuable prognostic indicators in tumour pathology. The aim of this study was to assess the proliferative activity of primary prostate cancer and lymph node metastases using the volume-corrected mitotic index (M/V). Mitotic figures were quantitated as number of mitotic figures/mm^2 of neoplastic epithelium in a series of 40 prostatic adenocarcinomas, and the results were related to clinical and histological features of the primary tumours. High-grade tumours showed higher mitosis counts than intermediate-grade tumours, and metastasis was related to mitotic indeces as well. The proliferative activity was significantly higher in lymph node metastasis than in primary tumours. The mitotic index did not correlate significantly with any clinical or histological feature of the primary tumours. However, the results show that mitotic index (M/V) can be considered an useful, quick, and efficient factor in evaluation of malignant tumor prolife rative activity, and could be an useful prognostic parameter in prostatic adenocarcinomas.

Keywords-mitotic count, progression, prostatic adenocarcinoma, volume-corrected mitotic index.

I. INTRODUCTION

There is a general acceptance that most neoplasias arise from a single altered cell. Tumorigenesis involves specific genetic changes that conferre on the progenitor cell a selective growth advantage, allowing its progeny to expand as a neoplastic clone of unicellular origin [1]. The subsequent alterations in cellular DNA lead to a variety of processes that commit transformed cells to continuous proliferation. Autonomy from normal growth regulatory mechanisms of the host and tumour progression leads to a sequential increasing of genetically altered subpopulations with new characteristics within the tumour. However, the genetic changes that result in uncontrolled proliferation associated with tumorigenesis do not, by themselves, produce the metastatic phenotype. The acquisition of this trait may require additional genetic changes beyond those related to tumor growth. As in growth control, these changes may involve activation of positive modulators (protooncogenes) or loss of negative effectors (tumour growth suppressors and invasion and metastatic suppressors) [2]. Invasion and metastasis can be facilitated by proteins that stimulate tumor cell attachment to host cellular or extracellular matrix components, tumor cell proteolysis of host barriers such as the basement membrane, tumor cell migration and tumor cell colony formation in the tissue of the secondary site. Facilitating proteins may act at many intracellular or extracellular levels, but are counterbalanced by factors that are able to block their production, regulation or action [2], [3].

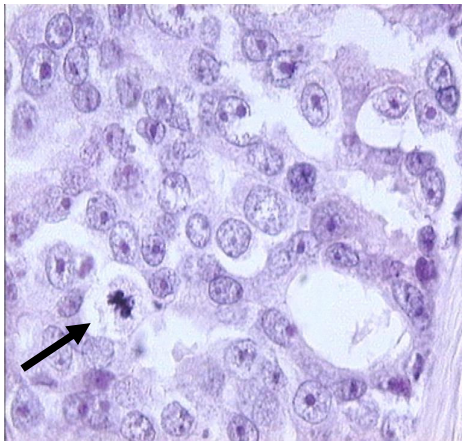

Fig.1. Mitosis in a Gleason grade 3 prostate adenocarcinoma (H&E, x40)

The comparative features of cells in primary tumours and their metastases have been discussed in numerous papers. The aim of this study was to investigate the proliferative activity in prostate carcinomas and their lymph node metastases.

The proliferative potential of neoplastic cells can be evaluated in several ways, including determination of the mitotic rate, e. g. by counting the mitotic figures, or determination of the fraction of cells in S-phase. The latter can be estimated by flow citometry, bromodeoxyuridine (BrdU) uptake, or Ki-67 and proliferating cell nuclear antigen (PCNA) expression assessment [4].

Counting of mitotic figures is the oldest way of assessing proliferation and has been applied as a diagnostic tool, especially in tumour pathology. The ease with which mitoses can be recognized without special equipment apart from a standard laboratory microscope and a well stained hematoxylin-eosin slide, has led to increasing popularity of this way of counting of mitotic figures up to the present [5], [6]. Strict morphological criteria should be applied for the recognition of mitotic figures. Mitoses can be defined as dark clots of chromosomes which can be often recognized by the presence of hairy extensions when focusing up and down, while the nuclear envelope is absent and the cytoplasm is basophilic rather than eosinophilic (fig. 1). These chromosomal clots can have the configuration of the metaphase, anaphase, or telophase [5].

