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Expression of MIB-1, mitotic index and S-phase fraction as indicators of cell proliferation in superficial bladder cancer

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Abstract Cell proliferation of transitional cell bladder cancer (TCC) was determined by MIB-1 immunolabeling, volume-corrected mitotic index (M/V index) and S-phase fraction measurement in 207 patients with superficial (Ta-T1) bladder cancer. The results were compared to T category, WHO grade and DNA-ploidy. The MIB-1 score was related to T category ($P < 0.001$), WHO grade ($P < 0.001$), DNA ploidy ($P < 0,0001$), M/V index ($P < 0.0001$) and fraction of cells in S phase ($P < 0.0001$). The mean MIB-1 score was 6.37% for G1, 14.59% for G2 and 28.59% for G3 carcinomas ($P < 0.001$). The MIB-1 score for Ta tumors was 9.24% and for T1 tumors 25.34% ($P < 0.001$). The M/V index was 3.9 for G1, 11.5 for G2 and 25.9 for G3 tumors ($P < 0.0001$). The M/V index for Ta tumors was 6.4 and 25.3 for T1 tumors ($P < 0.0001$). WHO grade 1 tumors had 7.7%, grade 2 tumors 13.8% and grade 3 tumors 21.8% of cells in S phase ($P < 0.001$). Of grade 1 tumors, 97% were diploid and 3% aneuploid, and 78% of grade 2 tumors were diploid and 22% aneuploid. Of grade 3 tumors, 30% were diploid and 70% aneuploid ($P < 0.001$). Of Ta tumors, 92% were diploid and 8% aneuploid, respectively, whereas 40% of T1 tumors were diploid and 60% aneuploid ($P < 0.0001$). The results show that quantitative cell proliferation indices are associated with T category and WHO grade in superficial bladder cancer. The prognostic value of the S-phase fraction and mitotic index has been demonstrated in several previous analyses of prognostic factors while the value of MIB-1 score on bladder cancer prognosis remains to be established in further follow-up studies.

Key words Superficial bladder cancer · MIB-1 · S-phase fraction · M/V index

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Introduction

The biological behavior of superficial bladder cancer (SBC) is variable but in general the prognosis is good. Five-year survival rates are higher than 80%. About one-half of patients will have recurrences and only 10% will have a tumor progression and eventually die of bladder cancer [36]. The main question in SBC is how to distinguish those tumors which will progress and should be managed with early radical surgery.

Various pathological and clinical parameters have been related to the likelihood of progressive disease. Ta and T1 lesions have different prognoses [17]. However, some reports suggest that the separation of these categories is unreliable in clinical staging [16, 32].

Histological grade of superficial tumors is closely related to the likelihood of progression [17]. However, the role of subjective histological grading as a prognostic factor is a matter of controversy due to low inter-observer reproducibility [31].

The presence of in situ atypia with grossly normal mucosa, size [17] and multiplicity of the tumors [30]

are also well-known prognostic markers. A large number of studies have shown that histoquantitative methods can accurately categorize bladder tumors into prognostic groups [5, 27, 8, 24]. Cellular DNA content determination by flow cytometry seems to be a useful prognostic tool, aneuploidy being related to invasive potential in SBC [38]. In particular, variables related to cell proliferation are accurate predictors of progression and survival [22, 26, 24].

One of the most widely recognized proliferation estimating methods has been the Ki-67 antigen, the expression of which has been shown to have strong prognostic value [6, 13]. The new Ki-67 antibodies monoclonal MIB-1 and MIB-3 [18], a polyclonal antiserum against Ki-67 antigen [19] and an antigen retrieval method, based on microwave heating, have made it possible to assess Ki-67 expression in sections obtained from normal paraffin blocks [9]. The role of MIB-1 expression, however, is still incompletely understood in transitional cell bladder cancer (TCC).

In the present prospective study, cell proliferation indices (volume-corrected mitotic index, S phase, MIB-1) were compared with established prognostic factors (stage, grade) and DNA ploidy in order to assess their potential usefulness as prognostic markers in SBC.

