Review

DNA Image Cytometry in Bladder Cancer: State of the Art

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Abstract. *The most recent Consensus Review of the Clinical Utility of DNA Cytometry in Bladder Cancer, which took place in Maine back in 1992, focused solely on flow cytometry results. Since then, there have been a significant number of articles published on the use of image cytometry to evaluate DNA content in bladder cancer. This has meant that a large proportion of the information collected is scattered across the published literature. The purpose of this article was to organize the data referred to in those articles published since the 1992 Consensus Review, and organise it under three major topic headings: a) DNA image cytometry versus flow cytometry, (b) specimen sources, and (c) its clinical utility with regard to improving prognosis and recurrence detection. A variety of factors and issues are discussed and points have been raised for discussion. Prospects for the future and potential research areas are also suggested.*

In 1992, a DNA Cytometry Consensus Conference (1) was held in order to evaluate the clinical applications and technical aspects of DNA ploidy analysis in patients with transitional cell carcinoma of the bladder (TCC). A highly significant review of the most representative results published on this issue took place at this gathering, which led to the establishment of a series of consensus conclusions and guidelines. However, flow cytometry (FCM) analyses were the only form of analysis considered, the reasoning behind this decision being a perceived paucity of reports on image analysis in bladder cancer.

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A number of guidelines were laid down with regard to the application of FCM in the DNA content analysis of bladder samples, specimen sources, preparation and staining, and data analysis. Various technical factors, such as optimal staining protocols, biological specimen and key factors of histogram interpretation were also discussed.

Following the recommendations that resulted from this Consensus Conference, the last fifteen years have seen the appearance of several published articles on the use of image cytometry (ICM) to evaluate DNA content in bladder cancer in a bid to define its usefulness from a clinical and prognostic perspective. Some studies compared ICM with FCM, while others examined its association with other biological markers. The purpose of this review was to help to organize this widely scattered collection of data and to discuss certain aspects of the correlations discovered and the prognostic value identified in the distinct studies, and then compare these findings with our own experience.

DNA Image Cytometry vs. Flow Cytometry

The DNA content of neoplastic cells is generally evaluated using FCM, though quantitative microscopic image analysis is a reliable alternative technique for quantifying of DNA content. Both methods have been used to measure the extent of genetic alteration in bladder lesions. Despite the positive overall correlation that the published literature draws between these two methods particularly when it comes to cytological specimens (2, 3), they each have their own particular advantages.

FCM is quicker to perform than ICM, enabling a greater number of cells to be measured and therefore resulting in a lower coefficient of variation (CV). Offsetting these positive features, the sensitivity of FCM analysis may suffer from a lack of necessary visual discrimination to enable non-tumour

cells and artefacts to be excluded from the measurements. At the aforementioned Consensus Conference (1), the need to enrich the proportion of tumour cells present in order to achieve an adequate level of sensitivity when using FCM was highlighted, especially with paraffin-embedded material where section trimming is sometimes required. The relatively small number of cells in each sample can also make the FCM analysis less reliable (4-6).

These issues can be avoided with ICM, by microscopically selecting only the tumour cells. ICM's higher sensitivity when used on small populations of aneuploid cells is noted by several authors (2, 3, 5-13), particularly in tetraploid cell clones (9, 10). Nevertheless, this relatively high resolution can wane in those histograms with near-diploid cell populations, which due to this technique's higher CV cannot be accurately classified as true near-diploid or right shifted diploid populations (2, 3, 6).

The need for ICM to be performed by skilled operators and the fact that it is a very time-consuming method are both significant limitations and combine to reduce its cost-effectiveness (6, 14). Moreover, the interactive operator-guided selection of the nuclei can cause selection bias and low reproducibility (6). Some authors claim that these disadvantages can be offset by an automated image cytometry system (6, 15). Baak *et al.* (6) proposed a highly automated system, developed in their own laboratory, based on an automated motorized microscope and scanning platform. Measurement efficiency is improved using densitometric and geometric filters based on quantitative cell features, such as area, integrated optical density, membrane roundness, convex perimeter and curve width. That said, these authors did mention that the system had some weaknesses, given that single-cell preparation is required and the resulting histograms can sometimes be inadequate. According to the authors, these kinds of automated systems can bring CV values and the time required for each measurement very close to those obtained using FCM analysis. In our opinion, the lower CV value and time required are of course important advantages of these more automated systems. However, operator experience is one of the most important features of ICM and should not be restricted by automatic analysis. This experience is vital for distinguishing between certain neoplasic and preneoplastic lesions, whether pathological or reactive cells, and offers a high level of sensitivity when it comes to detecting small aneuploid peaks. In future, image analysis systems should take these two differing points of view into consideration.

