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Quantitative Nuclear Grade (QNG): A New Image Analysis-Based Biomarker of Clinically Relevant Nuclear Structure Alterations

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Abstract This review addresses the potential clinical value of using quantitative nuclear morphometry information derived from computer-assisted image analysis for cancer detection and predicting outcomes such as tumor stage, recurrence, and progression. Today's imaging technology uses sophisticated hardware platforms coupled with powerful and user-friendly software packages that are commercially available as complete image analysis systems. There are many different mathematically derived nuclear morphometric descriptors (NMD's) (i.e. texture features) that can be calculated by these image analysis systems, but for the most part, these NMD's quantify nuclear size, shape, DNA content (ploidy), and chromatin organization (i.e. texture, both Markovian and non-Markovian) parameters. We have utilized commercially available image analysis systems and the NMD's calculated by these systems to create a mathematical solution, termed quantitative nuclear grade (QNG), for making clinical, diagnostic, and prognostic outcome predictions in both prostate and bladder cancer. A separate computational model is calculated for each outcome of interest using well-characterized and robust training, testing, and validation patient sample sets that adequately represent the selected population and clinical dilemma. A specific QNG solution may be calculated either by non-parametric statistical methods or non-linear mathematics employed by artificial neural networks (ANNs). The QNG solution, a measure of genomic instability, provides a unique independent variable to be used alone or to be included in an algorithm to assess a specific clinical outcome. This approach of customization of the nuclear morphometric descriptor (NMD) information through the calculation of a QNG solution mathematically adjusts for redundancy of features and reduces the complexity of the inputs used to create decision support tools for patient disease management. J. Cell. Biochem. Suppl. 35: 151–157, 2000.

Key words: prostate cancer; bladder cancer; nuclear morphometry; logistic regression; algorithms; image analysis; staging; prognosis

Numerous studies have demonstrated that transformation of a normal cell to a malignant cell requires a series of genetic changes such as mutations, methylation events in promoters or exons, chromosome deletions, insertions, and translocations [Fearon, 1991, 1994; Sidranski et al., 1991; Vogelstein and Kinzler, 1993; Spruck et al., 1994; Lalani et al., 1997; Steiner et al., 1997; Stein et al., 2000]. These genetic alterations have been assessed clinically using individual diagnostic and prognostic tests for specific genes, or more globally by applying modern gene chip and array technologies [Khan et al., 1997; Cairns and David, 1999]. Such sophisticated molecular assessments, though very informative, can be cumbersome. They generate complex data and currently tend to be expensive to perform. DNA ploidy using computer assisted image analysis or cytogenetics is a method of measuring abnormal DNA content, which represents rather large scale chromosomal alterations (i.e. tetraploidy, aneuploidy, hyperploidy, etc.) that usually reflect late stage changes of genetic instability in cancer cells [Bacus and Grace, 1987; Gibas and Gibas, 1997; Slaton et al., 1997; Steiner et al., 1997; Veltri et al., 1998; Wojcik et al., 1998]. Nuclear morphometry information can be derived from the cell images captured and analyzed for the DNA ploidy assessment. Morphometry mea-

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sures more subtle alterations in nuclear size, shape, DNA content, and chromatin organization in both intact (i.e. cytology) and cut (i.e. histology) cell nuclei, and reflects key pathologic alterations which signal genomic destabilization in both the morphologically malignant or non-morphologically transformed cells of an organ system [Bacus and Grace, 1987; Palcic, 1994; Palcic and MacAulay, 1994; Slaton et al., 1997; Veltri et al., 1998; Wojcik et al., 1998]. The application of non-parametric statistical modeling or construction of non-linear models using artificial neural networks (ANNs) based upon mathematical weighting of input variables have permitted new approaches to management of the complex data derived from such nuclear images, as well as clinical information, to create objective decision support tools [Badalament et al., 1996; Veltri et al., 1996, 1997, 1998, 2000, 2001; Wojcik et al., 1998; Potter et al., 1999; Reckwitz et al., 2000].

This article will illustrate the methodology and application of a new image analysis based biomarker termed Quantitative Nuclear Grade (QNG), a measure of genetic instability that uses nuclear morphometry, to make outcome predictions. We have chosen several examples to illustrate how QNG can be applied to histology, cytology, and creation of multivariate prediction algorithms, with a clinical emphasis on prostate and bladder cancer.