Several approaches may be followed when counting mitotic figures: number of mitoses expressed as the total number in a defined number (e.g., 10) high power fields, number of mitotic figures per unit area (e. g., 2 mm^2), number of mitoses per a certain number of tumour cells (e. g., 1000) or number of mitotic figures per area of tumour epithelium [5], [7], [8]

The value of the standardized mitotic index (volume-corrected mitotic index or M/V index) was evaluated giving the results in mitotic figures per square mm of neoplastic epithelium. Volume-corrected mitotic index (M/V) is estimated on a microscopic field at high magnification, expressing the number of mitoses per area of tumour epithelium (mm^2) [7]-[9].

II. METHODS

Patients

The histopathology files of Clinical County Hospital, Department of Urology, Brasov, Romania, were searched for all cases of prostate

cancer with lymph node metastasis accessioned between 1.01-31.12.2009, and a total of 40 cases were identified.

The histology slides from all cases were reviewed to confirm the diagnosis and to select an appropriate block representative of the dominant tumour pattern for mitotic counting.

Clinical information including follow-up data were obtained from review of the case records, and correspodence with patients' clinicians. Clinicopathological data regarding the primary tumours were derived from the original histopathology reports and from reviews of the slides.

The mean age at diagnosis of prostate cancer among patients was 68 ± 16.16 years (mean ± standard deviation [SD]).

The location and size of the tumour as well as the status of the regional lymph nodes were registered as described at operation, especially in examination of the resected prostate. The follow-up data including the time and location of metastases or other recurrence of the tumour were collected from the patients records.

Histological methods

The tumour samples were routinely fixed in 10% formalin and embedded in paraffin. Several original sections from each of the primary tumour were re-examined and the most representative tissue block was selected, cut at 5 μm thickness, and stained with hematoxylin and eosin (H&E). The original and new slides were examined by one experienced histopathologist (DD) while being unaware of the clinical data.

All tumours were classified according to the Gleason three-grade system corresponding to tumours that are well (combined Gleason grades 2 to 4), moderately (combined Gleason grades 5 to 7), and poorly differentiated (combined Gleason grades 8 to 10) [10]-[12].

Tumour invasion into the walls of veins, arteries and lymphatics or into the perineural space, as well as tumour necrosis, were registered in this new representative H&E section and graded as absent, weak, moderate or extensive (the latter groups were later combined to form one group of positive invasion).

The peri-tumor infiltration of lymphocytes was estimated avoiding ulcerated or necrotic areas and graded as weak, moderate or strong. Similarly, desmoplasia was graded as weak, moderate or strong.

Assessment of proliferation

Proliferative activity of tumour cells was estimated using the M/V index, expressed as number of mitotic figures/mm^2 of neoplastic epithelium, using the method described by Collan (1996) [8] and Haapasalo (1989) [9].

Counting was carried out in each primary tumor and each lymph node metastases, at thirty high power fields (at objective magnification x40). The tumour area was scanned and consecutive fields were studied following each other in a line or stow of lines trying to follow the most cellular fields. The microscope was equipped with a compensating measuring eyepiece x12.4 with a Weibel graticule for stereological measurements having 21 lines and 42 end points.

The Weibel graticule consists of short lines with interruptions the same length as the lines. Basically, the number of intersections falling over the short lines are counted and the number of endpoints falling on the end of the structure are determined [13], [14].

Mitotic figures were characterized by an absent nuclear membrane with clear, hairy extensions of nuclear material (condensed chromosomes) that were clumped in a plane, or in separate chromosomal aggregates. The basic idea was that at least one chromosomal end was seen in mitosis. Two parallel, clearly separate chromosome clumps were counted as one mitotic figure.