DNA Image Cytometry and Specimen Sources

According to the guidelines set out at the 1992 Consensus Conference, voided urine was not suitable for DNA FCM analysis due to poor cell yield and the cellular aggregation often presented in these samples (1).

The specific features of ICM, as mentioned in the previous section, make it a suitable method for studying cytological samples such as voided urine and bladder washings. Nevertheless, certain samples should be analysed with caution. A case in point are the nuclei of superficial (umbrella) cells that often have abnormal DNA content, which may cause abnormal DNA ploidy results in cytomorphologically normal bladder washes. Umbrella cells should be avoided in the evaluation of urine samples (16).

In the study carried out by Forte *et al.* (17), the DNA ICM results obtained from different specimen sources: cytologic smears, cytocentrifuge samples, tissue sections (5-micrometer sections) and nuclei extracted from paraffin-embedded tissue were compared. Using a binary DNA classification scheme (diploid or aneuploid), they found comparable DNA content results across the different samples: bladder washings and tissue section results coincided in 40 out of 43 patients.

Mainguene *et al.* (18) observed differing results when they compared touch imprints with formalin-fixed paraffin-embedded samples. They made imprints of fresh specimens from 31 urothelial carcinomas biopsies and cell suspensions were obtained from dewaxed tissue sections (7 microns) using the Hedley procedure (18). These authors stated that imprints were a simple and reliable procedure for DNA ploidy assessment which were particularly sensitive when it came to detecting small aneuploid peaks and multiploid tumours.

As far as our experience with a variety of biological tissues are concerned (*e.g.* breast, bladder and lymph nodes), cytological preparations, especially from fresh material, provide highly suitable source material for DNA ICM evaluation, with CV values near those obtained by FCM. However, in those studies where different compartments of the tissue must be evaluated, namely where the adjacent mucosa needs to be evaluated, then tissue sections must be used. However, there are certain technical problems that must be considered when using these tissue sections. These include: section thickness, sliced nuclei resulting in a low DNA ploidy estimate, overlapping nuclei that result in an overestimation of nuclear DNA content, and the time-consuming analysis that requires many nuclei be sliced apart or rejected (19). We believe that the 6-Bm section thickness used in our laboratory is a suitable compromise between the need for minimal nuclear overlap and sharp focusing, and the need for full nuclear thickness. Given the DNA content estimates made from sliced nuclei, internal reference cells should be used rather than external populations. These internal reference cells may be infiltrated lymphocytes or granulocytes present in the tissue, or normal epithelial and stroma cells as stained in the ESACP DNA Consensus in Image Cytometry (20). In our own experience, infiltrated

lymphocytes are a suitable reference cell population. When cytological specimens are used and external reference cells are required cells such as rat liver hepatocytes or lymphocytes, from the same animal species as the pathological cells, can be used. In a previous study using normal mice we evaluated imprints from a variety of sources (spleen, lymph nodes and thymus). We found that spleen samples were the material best suited to DNA content evaluation, due to the higher number of intact and non-overlapping lymphocytes and better-stained samples (21). These external reference cells should be prepared and fixed in the exact same way as the cells under analysis.

DNA Image Cytometry and Clinical Utility

Screening. One of the conclusions drawn by the Consensus Conference was that DNA FCM should not be used for screening for bladder cancer nor for investigation of microscopic hematuria. In spite of its higher sensitivity for detecting bladder cancer, in comparison with urine cytology, its specificity remains too low especially in low-risk patients (1). In the years that followed this Consensus a number of articles have evaluated the clinical utility of DNA ICM analysis, compared with conventional urinary cytology (11, 22-28) or with others methods such as fluorescence *in situ* hybridization (FISH) (5, 11, 29, 30), or immunocytology (24). In general terms, the authors agree that DNA ICM has a higher level of sensitivity than cytology, particularly in grade 1 tumours. Planz *et al.* (23) studied spontaneously voided urine from patients with histologically proven TCC and patients with benign bladder diseases and found that DNA ICM was significantly more sensitivity for detecting grade 1 tumours (70.4%) than cytology (26%).