MATERIALS AND METHODS

Bladder Cytology Cell Classifier

The cell nuclei for the bladder cytology cell classifier presented were captured from voided urine specimens obtained from 43 patients with biopsy-proven low-grade transitional cell carcinoma (LG-TCC) and 22 patients with no history of TCC, negative cystoscopic findings, and cytological atypia [Veltri et al., 1997; Wojcik et al., 1998]. The specimens were immediately ethanol-fixed (100 ml of urine in 100 ml of 50% buffered ETOH) and received in our laboratory 24–48 h after collection. Using a Feulgen stained imprint, two expert cytopathologists selected intact urothelial cell nuclei most representative of atypia (n = 1,893 nuclear images), and LG-TCC (n = 1,010 nuclear images). All images were captured and analyzed using a CAS-200 Image Analysis System (BD-CIS, San Jose, CA). A listmode, CAS-200 compatible, file conversion program, Cell Sheet v1.0 (JVB Imaging, Elmhurst, IL), was used to calculate 38 different NMD's for each nuclear image. These descriptors included 8 DNA content, 22 Markovian texture, and 8 nuclear shape features. All data were analyzed with the Stata v5.0 statistical analysis software program (Stata Corporation, College Station, and TX). Logistic regression analysis and backwards stepwise variable selection at a stringency of $P < 0.05$ was used to evaluate which of the NMD's contributed to a QNG model solution to accurately classify nuclei as either atypical or LG-TCC.

Bladder Cytology Patient Classifier

This small patient sample consisted of 31 non-recurring and 28 recurring patients with a cytopathological diagnosis of atypia who had been previously diagnosed with superficial TCC from the M.D. Anderson Cancer Center Department of Urology [Slaton et al., 1997]. These patients were followed with cystoscopy and/or biopsy for a minimum of 6-month period after the diagnosis of cytological atypia was made. The nuclear images (75–100 per case) were captured and analyzed using a CAS-200 Image Analysis System (BD-CIS, San Jose, CA). A listmode, CAS-200 compatible, file conversion program, Cell Sheet v2.0 (JVB Imaging, Elmhurst, IL), used to calculate 41 different NMDs, including 11 DNA content, 22 Markovian texture, and 8 nuclear shape features, were calculated for the nuclei in each case gallery. All data were analyzed with the Stata v5.0 statistical analysis software program (Stata Corporation, College Station, and TX). Backwards stepwise logistic regression analysis at a stringency of $P < 0.20$ was used to determine which NMD's multivariately contributed to a QNG solution for predicting recurrence.

Biopsy Staging Prediction: Clinical Stage T1c CaP

We prospectively enrolled 255 men between October 1998 and January 2000 (average age = 58.8±6 years) diagnosed with clinical stage T1c prostate cancer (CaP) that underwent radical prostatectomy (RRP) at a single institution [Veltri et al., 2000]. Exclusion criteria included neoadjuvant treatment or medications, which could effect serologic or histologic presentation of CaP. Pre-operative sera, biopsy histology slides, clinical information, RRP pathology, and gland weights were obtained.

Biomarkers assessed included total PSA (tPSA), complexed PSA (cPSA), free PSA (fPSA), f/t PSA ratio, cPSA-density, and biopsy Gleason score. We also assessed QNG, and the nuclear images (~125/case) were captured and analyzed using an AutoCyte Pathology Workstation with QUIC-DNA v1.201 (TriPath Imaging, NC). A total of 60 different NMDs, including 19 DNA content, 10 texture, 21 Markovian texture, and 10 nuclear shape features, were calculated for the nuclei in each case gallery. All data were analyzed with the Stata v6.0 statistical analysis software program (Stata Corporation, College Station, and TX). Backwards step-wise logistic regression analysis at a stringency of $P < 0.20$ was used to determine which NMD's multivariately contributed to a QNG solution for predicting pathologic stage, and also at a stringency of $P < 0.05$ to determine the most accurate combination of variables (including QNG) for predicting OC disease.