Only clearly identified mitotic figures within tumour cells were counted, and care was taken to exclude mitoses in reactive stromal and endothelial cells as well as apoptotic bodies.

Between 8 and 15 complete grids were assessed in each case (depending on tumour cellularity) such that a minimum of 1000 tumour cells were actually counted.

The number of mitotic figures in primary tumours and lymph node metastases was estimated in 30 consecutive microscopic fields (Table I).

Table I. Working sheet for M/V counting

Microscopic field no.	Primary tumour		Metastasis	
	MI	P_A	MI	P_A
1	4	20	2	23
2	1	16	2	26
3	1	14	0	26
4	2	17	1	31
5	0	13	5	31
6	1	20	0	28
7	1	14	2	23
8	2	22	2	15
9	1	25	3	24
10	2	9	1	28
11	1	18	2	19
12	3	20	3	16
13	2	21	3	16
14	3	23	6	30
15	2	14	4	17
16	2	19	5	25
17	3	31	5	23
18	2	30	1	19
19	1	23	3	18
20	1	32	0	22
21	3	19	3	30
22	1	21	2	19
23	1	28	2	19
24	0	24	3	22
25	2	20	3	25
26	2	18	2	27
17	2	33	4	23
28	5	30	2	16
29	1	27	0	18
30	0	22	2	19
Total	52	643	71	668
M/V (mitoses/mm^2)	**67.53**		**88.75**	

MI – number of mitoses/field, P_A – number of points superimposed on tumour cells

The M/V index was calculated using the following equation:

$$M/V = \sum MI \; / \; \sum a \times A_A \qquad (1)$$

where *MI* is the mitotic count in a microscopic field (number of mitosis/field), $\sum MI$ is the mitotic count in all studied fields, *a* represents the area of the microscopic field, A_A is the area fraction of neoplastic epithelium in a microscopic field, and $\sum a \times A_A$ is the sum of area fractions multiplied by the area of the microscopic field.

A_A can be easily calculated according to the following equation:

$$A_A / A = P_A / P \qquad (2)$$

where *P* is the total number of points counted (30 fields of 42 points = 1260 points), and P_A the number of points corresponding to neoplastic tissue (number of points superimposed on tumour cells) (fig. 2 and fig. 3) [13].

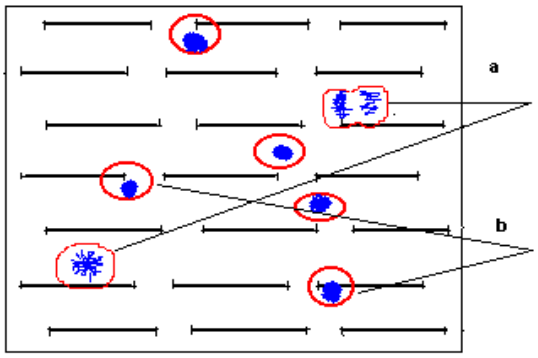

Fig. 2. Schematic representation of the Weibel graticule superimposed on the microscopic field (a = mitoses; b = points that superimpose tumor cells).

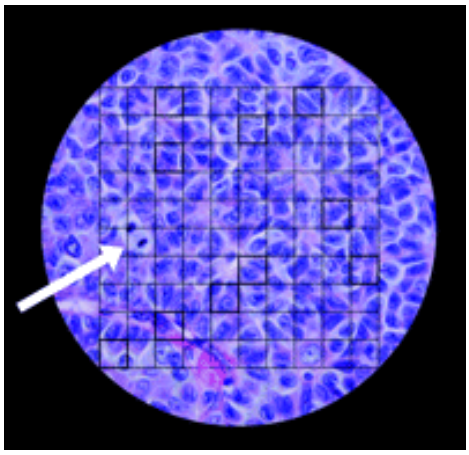

Fig. 3. Illustration of eye-piece graticule (H&E x 40). A single mitotic figure (arrow) is seen within the complete grid.