Materials and methods

Patients

From December 1991 to March 1994 23 Finnish hospitals randomized 273 patients with newly diagnosed superficial transitiocellular carcinoma of the urinary bladder to three different groups of treatment. In this Finnbladder III trial one group was treated by transurethral resection (TUR) alone, the second group of patients had an instillation of 50 million IU interferon alpha-2b (Introna, Schering-Plough) for 2 h after TUR and the third group had an instillation of 100 mg epirubicin (Farmorubicin, Pharmacia) for 2 h after TUR. The primary diagnostics and staging were done according to the UICC 1978 classification [40]. The initial staging was based on urethrocystoscopy, transurethral ultrasound of the bladder, cytological examination of voided urine and excretory pyelography. Pathological staging and grading were done according to the WHO [28]. Slides from tissue blocks from each participating hospital were sent to the referee pathologist (M.H.) to obtain a uniform diagnosis of T category and grade. The total number of eligible patients was reduced to 207 due to protocol violation, change in T category by the referee pathologist and insufficient sample material.

Histological methods

The histological samples were either preoperative bioptic or peroperative TUR specimens. They were fixed in buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin for histological examination. The samples were graded histologically according to the WHO [28] in a blinded manner, i.e., with no knowledge of the clinical data.

Mitotic index

The mitotic figures were counted using an objective magnification of ×40 (field diameter = 490 μm). The mitotic figures were identified as described earlier [2] from the most cellular areas of the tumor samples, avoiding necrotic areas in a blind manner (P. L.). The mitotic activity was measured using the M/V index method, which was originally described by Haapasalo and Collan [15]. In this method the fraction of neoplastic tissue is simultaneously recorded along with the mitosis count (in the present study ten consecutive fields correspond to 1.94 mm^2 in a section). The M/V index expresses the number of mitotic figures/square millimeter of neoplastic tissue. The reproducibility of the M/V index in TCC has been tested previously [10, 23]. The counting of mitotic figures could be reliably (the specimen was representative and contained sufficiently cancer tissue) done in 206/207 (99%) of the sections.

Flow cytometry

Fifty-micrometer-thick sections were prepared using a standard method described previously [24]. Sections were treated with 10 μg ml proteinase K (Sigma, St Louis, Mo., USA) for 30 min at room temperature. After incubation, the nuclei were treated with 10 μg ml RNAse and stained with 25 μg ml ethidium bromide (Sigma) for at least 1 h. The DNA was determined by flow cytometry (FACScan, Becton Dickinson, Mountain View, Calif., USA) using excitation at 488 nm at 200 mW. The total emission above 560 nm was recorded. As the staining intensity of fixed nuclei varies from one sample to another, no internal standard was added. The lowest peak was given a DNA index (DI) value of 1.00, and the DIs of other peaks were calculated using this as a reference. The histograms were interpreted by one of us (S. N.) in a blind manner. The percentage of cells in the S-phase fraction (SPF) was calculated by using the Cellfit program of the FACScan flow cytometer and manually by a modified rectilinear method [4, 7] in 165/207 (79%) of the tumors. If the automatic and the manual methods gave different results, the lower SPF was chosen. Tumors with a DI of 1.00 were designated diploid, and those with a DI > 1.00 were considered aneuploid. The DI was available in 166/207 (80%) of cases and S-phase fraction in 165/207 (79%) of cases.

Immunohistochemistry

Ki-67 nuclear antigen (MIB-1)

From routine processed representative paraffin blocks 5-μm sections were cut and placed on poly-L-lysine-coated slides. Drying of the sections at 37 °C overnight was followed by dewaxing and hydration. The sections were dewaxed in xylene and rehydrated in a graded series of ethanol to water. For the antigen retrieval, citrate buffer (pH 6.0) was used in a microwave processor. The sections were treated twice for 7 min at 850 W power in a household microwave oven, after which the sections were allowed to cool in the buffer for 30 min. For the immunostaining of Ki-67 antigen, the monoclonal antibody MIB-1 (IgG1, Immunotech SA, Marseille, France) was used at a 1:40 concentration. The sections were incubated overnight at +4 °C and the primary antibody was demonstrated with a streptavidin-biotin technique (Zymed Laboratories, Calif., USA). The counterstaining was done using 0.4% ethyl green in acetate buffer for 15 min.

Quantitation of immunohistochemistry

The stainings were evaluated by one observer (T. L.) using a computer-assisted image analysis system (CAS-200 Software, Beckton

Dickinson, USA). The microscope-based system was equipped with two cameras that convert the image of immunopositive areas (brown) and immunonegative areas (green) in nuclei to computer processing. The proliferation index was the percentage ratio of brown and green images. In this study, the index defines the percentage area of immunopositivity in nuclei (area-related proliferation index).