Radical Prostatectomy Biochemical Recurrence Prediction

A total of 214 men with prostatectomy Gleason scores of 5–7 and clinical stage T1b–T2c CaP were non-consecutively selected from a cohort of more than 1800 RRP patients treated between 1982 and 1996 at the Johns Hopkins Medical Institutions [Veltri et al., 1996; Potter et al., 1999]. Men were followed up with serum PSA measurements at 3-month intervals for 1 year, at 6-month intervals for an additional year, and yearly thereafter (after PSA testing became available in 1987). An annual interview and DRE were performed. Biochemical recurrence was defined as a postoperative serum PSA greater than 0.2 ng/ml. All non-progressors had a minimum of 5 years follow-up, and no patient received radiation or hormonal therapy before biochemical disease recurrence. The nuclear images (~150/case) were captured and analyzed using a CAS-200 Image Analysis System (BD-CIS, San Jose, CA). A listmode, CAS-200 compatible, file conversion program, Cell Sheet v2.0 (JVB Imaging, Elmhurst, IL), was used to calculate 41 different NMDs, including 11 DNA content, 22 Markovian texture, and 8 nuclear shape features, for the nuclei in each case gallery. All data were analyzed with the Stata v6.0 statistical analysis software program (Stata Corporation, College Station, and TX). Backwards step-wise logistic regression analysis at a stringency of $P < 0.20$ was used to determine which NMD's multivariately contributed to a QNG solution for predicting progression, and also at a stringency of $P < 0.05$ to determine the most accurate combination of variables (including QNG) for predicting biochemical recurrence.

RESULTS

Bladder Cytology Cell Classifier

The bladder cytology cell classification model for atypical cell nuclei vs. LG-TCC cell nuclei utilized 16 NMD's to calculate a QNG model with an area under the receiver operator characteristics curve (ROC-AUC) of 95.8% (Fig. 1). At a QNG cutoff of 0.50, the model accurately identified 89% of the nuclear images as being either atypical or LG-TCC with a sensitivity of 83% and a specificity of 93% ($P < 0.0001$).

Bladder Cytology Patient Classifier

In patients with a previous history of TCC, QNG, calculated from a model using 14 NMD's, was able to accurately predict 85% of patients with cytological atypia that would recur within 6 months (sensitivity = 86%, specificity = 84%, ROC-AUC = 94%, $P < 0.001$) (Fig. 2). In this same patient sample, DNA ploidy was only able to predict which patients with cytological atypia would recur within 6 months with an accuracy of 68% (sensitivity = 61%, specificity = 74%, ROC-AUC = 67%, $P = 0.006$) (Fig. 2).

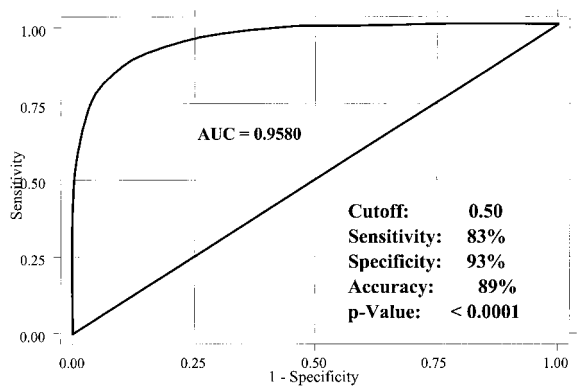

Fig. 1. This figure illustrates the ability of QNG, calculated from a multivariate logistic regression model using 16 of 38 NMD's, to differentiate between nuclear images captured from patients with cytological atypia (i.e., no cystoscopic evidence of disease) and LG-TCC. There were 1,893 atypical and 1,010 LG-TCC nuclear images analyzed in this study.

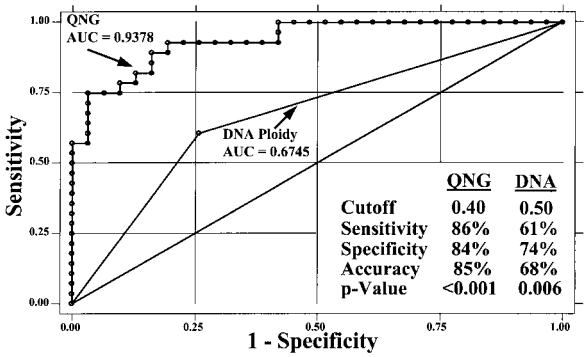

Fig. 2. This figure illustrates the ability of QNG, calculated from a multivariate logistic regression model using 14 of 41 NMD's, and DNA ploidy to predict recurrence in patients with a history of TCC and cytological atypia. There were a total of 59 patients, 28 of whom recurred within a 6 month period.