With the microscope that has been used in this study, the area of the microscopic field included in the Weibel graticule (A) had the value of 0.050625 mm^2.

Let us suppose that in the 30 fields which were scanned in a tumour there were 47 mitoses (M/V = 47) and that 975 points corresponded to neoplastic tissue.

In the 30 examined microscopic fields of the primary tumour 47 mitotic figures were counted ($\sum$IM = 47) and the number of points superimposed on the neoplastic tissue was 975; in this case the M/V index was calculated as follows:

$$A_A / A = P_A / P = 0.75 / 1260 = 0.77$$
$$M/V = 47 / (0.050625 \text{ x } 30 \text{ x } 0.77) = 47 / 1.17 = 40.2$$

i.e. in this case there are 40.2 mitoses/mm^2 of neoplastic tissue.

In a similar way, at 77 mitosis/30 microscopic fields, and 668 points superimposed on the neoplastic tissue, in the lymph node metastases the value of M/V was 88.75 mitosis/mm^2 neoplastic tissues.

The same method was applied to all studied tumours.

Inter-observer variation

The M/V$_v$ index was assessed independently by one additional observer (SD). Concerning the reproducibility of the method, consecutive measurements of the same cases showed excellent agreement (0.9-4% variation between two measures).

Statistics

For statistical analysis the Statistica for Windows (StatSoft Inc.) system was used. Correlation between quantitative parameters was analysed with the Student t test. Data were considered statistically significant at $p<0.05$.

III. RESULTS

III.1. Relation between M/V and clinical characteristics of the patients

The clinical characteristics of the patients related to M/V are summarized in Table II.

Table II. Clinical characteristics of the patients (n = 40) related to M/V

Parameter	*Number*	*Mean M/V$_V$ (± SD)*
Tumour location		
Transition zone	19	53.58 ± 12.45
Peripheral zone	11	69.64 ± 27.47
Central zone	10	64.75 ± 18.35
	p = NS	
Tumour size		
< 5 cm	11	55.78 ± 13.63
≥ 5 cm	29	62.87 ± 24.92
	p = NS	
Depth of invasion		
pT1	-	-
pT2	4	53.96 ± 13.56
pT3	23	65.91 ± 25.44
pT4	13	66.42 ± 23.39
	p = NS	
Nodal status		
N0	-	-
N1	-	-
N2	21	70.40 ± 25.81
N3	19	60.43 ± 19.95
	p = NS	
Metastases		
M0	16	61.85 ± 23.52
M1	24	64.91 ± 22.14
	p = NS	

NS = not significant

Tumours arising in the transition zone of the prostate showed a lower number of mitosis/mm^2 than those arising in the peripheral or central zone, but the differences between the groups were not significant.

Higher but not significantly different M/V values were noticed in tumours ≥ 5 cm in size.

Tumours limited to the prostate gland (T2) showed the lowest M/V index, while those who spread out the prostatic capsule (T3) or invaded the nearby structures (T4) had similar number of mitosis/mm^2, these differences being not significant.

All analyzed tumours had lymph node metastases, their number varying between 1 and 12. All tumours showed both pelvic and distant lymph node metastases. Cases were separated in two groups: with local, pelvic metastases (N), and with distance metastases (M)

respectively. Local metastases were either single nodes having dimensions between 2 and 5 cm (N2) or node greater than 5 cm (N3) ($p<0.18$). Most of the tumours, 24 (60%), had distance metastases (M1). Tumours with local metastases (M0) had a lower mean value of M/V.

III.2. Relation between M/V and histologic features analyzed

None of the analyzed tumours were well differentiated (Gleason score 2 to 4). Tumours with high malignancy (Gleason score 8-10) showed higher M/V values than those with medium differentiaton (Gleason score 5-7), but these mean values were not statistically different.

The tumour invasion to vessels and perineural space offers an additional route for tumour spread outside the prostate gland. The presence of vascular, lymphatic and neural invasion was not related to M/V ($p<0.51$). In addition, the presence of lymphatic invasion could not predict lymph node involvement.