The microscopic fields (×400 magnification, $n = 20$) had to be representative of the proliferative activity of the tumor tissue. Only neoplastic cells were included in the analysis. Necrotic and technically poor (damaged by electrocoagulation during TUR) areas were omitted. The quantitation of MIB-1 score could be reliably done (the result of immunostaining was acceptable, there was no confounding background staining and the specimen was representative, containing sufficient cancer tissue) in 196/207 (94%) of cases. The method has been tested in previous studies [34, 35].

Statistical methods

One-way analysis of variance (ANOVA) was carried out to study the difference in the proliferation status of various histological grades and stages. To compare associations between each pair of groups, the Bonferroni method was used. Associations between ploidy/aneuploidy with tumor histological grade and stage were evaluated by Fisher's exact test (χ^2-test).

Results

The clinical data and the distribution of patients in the pathological stages and WHO grades are shown in Table 1. Mitotic index was highly significantly related to tumor grade ($P < 0.001$, $F = 45.64$, Table 2) and T category ($P < 0.0001$, $F = 104.68$, Table 3). There was a considerable variation of mitotic activity within the WHO grades and the variation was largest within grade II tumors (M/V index between 0 and 89). The distribution of tumor ploidy in different grades is shown in Table 2. The ploidy of the tumors correlated highly significantly with tumor grade ($P < 0.00069$, G1 vs G2, $P < 0.00014$, G2 vs G3), high-grade tumors being more often aneuploid. In addition T1 tumors were significantly more often aneuploid than Ta tumors ($P < 0.0001$, Table 3). There were significantly more cells in S phase in grade II than grade I tumors ($P < 0,001$) and correspondingly the same observation could be made between grade II and grade III

Table 1 Clinical data and the distribution of patients into pathological stages and WHO grades

Number of patients	207		
Mean age at diagnosis (years)	65.7 (range 30–89)		
Females/males	55/152		
Histological grade	Ta	T1	Total
1	104	–	104
2	57	20	77
3	8	18	26
Total	169	38	207

($P < 0.001$, $F = 61.12$, Table 2). S-Phase fraction also correlated significantly with T category ($P < 0.0001$, $F = 68.73$, Table 3).

The percentage of MIB-1 positive cells varied between 0% and 66.9%. In low-grade tumors only a few nuclei are stained positively (Fig. 1), whereas in aggressive tumors most of the nuclei were positive for MIB-1 (Fig. 2). The mean values were 6.4% (grade I), 14.6% (grade II) and 28.6% (grade III). The MIB-1 score correlated highly significantly with tumor grade ($P < 0.001$, $F = 61.39$, Table 2) and T category ($P < 0.0001$, $F = 74.23$, Table 3). Also the MIB-1 score was variable in all WHO grades and the largest variation was seen in grade II tumors (range 0.7–48.9). Many overlaps were found between grade I and grade II cases but only a few overlaps were seen between grade III and grade I/II tumors.

MIB-1 score correlated highly significantly with S phase ($N = 165$, $P < 0.0001$, $F = 42.21$), with DI ($N = 166$, $P < 0.0001$, $F = 52.22$) and with M/V index ($N = 196$, $P < 0.0001$, $F = 126.59$).

Discussion

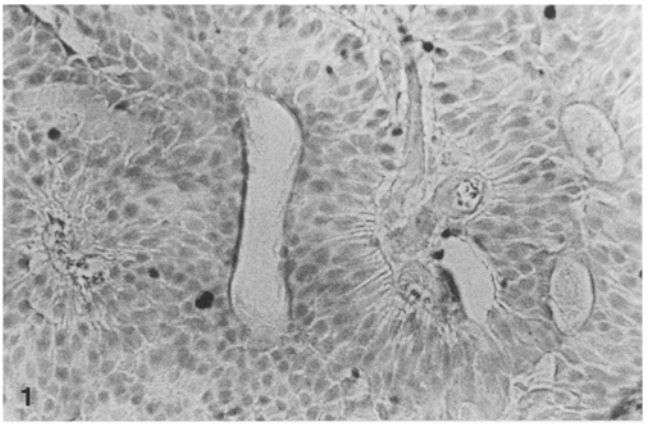
Cell proliferation in bladder tumors is related to histological dedifferentation and prognosis [26]. Methods that have been used to measure cell proliferation, so far, include flow cytometry [5, 38], mitotic indices [26], Ki-67 immunostaining, bromodeoxyuridine incorporation [39], proliferating cell nuclear antigen (PCNA)

