IBD, but may hopefully lead to the development of highly sensitive, specific, and reproducible assays.

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References

Survivin mRNA Copy Number in Bladder Washings Predicts Tumor Recurrence in Patients with Superficial Urothelial Cell Carcinomas, Iman J. Schultz,1,2 Lambertus A. Kieneman,2,3 Herbert F.M. Karthaus,4 J. Alfred Witjes,3 Johannes L. Willems,2 Dorine W. Swinkels,2 Jacqueline M.T. Klein Gunnewiek,1 Jacques B. de Kok1 (Departments of 1 Clinical Chemistry, 2 Epidemiology and Biostatistics, and 3 Urology, University Medical Center Nijmegen, Nijmegen, The Netherlands; 4 Department of Urology, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands; * address correspondence to this author at: AKC/564, University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands; fax 31-243541743, e-mail i.schultz@akc.umcn.nl)

Urothelial cell carcinoma (UCC) is the most common form of bladder cancer (1). Approximately 70% of patients diagnosed with UCC have superficial tumors (designated Ta or T1) and are treated by transurethral resection of the tumor (TURT). Most of these patients develop recurrences after TURT and therefore need to be followed intensively. Cystoscopy is the gold standard to check a patient’s bladder for recurrences. Unfortunately, cystoscopy is invasive, labor-intensive, and costly. Accurate prediction of UCC recurrence could significantly reduce the number of cystoscopies performed during patient follow-up.

Currently, the major determinants of a patient’s follow-up scheme and treatment procedures are pathologic tumor stage and grade. However, pathology-based assessment of bladder tumor stage and grade is subject to variability (2, 3). Furthermore, it is important to address other disease characteristics, such as tumor multiplicity and tumor size, to carefully assess the risk of recurrence in patients with superficial UCC (4–6). As a possibly better and more standardized estimation of the risk of recurrence in patients with UCC, molecular biological alterations have been studied (7–10). An important molecular tumor marker that has emerged is survivin (11). The concentrations of both survivin protein and mRNA in tumor tissue from patients with superficial UCC are indicative of the risk of tumor recurrence (12, 13).

Assessment of prognosis in UCC is usually restricted to analysis of the resected tumor tissue. However, routine cystoscopy frequently overlooks carcinoma in situ and...
small, solid high-grade or papillary tumors (14, 15). These
lesions may thus not be removed during TURT, and
consequently, prognostic information may be missed. As
an advantage over tumor biopsies, bladder washings
cover the entire urothelium and may reflect the general
molecular biological status of the bladder (16–19). They
are easily obtained during cystoscopy or before TURT.

We quantified survivin mRNA in bladder washings by
real-time quantitative reverse transcription-PCR. The data
were correlated with histopathologic characteristics and
with time to first recurrence in patients with superficial
UCC.

We collected thirty-seven 50-mL bladder washings
[performed with phosphate-buffered saline (PBS)] before
TURT from 36 patients with UCC. The patients were
asked to participate in the study and gave oral informed
consent. After collection, the washings were immediately
cooled on ice, and the cells were harvested by centrifugation
at 4 °C for 10 min at 800g. The cell pellet was washed
twice with ice-cold PBS and subsequently resuspended in
ice-cold PBS containing 15 mL/L fetal calf serum. We
enriched the bladder washings for urothelial cells to
eliminate possible contamination with (white) blood cells,
which could interfere with normalization of gene expres-
sion, by adding 30 μL of magnetic beads (3 × 10^7
particles) coated with an antibody (Ber-EP4) against epi-
thelial cells (Dynal). After incubation at 4 °C for 30 min,
the bead-bound urothelial cells were washed three times
with PBS containing 15 mL/L fetal calf serum, frozen in
liquid nitrogen, and stored at ~80 °C.

The histopathologic characteristics of the resected tu-
more tissues were evaluated by a pathologist according to
the WHO criteria for tumor grade (20) and the TNM
classification for stage (21). The tumors of two patients
were classified as superficial and low grade, but the
pathology report did not specify stage (Ta, T1) or grade
(grade I, grade II). Patients were followed clinically from
the time of tumor resection. The mean follow-up period
was 32.6 months (range, 1–45 months). Thirteen of the 27
patients with superficial (Ta/T1) UCC received adjuvant
treatment after TURT. The time to first recurrence was
determined only for patients with superficial UCC.