Perineural invasion was observed in a half of the cases, not statistically different from those without perineural invasion ($p<0.86$).

The association between M/V and histological tumour type, tumour necrosis, and desmoplasia was not significant.

Lymphocytic response around and in the tumour usually reflects tumour-host interaction and is associated with prognosis in prostate cancer. A dense infiltration usually indicates a more favourable prognosis. In our study, we found no relation between the density of lymphocytic infiltration and number of mitoses/mm^2.

The histological features related to M/V are summarized in Table III.

Table III. Histological features related to M/V

Parameter	*Number*	*Mean M/V (± SD)*
Histologic tumour type		
Acinar carcinoma	24	59.83 ± 20.86
Ductal carcinoma	14	73.57 ± 21.66
Small cell carcinoma	2	55.53 ± 31.12
	p = NS	
Gleason score		
Gleason 2-4	-	-
Gleason 5-7	32	61.42 ± 21.73
Gleason 8-10	8	76.45 ± 23.93
	p = NS	
Vascular invasion		
Negative	33	65.35 ± 23.43
Positive	7	59.18 ± 19.55
	p = NS	
Lymphatic invasion		
Negative	13	60.17 ± 17.50
Positive	27	66.47 ± 24.87
	p = NS	
Perineural invasion		
Negative	20	63.78 ± 20.08
Positive	20	65.05 ± 25.58
	p = NS	
Lymphocytic infiltration		
Weak	15	62.48 ± 25.03
Moderate	22	65.47 ± 22.46
Strong	3	65.92 ± 17.95
	p = NS	
Tumor necrosis		
Absent	7	59.17 ± 16.32
Weak	5	62.49 ± 15.10
Moderate	12	64,58 ± 21.70
Strong	16	67.21 ± 28.38
	p = NS	
Desmoplasia		
Weak	9	67.73 ± 31.25
Moderate	22	63.44 ± 20.80
Strong	9	63.50 ± 19.62
	p = NS	

NS = not significant

III.3. Comparison between mitotic activity in primary tumours and metastases

The mean value of M/V (±SD) in the studied group was 64.42 ± 22.70 in primary tumours, and 75.66 ± 25.06 in lymph node metastases (Table IV).

Table IV. Mean values of M/V in primary tumours and metastases

Tumour type	*No.*	*Mean M/V (± SD)*	*Limits*	
Primary tumour	40	64.42±22.70	Minimum	33.53±27.61
			Maximum	132.59±81.87
Metastasis	40	75.66±25.06	Minimum	28.79 ± 36.6
			Maximum	142.25 ± 86.39

The statistical analysis (one-tailed paired *t*-test) showed significant differences between the two groups. In 75% of the cases M/V values in lymph nodes were higher than in primary tumours. In metastases, M/V was significant higher than in primary tumours ($p=0.0017$).

Statistical analysis of M/Vvalues using linear regression method revealed a strong correlation of the M/V in the two series, especially for values between 40 and 80 mitosis/mm^2 (fig. 3).

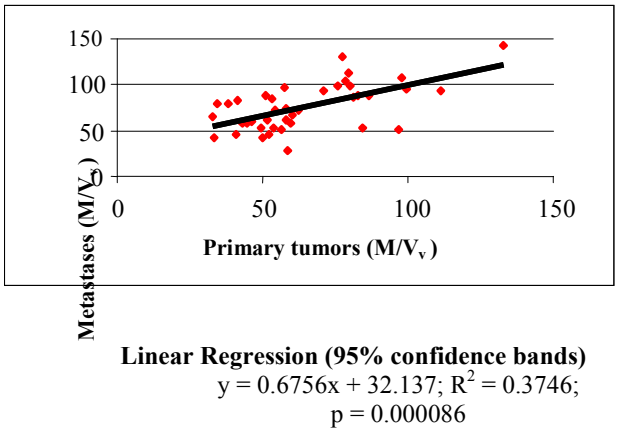

Fig. 3. Linear regression analysis of the M/V$_v$ values in primary and metastatic prostate carcinoma

