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Determination of DNA ploidy and ABH antigen reactivity in both frozen and formalin-fixed bladder tumor tissue

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Summary. In a prospective series of patients with transitional-cell bladder carcinomas, DNA ploidy and ABH antigen reactivity were determined in both frozen tissue and formalin-fixed paraffin blocks. ABH antigen measurement in frozen tissue was hampered by methodological problems, especially insufficient morphology, which made quantification difficult. In only 38/55 (69%) cases was the same ABH reactivity found. The knowledge of secretor status was not helpful in the interpretation of these results. In 53/59 (90%) cases the same DNA ploidy was achieved, resulting in good correlation between flow cytometric DNA measurements in formalin-fixed and frozen tissue. The deviations occurred mainly in cases exhibiting a tetraploid DNA profile. DNA assessments using image cytometry on imprints of fresh tissue was a rapid and reliable method in our hands, with identical results being obtained in 32/36 (89%) cases.

Key words: Bladder carcinoma – DNA ploidy – Blood group isoantigens – Fixation methods

Bladder cancer is a heterogeneous disease with an unpredictable clinical course. New treatments for bladder carcinoma demand better selection of patients at risk for invasion and metastasis. In addition to clinical staging, histopathological grading is a cornerstone in clinical decision making regarding treatment. However, because of limitations in these prognostic indices, many other biological markers have been tested.

We have previously reported the results of a follow-up study using the combination of blood-group ABH antigen status and DNA ploidy as prognostic factors in a retrospective consecutive series of 230 patients with transitional-cell bladder carcinoma [7, 10]. These determinations were done in formalin-fixed tissue, which is considered to be less suitable than fresh or frozen tissue. Moreover, the secretor status of the patients, a factor that influences ABH antigen reactivity, was not known.

The purpose of the present study was to evaluate these factors in prospective material with the aim of determining blood-group ABH antigen status and DNA ploidy in frozen and formalin-fixed tissue from patients with known secretor status.

Materials and methods

The present study included all patients who had been newly diagnosed as having bladder tumors and for whom histological material was available at Uppsala University Hospital during the period 1985–1989. The total series consisted of 113 patients.

The immunostaining method for the A, B and H isoantigens employed mouse monoclonal antibodies against the A and B antigens and the *Ulex europaeus* lectin for identification of the H substance. A biotinylated rat monoclonal antibody against mouse immunoglobulin was used as the second layer and a commercial avidin-biotin-peroxidase complex was applied as the third. Evaluation of the results was based on both the extent and the intensity of the staining. A semiquantitative scale was used, each tumor being given a score of between 0 and 12. Tumors with scores of ≤ 4 represented only weak, if any, expression and were classified as ABH-negative; those with a score of >4 represented strong expression and were categorized as ABH-positive. Details regarding this method have been described elsewhere [2].

For determination of DNA ploidy, fresh tissue specimens were stored at −70°C until the time of analysis. DNA staining was done as described by Vindelöv et al. [13], using propidium iodide (PI) containing a detergent (Nonidet P40) and RNAse. For determination of DNA ploidy in fixed tissue, sections measuring 100 μm in thickness were cut from paraffin-embedded blocks. The sections were immersed in xylol and rehydrated with alcohol and, finally, with distilled water [3]. The tissue was minced into small pieces, and enzymatic dispersion and digestion were achieved using 0.2% protease (type 7 from a strain of *Bacillus subtilis;* Sigma, St. Louis, Mo., USA). The suspension was vortexed and tissue clumps were removed by filtration. The single-cell suspensions were centrifuged, the supernatant was removed and the pellet was resuspended in phosphate-buffered saline (PBS). The suspension was added to a 400-μl Percoll solution (Pharmacia AB, Uppsala) 45% in PBS and then centrifuged. Three layers were formed: a supernatant, a thin nuclear band and the Percoll cushion. The fractions were separated and diluted, following which 15 μl PI was added. After 15 min the samples were ready to be measured. The thin band usually gave the best histogram. The cellular DNA content was measured with a

Table 1. Correlation between ABH measurements in 55 frozen and formalin-fixed bladder tumors

		Formalin-fixed		
		ABH-positive	ABH-negative	Totals
Frozen	ABH-positive	19	11	30
	ABH-negative	2	23	25
	Totals	21	34	55

Table 2. Correlation between DNA ploidy in 59 frozen and formalin-fixed bladder tumors

		Formalin-fixed		
		Diploid	Aneu-ploid	Totals
Frozeny	Diploid	22	1	23
	Aneuploid	5	31	36
	Totals	27	32	59

Leitz flow cytometer. Details regarding the method have been described elsewhere [9].