Isolation of total RNA from the bladder washing cells
and cDNA synthesis were performed as described previ-
ously (22). Subsequent real-time PCR quantification of
survivin mRNA and, for normalization of expression, of
the housekeeping gene cyclophilin A was performed as
described previously in detail (13). The primers and the
probe for survivin were chosen such that all three known
splice variants (23) were detected. Real-time quantitative
PCR experiments were performed with an ABI Prism 7700
Sequence Detection System (PE Applied Biosystems). We
quantified survivin and cyclophilin A mRNA copy num-
bers by constructing linearized plasmids containing either
of the amplicons. A triplicate 5-log-range calibration
curve containing 10 to 10^6 copies of either survivin or
cyclophilin was included in each real-time PCR assay.

Statistical analyses were performed with the Statistical
Package for Social Sciences (SPSS), release 10.0.7 (June
2000). Only bladder washings with a cyclophilin A copy
number ≥5 were included in the analyses because lower
numbers indicated insufficient yield of cells from the
bladder washings and thus unreliable quantification of
survivin mRNA copy number. Four of the 37 bladder
washings did not meet this criterion and were excluded
from further analyses.

We first investigated the correlation between normal-
ized survivin mRNA copy number and tumor pathology
characteristics, using the Kruskal–Wallis test (Fig. 1A).
The two patients for whom tumor stage and grade were
not specified were excluded from this analysis. Although
the Kruskal–Wallis P values were 0.001 and 0.004 for
tumor stage and grade, respectively, indicating significant
differences in expression (Fig. 1A), we observed no linear
correlation between survivin mRNA copy number and
increasing stage or grade.

We subsequently investigated whether normalized sur-
vivin mRNA copy numbers in bladder washings of pa-
tients with superficial UCC could be used to distin-
guish patients with a high risk of recurrence from patients with
a low risk. One patient with superficial UCC died 1 month
after TURT and was excluded from the analysis because
the first control cystoscopy for detection of tumor recur-
rence is not performed earlier than 3 months after TURT.
The two patients for whom the tumor stage and grade
were not specified were included in this analysis because
their medical files clearly stated that they suffered from
superficial UCC. The median normalized survivin mRNA
copy number for patients with superficial UCCs was 0.13,
which was used as the cutoff value. Kaplan–Meier curves
were constructed for patients with a normalized survivin
mRNA copy number above (n = 13) and below (n = 13)
the cutoff (Fig. 1B). The difference between the curves was
statistically significant (log-rank test, P = 0.018). The
median times to recurrence for patients with a normalized
survivin mRNA copy number above and below the me-
dian were 10 and 22 months, respectively.

In the low- and high-risk groups, 3 and 10 patients,
respectively, received adjuvant treatment after TURT. Of
the patients with superficial UCC and a normalized survivin
mRNA copy number above the cutoff (n = 13), 3
patients died recurrence-free (after 3, 11, and 14 months).
Eight patients had recurrences within 1 year, and one had
a recurrence after 14 months. Another patient remained
recurrence-free during the follow-up. Of the patients with
superficial UCC and a mRNA copy number below the
cutoff (n = 13), 1 patient died after 11 months (recurrence-
free). Of the remaining 12 patients, only 1 had a recur-
rence within 12 months (after 10 months).

The results show that bladder washings provide good-
quality patient material: 33 (89%) of our bladder washings
yielded sufficient amounts of cells for reliable quantifica-
tion of mRNA. The poor quality of four bladder washings
may have been attributable to insufficient rinsing of the
bladder and, consequently, a low yield of cells.

In addition, we investigated the possible relationship
between survivin mRNA copy number in bladder wash-
ings and tumor pathologic grade and stage. Although
previous studies showed that mRNA or protein concentrations of other molecular markers found in bladder washings can indicate UCC stage or grade (22, 24), we observed substantial overlap in the survivin mRNA copy numbers between the different tumor stages (e.g., between Ta and T2) and grades (e.g., between grade I and grade II). Therefore, in our study, survivin mRNA copy numbers in bladder washings did not seem directly indicative of UCC stage or grade.

We also investigated whether survivin mRNA could be detected in the urine from our patient group because collection of urine would circumvent cystoscopy. Despite immediate processing, mRNA could not be detected in 50% of the urines, and in cases in which mRNA was quantified, we found correlations with neither pathology nor clinical characteristics (data not shown).

Thus, bladder washings provide good-quality patient material, and quantification of survivin mRNA therein may indicate whether a patient needs early or late cystoscopy after TURT. If this can be confirmed in a larger cohort of patients, it may help to reduce the number of cystoscopies in patients with superficial UCC.