IV. DISCUSSION

The study of DNA patterns has revealed interesting data. The patterns with diploid DNA tumours have a better overall survival than those with aneuploid tumours [15], [16]. Numerous primary tumours were found to have multiple DNA stemlines [17]. Comparison of the DNA pattern obtained from the primary tumour and the corresponding metastases showed very close correlations [18]. DNA aneuploidy is not uniquely associated with metastasizing capacity since there are many cases of DNA diploid metastases associated with either DNA diploid primaries or heterogenous primaries with DNA aneuploid and DNA diploid tumor cell populations [18]-[24]. The preservation of DNA stemlines after metastasing in lymph nodes suggests a degree of karyotype stabilization in tumor cell clones with metastatic involvement [25].

The situation is even more complicated as Kerbel *et al.* (1988) [26] showed a clonal domination of primary tumour by metastatic cells. Their results show unequivocally that spontaneous metastases can develop from a genetically distinct subpopulation of cells in a non-random (i. e. selective) manner. However, because in some cases primary tumors can gradually become overgrown by the progeny of a metastatic clone, results of any comparison of the properties of a primary tumor with a distant metastasis can be profoundly affected by the stage at which the primary tumor is removed and analyzed. Thus, in an early stage, primary tumours may have none or only very small proportion of metastatic cells, but the same tumor may be the biological equivalent of a metastasis at a later, more advanced stage of growth. Consistent with this hypothesis, Morikawa *et al.* (1988) [27] concluded that late stage human tumours contain proportionally more metstatic cells than earlier stage tumours.

The results concerning the proliferative activity are rather conflicting. Chang *et al.* (1993) [25] found that the primary breast cancers and their lymph node metastasies had similar DNA model values and S-phase fractions (commonly used to characterize proliferative activity in human tumours). Goodson *et al.* (1993) [28] demonstrated a strong correlation between the in vivo BrDU labeling index of a primary breast cancer and the simultaneous labeling index of regional lymph node metastases. Daidone *et al.* (1990) [29] found that the thymidine labeling index of primary tumours and regional lymph node metastases were also concordant. However, discordant data have been published by other authors. Thus, Feichter *et al.* (1989) [30] noted that the agreement of S-phase fraction on primary breast tumours and metastases was less consistent than the DNA index. In only a few cases the analysis of case-related matched pairs revealed higher S-phase fractions in the metastases than in the primary tumour (16%) and also in some cases less S-phase fractions in the metastases than in the primary tumour. Olszewsky *et al.* (1982) [31] reported similar observations, particularly a significant decrease of the S-phase fraction in the metastases of tumours with a positive content of estrogen receptors. As tumors with a low mitotic index can metastasize to regional lymph nodes, a high growth rate seems not to be prerequisite for the development of regional metastases.

The assessment of cellular proliferation is widely used in the assessment of tumours, not only for primary diagnostic purposes but also as a guide to prognosis. Since tumours that exhibit increased proliferation tend to be more aggressive clinically, measures of proliferation are often incorporated into histological grading systems. The simplest and most widely used method is the mitoses counting, which despite many potential technical limitations correlates with prognosis in many neoplasms [32].

There are several methods for the estimation of the mitotic rate and truly comparable figures are not available. Most often, the number of mitoses is expressed as the total number in a defined number high power fields (HPF). The exact area of the HPF must, however, be defined to be able to compare results from different studies, since the area of field of vision can vary considerably between different objectives. Unfortunately, most workers have failed to do so in the past, which has led to much criticism. Therefore, some use the number of mitotic figures per unit area (e. g., 2 mm^2). It may be also useful to correct for the actual content of tumour cell within the slide by expressing the number of mitoses per a certain number of tumour cells (mitotic rate), or to correct the number of mitotic figures for the area percentage of epithelium, (M/V index, volume-corrected mitotic index) [8], [9]. These methods are potentially time consuming, but stereological sampling approaches are quite useful to keep the extra time spent within acceptable limits [33], [34].