Table 2 M/V index, MIB-1 score, S-phase fraction, Mean + SD and DNA ploidy in grade 1, 2 and 3 bladder carcinomas

Variable	All series	Grade 1	Grade 2	Grade 3	*P* value
M/V index	9.6 ± 12.9 (range 0–89)	3.9 ± 5.3 (range 0–42)	11.5 ± 14.7 (range 0–89)	25.9 ± 13.4 (range 1–63)	<0.001
MIB-1 score	12.3 ± 12.2 (range 0–66.9)	6.4 ± 6.6 (range 0–38.3)	14.6 ± 11.3 (range 0.7–48.9)	28.6 ± 14.7 (range 4.2–66.9)	<0.001
S-phase fraction	11.3 ± 7.8 (range 0.5–42.9)	7.7 ± 3.9 (range 0.5–18.8)	13.8 ± 7.9 (range 1.9–42.9)	21.8 ± 8.7 (range 6.4–38.2)	<0.001
DNA ploidy	Diploid 136	Diploid 80	Diploid 50	Diploid 6	<0.00069 G1 vs G2
	Aneuploid 30	Aneuploid 2	Aneuploid 14	Aneuploid 14	<0.00014 G2 vs G3

Table 3 M/V index, MIB-1 score, S-phase fraction (mean, SD) and DNA ploidy in Ta and T1 bladder carcinomas

Variable	Ta	T1	*P* value
M/V index	6.4 ± 8.7	25.3 ± 16.7	<0.0001
MIB-1 score	9.24 ± 7.98	25.34 ± 14.87	<0.0001
S-phase fraction	9.3 ± 5.5	19.0 ± 10.4	<0.0001
DNA ploidy	Diploid 122 Aneuploid 9	Diploid 14 Aneuploid 21	<0.0001



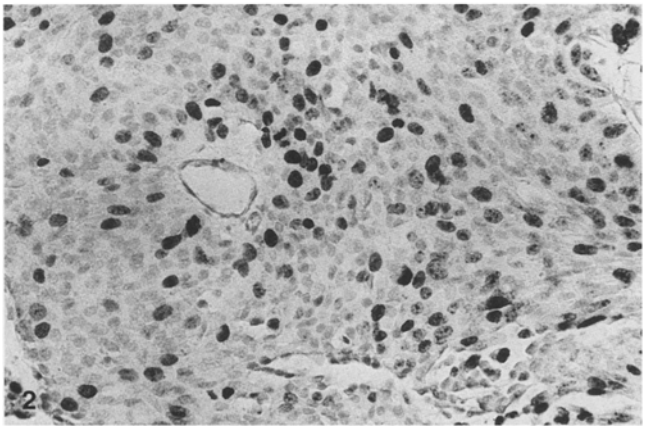


Fig. 1 A papillary WHO grade 1 TCC with only a few MIB-1-positive nuclei, ×400

Fig. 2 A WHO grade 3 TCC, in which most of the nuclei are positive for MIB-1, ×400

expression and silver-stained nucleolar organizing regions (AgNORs) [22, 25]. It has been shown that the importance of histological grading increases as the disease becomes invasive, whereas in local, superficial tumors the proliferation rate seems to give the best prognostic estimates [26]. These conclusions are based on analyses of retrospective patient cohorts.