Biopsy Staging Prediction: Clinical Stage T1c CaP

A total of 49/255 (19%) of the clinical stage T1c CaP patients had pathologically non-organ-confined (NOC) disease. Table I presents the results of the univariate and multivariate logistic regression analysis for the prediction of organ confined disease. The univariate analysis of the pre-treatment variables showed that QNG, biopsy Gleason score, tPSA, cPSA, f/t PSA ratio, and cPSA density were significant ($P < 0.05$). The QNG variable was calculated from 26 multivariately significant NMD's (stringency $P < 0.20$). Using backward stepwise logistic regression at a stringency of $P < 0.20$, only QNG, cPSA-density, and the biopsy Gleason score remained in the multivariate model and yielded ROC-AUC of 82.4%. The sensitivity, specificity, negative predictive value, and accuracy of the model at a cutoff of 0.19 were 77.6, 81.6, 93.9, and 80.8%, respectively. The QNG variable was the strongest contributor in this CaP pathological staging prediction model (univariate ROC-AUC = 80.6%).

Radical Prostatectomy Biochemical Recurrence Prediction

A total of 149 patients (70%) had NOC CaP, with 66 (31%) of these NOC tumors having positive surgical margins. The remaining 65 patients (30%) were organ confined. A total of 84 men (40%) developed biochemical progression within a median of 4 years (range = 1–14 years), 67% of these men having a prostatectomy Gleason score of 7. Table II shows the univariate and multivariate logistic regression analysis results for the independent variables assessed to predict biochemical recurrence. Univariately, QNG and prostatectomy Gleason score were fairly equal in their ability to predict progression. When combined, QNG and the Gleason score increased the sensitivity and accuracy to predict biochemical recurrence.

DISCUSSION

One method to assess the relationship between altered nuclear structure and cancer biology is through the use of computer-assisted image analysis of cytological and histological patient material [Bacus and Grace, 1987; Palcic, 1994; Palcic and MacAulay, 1994; Badalament et al., 1996; Slaton et al., 1997; Veltri et al., 1996, 1997, 1998, 2000, 2001; Wojcik et al., 1998; Potter et al., 1999; Reckwitz et al., 2000]. The required engineering platform for an image system capable of capturing nuclear images, storing them, and subsequently processing the data to derive feature measurements is a computer with a frame grabber, high resolution camera, microscope with quality optics, and a custom software program to process the data. In addition, it is very critical that the reproduci-

TABLE I. Ability to Pre-Operatively Predict Prostate Cancer Stage in 255 Men with Clinical Stage T1c Disease

Variable(s)	*P*-value	ROC-AUC	Sensitivity*	Specificity*	Accuracy*
Age	0.2905	54.4%	42.9%	68.9%	63.9%
Pre-Op tPSA	0.0002	63.7%	40.8%	77.2%	70.2%
Pre-Op cPSA	0.0001	65.1%	42.9%	77.2%	70.6%
cPSA density	<0.0001	67.3%	46.9%	76.2%	70.6%
Biopsy Gleason Score	0.0003	61.9%	34.7%	89.8%	79.2%
QNG	<0.0001	80.6%	67.4%	80.6%	78.0%
QNG & cPSA density	<0.0001	81.7%	67.4%	81.6%	78.8%
QNG, Gleason, cPSA density	<0.0001	82.4%	73.5%	83.0%	81.2%

*Using univariate and multivariate model cutoffs of 0.20.

TABLE II. Ability to Predict Prostate Cancer Progression Post-Operatively in 214 Men with ≥5 Years Follow-Up

Variable(s)	*P*-value	ROC-AUC	Sensitivity*	Specificity*	Accuracy*
DNA ploidy	0.0479	56.9%	60.7%	53.1%	56.1%
Age	0.0320	56.9%	54.8%	57.7%	56.5%
Clinical stage	0.1564	57.3%	45.2%	69.2%	59.8%
OC status	0.0203	57.4%	78.6%	36.2%	52.8%
Post-Op Gleason Score	<0.0001	73.6%	66.7%	74.6%	71.5%
QNG	<0.0001	79.2%	64.3%	78.5%	72.9%
QNG & Gleason	<0.0001	84.3%	75.0%	80.0%	78.0%

*Using univariate and multivariate model cutoffs of 0.40.

bility of the nuclear staining technique be standardized and quality controlled. The imaging platform should be commercially viable in terms of availability of all of necessary components with service support from a software and hardware perspective. The image capture and processing system must perform well with intact cells captured from both cytologic and histologic specimens, providing erosion algorithms that address overlapping or degenerate cells. Another important characteristic of the imaging system is the number of pixels per unit area as well as its size and shape (small and square) providing sufficient resolution of the nuclear image to be able to conduct sophisticated pixel intensity and distribution analysis of nuclear size, shape, and texture. This feature analysis may be accomplished using a variety of equations, but those that are especially important solve for specific nuclear chromatin texture using Markovian and non-Markovian equations [Veltri et al., 1998, 2001]. The operator is another important factor to assure reliable results and this individual must be thoroughly trained and certified to conduct these analyses.