The reproducibility of mitosis counting has been questioned. In several tumours, a significant variation in the mitotic rates was noticed in different areas of the same tumour [35]. The variation in mitotic rates reflects intratumoral heterogeneity, which can reduce the reproducibility of the method. Poor reproducibility may also be caused by hypoxia during the operation or variations in the fixation [36]. Indeed, when standardised methodology is not used quite variable results may be obtained on the same material by different observers [8], [37], but after thorough training and following a strict protocol, excellent inter-reproducibility results have been obtained [37]-[39]. By using a standardised method like the M/V index, the interobserver variation in the mitosis counting can be partly controlled [9], [40]. Since counting of mitotic figures is not entirely objective, attempts have been made to automate the counting of mitotic figures [41].

Proliferation markers, especially the mitotic index, has been shown to posses significant prognostic value in several human malignancies, e.g. breast [42]-[44], gastric [45], [46], pulmonary [14], pancreatic [47], [48], ovarian [32], [49], [50], urinary bladder [47], [51], hepatocellular [39] cancer, as well as in melanomas [52], [53], smooth muscle tumours or leiomyosarcomas of the uterus [54], [55], glial tumours [56] or other cancer types.

Concerning prostate adenocarcinomas, in the literature there are only a few and no concluding data about the influence of mitotic index on prognosis. The results concerning the proliferative activity are rather conflicting.

Mitotic figures are rarely found in tissue sections in normal or hyperplastic prostatic epithelium. Therefore, S-phase markers are commonly employed as surrogate markers for estimating proliferation rates [4]. In most studies, the number of mitotic figures increased progressively from benign epithelium through prostate intraepithelial neoplasia (PIN) to cancer [57]. Adenocarcinomas composed of solid areas of undifferentiated tumour cells contained most mitotic figures [4], [57]. Additionally, the number of mitotic figures correlated with cancer stage and grade [4], [58], and also with progression and progression-free survival [57]. Androgen deprivation therapy results in dramatic decline in tne number of mitotic figures in prostate cancer, whereas normal prostate epithelium undergoes apoptosis-mediated involution [59]. Thus it appears that elevation in epithelial cell proliferation parallels cancer progression and that determinants of elevated cellular proliferation have significant potential value as prognostic marker.

A given prostate cancer patient's response to therapy may be predicted by following mitotic activity [60]. Histologic evaluation of mitotic and apoptotic index, Ki-67, and p53 contributes to predicting the value of actual treatment and are recommended as histologically detectable predictive factors in prostate carcinomas [61], [62]. DNA ploidy combined with a proliferation index yields additional prognostic information in patients with Gleason score 5-7. Diploid

tumors with a low proliferation index are associated with a low risk of disease progression [63].

Some studies, analysing the mitotic and the apoptotic index in comparison with different prognostic factors, showed that mitotic indeces are useful prognostic parameters in prostate carcinoma, but only in addition to the conventional histologic grading system [57], [64]. Howe *et al.* (2005) [65] showed a decrease of the mitotic index after radiation exposure, while other authors describe a decrease of the apoptotic index, without significant changes of mitosis after experimental castration [66].

The investigations made in this study have shown that there are significant differences in the volume fraction-corrected mitotic index in primary tumours and metastases. Probably these differences could be explained by different conditions of vascularization and nutrition in the lymph node, and on the other hand by clonal selection, which could lead to the growth of clones with particular kinetic properties in the lymph node metastases. A decrease of the growth rate in the metastases could be the result of local inhibitory mechanisms, such as cell mediated inhibitory mechanisms. Finally, the differences between primary and metastatic tumours may be explained by the heterogeneity of the tumours [30].

In conclusion, the estimation of the proliferative activity of tumours by well-standardized mitotic counting techniques should have a central position in histopathology research and practice. In the application studied so far, these methods are easy, rapid, and inexpensive, and have great prognostic power.

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