Despite these results, Shabaik *et al.* (22) alert readers to the fact that some low-grade lesions with DNA diploidy and a low proliferation index can pass undetected by ICM. Furthermore, Katz *et al.* (31) point out that in patients with no history of TCC, abnormal DNA ICM patterns can occur after chemotherapy, radiation therapy, or viral infection. They suggest that a combined approach using both cytology and DNA ICM, alongside an accurate record of the clinical history is essential for correct diagnosis.

DNA ICM sensitivity can be increased with the use of other stemline analysis systems, when compared with the conventional classification based on the 2c deviation index (23). Some authors suggested the stemline interpretation system presented by Böcking *et al.* (32) which used the Kolmogoroff-Smirnow test (23, 33, 34), while others developed their own analysis system (27).

An image analysis system described in the literature and referred to as a test for bladder tumours is the Quanticyt Karyometric System. This automatic image analysis system evaluates the nuclear shape and DNA content of exfoliated cells in bladder wash specimens to detect bladder cancer and/or identify individuals at low or high risk for bladder cancer development (13). According to Lokeshwar *et al.* (25) this system may overestimate the risk of urothelial abnormalities and, therefore, have a lower specificity than bladder wash and urine cytology. Furthermore, the low number of urothelial cells or an abundance of leucocytes and erythrocytes present in the sample may limit its use.

Urine cytology is still very widely used for diagnosing bladder cancer, in spite of its limited sensitivity and subjective nature, due to its high specificity. Therefore, cytology could be used in combination with DNA ICM and others methods such as FISH to improve overall sensitivity levels without affecting the specificity. More studies ought to be carried out to evaluate this combined method given the relatively expensive and time consuming nature of ICM and FISH, prior to these methods being established as clinically routine.

Prognosis. Transitional cell carcinoma of the bladder is a tumour with varying biological potential and, therefore, with a wide spectrum of risks of recurrence or progression. The recurrence rate ranges between 50% to 70% and as many as 19% of pTa and 34% of pT1 tumours will progress into muscle-invasive tumours (14). With regard to this group of superficial TCC (sTCC), excluding the high-risk tumours (T1/G3 and *in situ* tumours), there is no established consensus treatment, and the classical histopathological markers do not define risk profiles. Consequently, there are no optimal treatment approaches or follow-up schedules (35). Several biological variables have been studied in order to try and obtain additional information that would facilitate patient treatment selection and improve patient survival.

Based on a review of the available literature and in reference to sTCC (Ta, T1 TIS), the Consensus Conference stated that DNA FCM correlates with grade and stage, and provides prognostic information regarding stage progression, muscle invasion and metastasis. The authors stated that the DNA ploidy status listed in order of decreasing risk of progression was: multiple DNA aneuploid populations > single DNA aneuploid populations > DNA "tetraploid" population > DNA diploid populations (1). With regard to muscle-invasive tumours (T2-T4), the Consensus concluded that the data concerning the role of DNA FCM was inconclusive and therefore did not provide a sound basis for treatment recommendations (1).

Over the last fifteen years, several studies have evaluated the prognostic value of DNA ICM in sTCC. A variety of cytometric parameters have been singled out as being useful in predicting survival and progression-free survival in this group of bladder cancer patients: DNA ploidy (6, 14, 36-43), mean ploidy (44), 2cDI (8, 44-46), proliferation index (47), DNA index (6), percentage of hyperdiploid and hypertetraploid cell

nuclei (48), and 5cER or percentage of polyploid cells (36, 46, 49). Some authors wrote that this prognostic value is particularly important in grade 2 tumours (17, 48, 50). This group of tumours is very heterogeneous in respect of their clinical behaviour and DNA ploidy, although they exhibit the same degree of histomorphological differentiation. According to al-Abadi and Nagel (50), these tumours can be subclassified as aneuploid (biologically aggressive), diploid or tetraploid (biologically less aggressive). Decaestecker *et al.* (48) evaluated nine different ploidy-related parameters *via* a computer-assisted method, selecting two of them (percentage of hyperdiploid and hypertetraploid cell nuclei) which enabled the identification of two subgroups within grade 2 tumours: ones which exhibit clinical behaviour similar to grade 1 tumours and a second subgroup which exhibit clinical behaviour similar to grade 3 tumours.

Our group, based on the findings of experimental studies on laboratory animals (rats and mice), also reported this discriminatory potential between lesions with the same degree of histomorphological differentiation showed by DNA ICM: the same histological lesion revealed differing aneuploidy frequencies depending on the length of exposure to *N*-butyl-(*N*-4-hydroxybutyl) nitrosamine (BBN) (51-54).