Several publications have reported Ki-67 expression in a large variety of neoplasms. Ki-67 antigen has recently been characterized as a bimolecular complex of molecular weight 345 and 395 kDa [14]. The gene encoding the human Ki-67 antigen has been cloned and sequenced, and it has been assigned to chromosome 10 (10q25) [12]. Ki-67 is a requirement for DNA synthesis and the antigen is expressed in all active phases of the cell cycle (G1, S, G2 phases and mitosis) [13]. Despite the generally accepted reliability of Ki-67 immunohistochemistry, recent observations have shown that the method overestimates growth fraction in vivo and that there is a loss of correlation between Ki-67 expression and proliferation in nutritionally deprived cells [3, 37]. Also, Ki-67 immunoreactivity (MIB-1, respectively) has been shown to be weakened by prolonged fixation times [29]. Sallinen et al. compared Ki-67 (MIB-1), PCNA and S-phase fraction in prediction of tumor proliferation and prognosis of brain tumors [35]. The authors concluded that the MIB-1 immunohistochemistry was the most informative of the methods analyzed in the assessment of tumor proliferation. MIB-1 generally showed the best sensitivity and specificity in placing patients correctly into the groups of survivors and nonsurvivors.

After new monoclonal antibodies (MIB-1–3) had been developed [18] and an antigen retrieval method [9] was available, it was tempting to test how MIB-1 score, M/V index, S-phase fraction and DNA ploidy correlated with established prognostic factors in superficial bladder cancer. To our knowledge only one study has reported data on MIB-1 expression in transitional cell carcinoma of the bladder. In that small series of 50 patients, Pich et al. compared different quantitative proliferation indices with tumor grade and invasion and found a significant relation [33].

In the present study of 207 superficial TCCs of the urinary bladder, the association of MIB-1 score, M/V index, flow cytometric S-phase fraction and DNA ploidy with histological grade and T category was highly significant. The proliferation indices and DNA ploidy were also highly significantly interrelated.

Flow cytometric quantitation of proliferation (S-phase fraction) is very cell cycle-specific, semiautomatic and fast. It also uses a large number of nuclei in the analysis. However, the analysis lacks morphological control. Therefore, intratumor variation, tumor necrosis and non-tumoral cells may significantly weaken the reliability of the method. The method also needs expensive equipment and somewhat complicated tissue processing.

It has been suggested that the grading of superficial TCCs could be based on the M/V index alone [26]. An advantage of the M/V index method is its high reproducibility and the simplicity of the assessment [15, 23]. The M/V index can be determined in routinely processed paraffin-embedded sections in 2–8 min depending on mitotic frequency. Moreover, the M/V index method also allows the visualization of proliferating cells in their local context and thus an adequate histopathological assessment is possible simultaneously.

The subjective grading systems are based on the evaluation of special cellular features and some estimate of the presence of mitotic figures in the sections [28]. They all share the common characteristic of being variable between observers and thus the prognostic estimates are also variable [31]. In superficial bladder carcinoma, routine histopathological parameters fail to differentiate those cases which are unlikely to progress, and thus could be treated conservatively, from those cases which will progress and need early aggressive therapy. In TCC [38, 5, 27, 26, 8] as well as in several other neoplasms [11, 1] there is a rather benign group of tumors and a highly malignant one. Tumors placed in the grade 2 category create a problem for the clinician. Therefore two-grade systems have been presented. A two-grade system might provide a more reliable basis, since there is only one grade limit in comparison to the three-grade system [10].

A number of two-grade systems have already been presented for TCC that are based on flow cytometry [38, 20, 27, 5] or morphometry [5, 21]. Carbin and coworkers presented a modified Bergkvist grading system, including subjective and quantitative elements, which correlated well with prognosis [8]. Malmström et al. combined flow cytometric data and the results from blood group immunohistochemistry into a new grading system for TCC [27]. Lipponen et al. found that WHO grade 2 tumors can be efficiently categorized into prognostic groups by the M/V index [26]. In superficial TCCs the M/V index appeared to be the best histological variable for prognostic purposes [26].

In the present study MIB-1 immunohistochemistry seemed to be a promising method in the evaluation of tumor proliferation. MIB-1 immunostaining is inexpensive and quantitation can be done by a relatively simple procedure [35]. It also allows the simultaneous histopathological evaluation of tumor morphology. Computer-assisted image analysis system seems to improve the reproducibility of the assessment, making the analysis faster although more expensive [34]. In this study there was a wide range of MIB-1 immunoreactivity especially in moderately differentiated bladder carcinomas. This might contribute to a more reliable placement of bladder cancers in different prognostic groups.

In conclusion, the results of this study show a highly significant correlation between quantitative cell proliferation indices and histological grade of urinary bladder transitional cell carcinomas. The prognostic potential of the new proliferation marker MIB-1 remains to be seen after a follow-up period in this same prospective material.

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