The imprints were made from fresh tissue and air-dried. The nuclei were later fixed in 10% formalin and then Feulgen-stained. DNA measurements were carried out using computer-assisted image cytometry (Imtec, Uppsala).

Results

Sufficient histological material for the four different measurements was available from 59 patients. In 55 cases, ABH reactivity could be determined in both frozen and formalin-fixed tissue; 4 were excluded because of equivocal antigen reactivity. In 42 cases similar results were achieved. Of the remaining 13 cases, 11 lost their ABH antigen on formalin fixation (Table 1); in 5 of the latter, the secretor status was known and all but 1 were secretors. This is an indication that false-negative findings in formalin-fixed tissue cannot be explained by secretors status.

In material from 59 patients, DNA ploidy could be determined in both frozen and formalin-fixed tissue (Table 2). Identical DNA histograms were obtained in 53 cases. In 1 specimen, formalin-fixed material was judged to be aneuploid; on review, it was found to be a small tumor that was not represented in the frozen tissue. The remaining 5 cases that were considered to be diploid in formalin-fixed tissue and non-diploid in frozen tissue proved to be peritetraploid, exhibiting small peaks.

In material from 36 patients, DNA ploidy was also measured on imprints from fresh tissue. In 32 (89%) cases, the DNA histograms were identical with those obtained from flow cytometry in frozen tumor tissue; 2 cases that were judged to be diploid using the single-cell method and non-diploid on flow cytometry proved to be peritetraploid, displaying small peaks. The remaining 2 cases were non-diploid as determined by single-cell cytometry and diploid as assayed by flow cytometry. The reason for the difference was the DNA heterogeneity, which created a selection fault.

Discussion

Since 1961, when Kay and Wallace [5] reported the correlation of ABH antigen deletion with tumor aggressiveness, numerous studies on this subject have been conducted. The earlier studies used the specific red-cell adherence test and underscored the predictive value of these determinations. However, the reproducibility of this test was limited and the amount of clinical material studied was small. Later studies [1, 8] used monoclonal antibodies and a standardized technique, but the prognostic value became more doubtful. These studies were mainly performed on formalin-fixed tissue.

The heterogeneity in antigen reactivity, as with most immunohistochemistry, makes quantification difficult. Deletion also occurs in normal urothelium and benign disease [2, 11]. A prospective study [1] using frozen sections recently reported that ABH expression was of no value in predicting the prognosis for an individual patient.

In the present study, ABH antigen measurement in frozen tissue was hampered by methodological problems, especially poor morphology, which made quantification difficult. We found a poor correlation with determination of ABH expression in formalin-fixed tissue. The knowledge of secretor status did not influence these results. Thus, ABH antigen determinations showed no proven clinical value in our hands.

There are important differences between the methods used in paraffin-embedded material and those used in fresh or fresh, fixed tissue for flow cytometric measurements. The advantages of paraffin-embedded methods include the possibility of retrospective studies as well as prospective investigations that do not require immediate handling of the specimen. They also enable the accurate selection of regions to be studied by microscopic review because of good morphology.

The disadvantages of using paraffin-embedded rather than fresh material for DNA determintions are [12]:

1. Such methods are time- and labor-intensive.
2. The quality of DNA histograms are usually inferior, and 10%–15% of specimens are of such poor quality that they are considered to be uninterpretable.
3. The degree to which a specimen enables access of the DNA stain to nuclear DNA molecules is highly variable, which precludes the use of an external diploid standard.
4. The need for thick tissue sections may result in the rapid consumption of paraffin blocks.

In spite of these observations, in the present study we found good correlation between DNA measurements in formalin-fixed and frozen tissue, which confirms our previous results [9]. Image cytometry using imprints from

fresh tissue was reliable in our hands. This technique requires relatively few cells and is rapidly and easily carried out, which makes it convenient for routine clinical use.

There is no universal agreement on the prognostic value of DNA measurements in bladder cancer. In fact, several recent studies have found that this technique failed to provide additional information [4, 6]. Tumor markers are generally tested against histological grading and T staging. However, both grading and T staging are hampered by poor reproducibility; thus inconsistent grading and staging can give a new tumor marker false-negative or false-positive prognostic value. To improve the prognosis for bladder cancer, we believe that first of all, histological grading and T staging need to be better standardized and made objective. We are currently investigating the use of computer-assisted image analysis of patterns and mitotic frequency to improve grading and the application of transmural needle biopsy to render staging more valid. Second, new tumor markers should be tested against stage and grade in large prospective studies in which the treatment has been standardized so as to exclude the latter as a confounding factor.

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