From a review of the literature, it is possible to conclude that the prognostic value of DNA ICM variables in sTCC could be significantly enhanced when associated with others biological parameters, such as morphometric features (13, 37, 38, 42, 43, 45, 55). This advantageous possibility is well-described in studies by van Velthoven's group (37, 38, 42). They used different morphonuclear parameters evaluated by digital cell image analysis in relation to the chromatin pattern. When the information contributed by this quantitative chromatin pattern description was added to DNA ploidy level, histological grading and clinical staging, they obtained a higher prediction value for biological behaviour in sTCC (38).

Another biological marker that has been studied alongside the DNA content status of the sTCC is the proliferation rate. Different proliferation markers have been evaluated such as proliferating cell nuclear antigen expression (39, 40, 46, 56), Ki-67 labelling index (52, 57-60) and mitotic index (58). In general, all these studies refer to an improvement in the predictive value of cytometric features with respect to the potential malignancy of sTCC when measured simultaneously with proliferation markers. They also state that recurring cases are less frequently found in the group of DNA euploid tumours exhibiting a low proliferation rate, in contrast with a very high percentage of recurrence in patients with DNA aneuploid tumours with a high proliferation rate.

As regards muscle-invasive TCC, the prognostic value of DNA ICM is as controversial as DNA content evaluation by FCM. Cai *et al.* (14), based on 10 years of follow-up examinations after surgery on 8 invasive bladder tumours, found no significant difference between patients with a normal DNA content and those with aneuploid DNA content. In contrast, Decaestecker *et al.* (61) state that nuclear DNA content and chromatin pattern-related parameters can provide prognostic information for this TCC group. These authors studied 41 patients and, using a number of image-generated variables produced *via* a computer-assisted methodology, they were able to characterise the tumours associated with a "bad" prognosis in the T2 sub-group and the tumours associated with a "good" prognosis in the T3-T4 one.

These conflicting results in the invasive TCC may be partially related to the kind of patients included in each study. Rotterud *et al.* (62). evaluating 94 patients treated with external pelvic radiotherapy, found a better rate of survival in the aneuploid tumours group compared with their diploid tumours counterparts. According to these authors, this discovery could be due to the increased radiosensivity of aneuploid cells, as demonstrated by others (63-65). More studies with comparable patient groups and treatment schemes are required in order to clarify the prognostic potential of DNA ICM in invasive TCC.

Detection of recurrence (follow-up). Cystoscopy remains the gold standard in the detection of TCC recurrences. Other methods have been studied in an attempt to reduce the frequency of follow-up cystoscopies in patients with a low risk of tumour recurrence, and thus reduce costs and patients discomfort, and can help in the stratification of treatment modalities (13).

For patient follow-up, the Consensus Conference established that taking a bladder irrigation specimen was their preferred technique of sampling the urothelium in DNA FCM analysis. Of the differing cytometric variables related to DNA content, a clear "nontetraploid" DNA aneuploid histogram proved to be the most informative parameter, as far as tumour recurrence and treatment failure were concerned (1). A DNA diploid histogram, however, does not exclude tumour recurrence and should be evaluated carefully (1).

With regard to DNA ICM, a variety of urine sample types have been studied and shown to be suitable for evaluating the DNA content of urothelial cells, such as voided and catheterized urine and bladder irrigation (5, 10, 12, 28, 29, 31, 66-69). As previously mentioned, this is related to the ability of ICM to measure fewer cells in smaller samples and its higher sensitivity for detecting aneuploid cells at extremely low frequency levels, when compared with FCM.

As previously mentioned, a combination of visual cytology of urine specimens with DNA ICM offers a better method for the early detection of recurrent TCC, than cytology alone (5, 12, 29, 31, 66, 67, 70, 71). In the literature, sensitivity

values of between 76-93% were reported as well as a specificity of between 41-94% (12). Different patient selection methods and a different histogram analysis scheme may explain this variation (various cytometric parameters have all been shown to be informative: DNA ploidy, 2cDI, hyperdiploid fraction and 5cER).

Authors are in agreement on one issue, an abnormal DNA content (nontetraploid histogram) is 100% specific for tumour recurrence. For negative cytology and normal DNA content cases, this value is remarkably lower. More sensitive diagnostic tests such as chromosomal abnormalities by *in situ* hybridization are suggested for use in these cases (5, 31, 72). A case in point is the multitarget FISH probe UroVysion, which includes probes for the centromeres of chromosomes 3, 7 and 17, and for the p16 gene at chromosome 9p21 (5). Nevertheless, there are interpretative challenges involved with this kit that are caused by signal overlaps in highly aneuploid samples, while focal plane distortions and high cost place limits on its clinical applications (30).