In general, normal cell nuclei tend to be round, or at least smoothly curved, and the DNA chromatin tends to be evenly distributed. This is not so for cancer cells, which tend to be irregularly shaped and their DNA chromatin distributed in clumped and disordered patterns [Diamond et al., 1982; Partin et al., 1989; Veltri et al., 1998, 2001].

The new QNG variable is a measure of the variance of size, shape, DNA content, and chromatin complexity in a given population of cancer cell nuclei (i.e., genetic instability). QNG is derived using nuclear images captured either from a single Feulgen stained five-micron tissue section with the most representative pathology (i.e., highest Gleason score) from prostate histology (i.e., needle core biopsies and radical prostatectomy specimens) or cytology specimens (i.e., voided urine and bladder washes) [Bacus and Grace, 1987; Palcic, 1994; Palcic and MacAulay, 1994; Badalament et al., 1996; Veltri et al., 1996, 1997, 1998, 2000, 2001; Slaton et al., 1997; Wojcik et al., 1998; Potter et al., 1999; Reckwitz et al., 2000]. Any image analysis technology may be used to generate such a variable, however, it is the "expert patient training set" that ultimately determines the clinical value and potential applications to predict patient-specific disease outcomes.

In the case of cytology, the QNG biomarker has been applied in the prediction of tumor recurrence [Slaton et al., 1997] and also to differentiate bladder cells based upon cytopathological classification of normal cells from low or high-grade cancer [Wojcik et al., 1998]. Additionally, the QNG biomarker has been applied to identify cancer nuclei in bladder cell populations cytopathologically classified as atypical [Veltri et al., 1997]. Although preliminary and requiring additional confirmation, this application of the QNG biomarker in the detection of abnormalities in cytology preparations is one that requires more attention in the future.

The first commercial application of the QNG technology was introduced in a statistical-based algorithm referred to as UroScore, which significantly enhanced the prediction of pre-operative stage using the QNG biomarker and information from a patient's biopsy specimen [Badalament et al., 1996]. Other applications of the QNG biomarker involve prediction of both stage and progression using QNG alone [Veltri et al., 2001]. The QNG variable has also been recently applied to predict pathological stage of men with non-palpable T1c prostate cancer, a new clinical challenge in men diagnosed with prostate cancer today. In a group of 255 men

with T1c CaP, the QNG biomarker alone was able to predict pathologic stage in T1c patients with an accuracy of 81% and an AUC of 78% at a cut-off of 0.5 [Veltri et al., 2000]. We have also utilized the QNG biomarker for the prediction of biochemical recurrence in patients that underwent prostatectomy and for whom we had greater than ten years follow-up [Veltri et al., 1996, 1998, 2001; Potter et al., 1999]. In the biochemical recurrence prediction example presented here, QNG was the strongest independent variable and the multivariate model had a sensitivity of 86% and specificity of 83% (AUC = 86%) [Potter et al., 1999]. Hence the QNG biomarker is capable of improving the prediction of both stage and recurrence in prostate cancer based upon imaging information extracted from a single section of either a needle core biopsy or a radical prostatectomy block.

Our results confirm that quantitative nuclear morphometry provides valuable information that indicates the pathological status of the disease. Additionally, we believe that these alterations in nuclear structure, as measured through QNG, are closely related to numerous alterations in gene expression that occur in the malignant process [Fearon, 1991, 1994; Sidranski et al., 1991; Vogelstein and Kinzler, 1993; Spruck et al., 1994; Davie et al., 1999; Holth et al., 1998; Stein et al., 2000]. We also believe that quantitative morphometry may be useful in the assessment of treatment efficacy associated with the restoration of normal gene expression patterns and cellular functions.

Finally, we need to take a mechanistic approach for confirmation of the QNG biomarker as a measure genomic instability, which will require studies that combine genetic analysis with assessment of morphometric changes on the same clinical specimens. This quantitative approach to assess nuclear structure and function changes in cancer cells using both molecular biology and image analysis offers an opportunity to create new clinical tools for disease management.

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