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Biological Variation of Plasma \( F_2 \)-Isoprostane-III and Arachidonic Acid in Healthy Individuals, Xiongwen Yu, Chung S. Ho, and Christopher W.K. Lam (Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Hong Kong, People's Republic of China; address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, People's Republic of China; fax 852-2636-5090, e-mail chungshunho@cuhk.edu.hk)

\( F_2 \)-Isoprostanes (iPF\(_{2α-III}\)) are formed by free radical peroxidation of esterified arachidonic acid (AA) in situ on phospholipids and are subsequently released in free form by the action of phospholipases (1). One of the iPF\(_{2α}\) isomers, iPF\(_{2α-III}\), is recognized as a sensitive and reliable index of lipid peroxidation in vivo. iPF\(_{2α-III}\) is increased in conditions associated with increased oxidative stress and decreased after dietary supplementation with antioxidants (2). Despite its increasing usage in clinical and nutritional studies, there has been no report on the biological variation in plasma iPF\(_{2α-III}\). The aims of this study were (a) to investigate the biological variation of plasma total iPF\(_{2α-III}\) in healthy individuals over a period of 4 months, and (b) to determine whether the use of the plasma iPF\(_{2α-III}/AA\) ratio can facilitate interpretation and improve clinical utility.

We recruited 20 healthy nonsmoking Chinese individuals (10 women and 10 men; age range, 31–51 years) for this study. Medical histories were obtained from a questionnaire survey. Comprehensive profiles including commonly used blood and urine tests were performed to exclude common diseases. Other exclusion criteria included pregnancy, hormonal therapy, and body mass index ≥25. These individuals maintained their usual lifestyle throughout the study and were not taking any other medications (including antioxidant supplements).

The study protocol, approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong, was explained thoroughly to the participants before their informed consent was obtained.

Once a month for 4 months, the same phlebotomist collected venous blood from each participant after an overnight fast. Blood (4 mL) was collected into lithium-heparin tubes containing 15 \( \mu \)mol/L indomethacin as a cyclooxygenase inhibitor. Blood samples were centrifuged at 2400 \( g \) for 10 min at 4 °C. Aliquots (1 mL) of plasma were transferred to Eppendorf tubes containing 20 \( \mu \)mol/L butylated hydroxytoluene as a free-radical scavenger. The samples were stored at −70 °C until analysis.

Our use of this standardized procedure allowed us to consider preanalytical variation as negligible. Samples from each participant were analyzed once in the same batch after all samples had been collected, thereby eliminating between-run analytical variation.

Plasma total iPF\(_{2α-III}\) was isolated by immunoaffinity extraction. iPF\(_{2α-III}\)-d\(_4\) (1 ng as internal standard) was added to 0.5 mL of plasma and hydrolyzed in the presence of 1 mol/L potassium hydroxide at 40 °C for 30 min. The hydrolyzed plasma sample was purified by affinity chromatography on a Gilson ASPEC XL fully automated workstation (Gilson). The affinity column contained anti-iPF\(_{2α-III}\) antibody immobilized on cyanobromide-activated Sepharose 4B gel supplied by Assays Design Inc. iPF\(_{2α-III}\) was quantified by isotope-dilution capillary gas chromatography–negative-ion chemical ionization mass spectrometry (GC-NICI-MS) according to the method of Zhao et al. (3).

To measure AA, we added 100 \( \mu \)L of distilled water, 10 \( \mu \)L of concentrated hydrochloric acid, 400 \( \mu \)L of ice-cold Folch solution (chloroform–methanol, 2:1 by volume), and 50 ng of AA-d\(_3\) (internal standard) to 10 \( \mu \)L of hydrolyzed sample. After thorough vortex-mixing, the sample was centrifuged at 2400 \( g \) for 5 min. The lower organic layer was transferred to another Eppendorf tube and evaporated under nitrogen. The residue was then dissolved in 200 \( \mu \)L distilled water, and AA was extracted by the addition of 400 \( \mu \)L of hexane. The hexane extracts were dried under nitrogen. AA was analyzed as the pentfluorobenzyl ester by GC-NICI-MS, according to the modified method of Hadley et al. (4).

To evaluate the imprecision of iPF\(_{2α-III}\) and AA measurements, we processed two plasma samples, collected from healthy laboratory staff, according to the same procedure as study samples. These samples were used for internal quality control and were included in each batch analysis. Within-run imprecision was estimated from 12 determinations within a single batch. Between-run imprecision was estimated from nine determinations over different batches. All data were processed with SPSS 10.0 for Windows (SPSS Inc.). Data distribution was evaluated by the Shapiro–Wilk test. The distribution of plasma total iPF\(_{2α-III}\) and AA and the iPF\(_{2α-III}/AA\) ratio were gaussian, and the data are expressed as the mean (SD). Para-