According to Richman *et al.* (12), when the detection capacity of cytology and DNA ICM are used in combination with each other, their detection capacity is greatest when urinary samples are obtained as close as possible to the moment the disease becomes clinical by evident. The authors stated that the combination can be of predictive value up to three months prior to clinical diagnosis. In this way, patients can be treated earlier and more conservatively.

The literature also made reference to another issue that can influence diagnosis accuracy. This issue is related to the DNA tetraploid tumours, the aneuploid population most frequently missed by FCM. Kline *et al.* (10) found in their study that 45% of cases, within this tetraploid group, exhibit no clinical (cystoscopic) or pathological (cytologic and histologic) evidence of neoplasia, and none of these patients developed tumours during follow-up. The authors suggested that the presence of DNA tetraploidy in cytologically negative cases should be interpreted cautiously. These findings are in agreement with the known occurring physiological polyploidy of urothelial cells. According to Planz *et al.* (23), for example, 4c and 8c ploidies could be detected in 90% of normal urothelial tissue samples. The use of stemline interpretation instead of single-cell interpretation could be used to overcome difficulties in discriminating between physiological polyploidy and the real abnormal tetraploid DNA content. The DNA stemline is defined as the most frequently occurring c value in a tumour cell population, if accompanied by cells at its twofold DNA value (23).

From literature reviewed in this work, it is possible to conclude that DNA content evaluation using ICM provides prognostic information in TCC, especially if other biological parameters, such as morphometric features and proliferation markers, are studied alongside it. This clinical value is not so evident when used in screening and recurrence detection due to the limited specificity of DNA ploidy. However, when combined with visual cytology, it offers a better method of bladder cancer detection than cytology alone.

In spite of the clinical utility showed by the DNA ICM, the heterogeneity between the different DNA parameters and the DNA histogram interpretation system included in each study contributes to some variation in the inter-laboratory reproducibility of the DNA content results. Moreover, the routine clinical use of current image analysis systems is limited because it remains a very time-consuming method and is dependent on the operator's skill level. The more automated image cytometry systems that have been developed and referred to above may overcome some of these drawbacks. However, further improvements are required to reduce the costly and elaborate preparatory procedures needed to produce cellular specimens suitable for automated analysis. These new systems must also consider operators as factors for potential improvement as opposed to limiting variables. This is because operator experience is the single biggest contributor to ICM's sensitivity and discriminatory potential.

DNA ploidy alterations are a global marker of genomic instability and chromosomal derangement. ICM is a suitable method of evaluating this genetic instability in distinct areas of a tissue section. This feature could prove to be a powerful tool in the study of biological mechanisms in the carcinogenesis process.

There is a great deal of published literature related to the study of DNA content in TCC. Most of this was focused on clinical and prognostic correlations rather than the biological mechanisms and possible role of DNA ploidy changes in the therapeutic response level. Following on from these areas of research, our group has published some data that could identify subgroups of urothelial cell carcinomas that correlate with a poor response to current therapies (surgical and chemotherapy approaches) and a less favourable clinical prognosis. This is the case with DNA diploid tumours induced by BBN in rats which were less sensitive to mitomycin C and bacillus Calmette-Guérin instillation (52), and a subgroup of invasive tumours in a series of human TCC that exhibited a decrease in proliferation rate, as measured by Ki-67 immunohistochemistry, despite their more frequently occurring DNA aneuploid pattern (60). The latter group of tumours was confirmed by the results found by Grossman *et al.* (73). In a series of locally advanced bladder cancer that were randomly treated using either neoadjuvant chemotherapy plus cystectomy or cystectomy alone, the results showed a less favourable progression-free survival rate for those patients whose tumours exhibited a lower expression of Ki-67, although it was only marginally significant.

As regards the therapy schemes now available for superficial and invasive TCC, it will be interesting to discover what the distribution of the DNA content pattern really means and if this biological information could help us to better understand and identify those cases which respond poorly to treatment. The issue lies in discovering if DNA ploidy changes could be a critical variable in designing a bladder nomogram, which would help physicians to decide which treatment method will be of the greatest benefit to their patients. For those tumours that do not respond to treatment, other therapeutic targets must be investigated in due course in order to lay down alternative treatment approaches.

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