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

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Survivin mRNA Copy Number in Bladder Washings Predicts Tumor Recurrence in Patients with Superficial Urothelial Cell Carcinomas

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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 expression, by adding 30 μL of magnetic beads (3 $\times$ 10^7 particles) coated with an antibody (Ber-EP4) against epithelial 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 tumor 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 previously *(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 numbers 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 $\geq$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 normalized 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 survivin mRNA copy numbers in bladder washings of patients with superficial UCC could be used to distinguish 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 recurrence 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 median 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 recurrence 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 quantification 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 washings and tumor pathologic grade and stage. Although
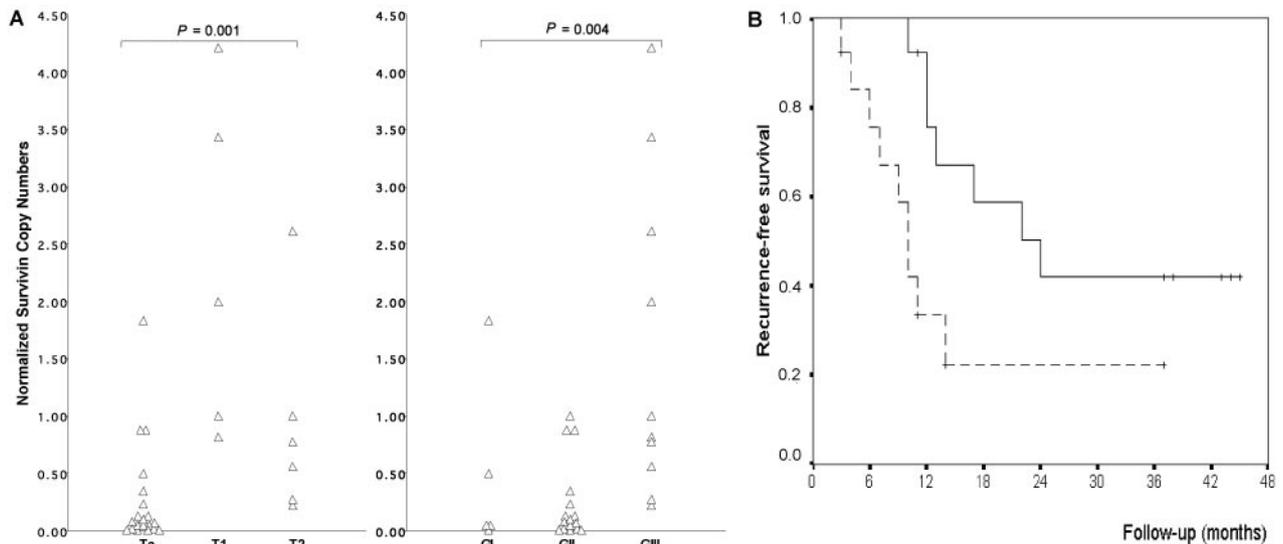
Fig. 1. Normalized survivin mRNA copy numbers in bladder washings (*A*) and Kaplan–Meier curves for recurrence-free survival in patients with superficial UCC (*B*).

(*A*), patients are grouped according to tumor stage and grade. *Ta* and *T1*, superficial tumors; *T2*, muscle-invasive tumors; *GI* and *GII*, low-grade tumors; *GIII*, high-grade tumors. Median normalized survivin mRNA copy numbers (range in parentheses) for tumor stage and grade are as follows: Ta, 0.08 (0.01–1.84); T1, 2.00 (0.82–4.22); T2, 0.68 (0.23–2.62); grade I (*GI*), 0.05 (0.01–1.84); grade II (*GII*), 0.1 (0.01–1.01); and grade III (*GIII*), 0.92 (0.23–4.22). Survivin mRNA was detected in all bladder washings. Normalized survivin mRNA copy number = survivin mRNA copy number/cyclophilin mRNA copy number. (*B*), the *solid line* and the *dashed line* indicate recurrence-free survival for the patients whose bladder washing had a normalized survivin mRNA copy number below (n = 13) or above (n = 13) the median cutoff value of 0.13, respectively. The difference in recurrence-free survival between the two groups is statistically significant (log-rank test, $P = 0.018$).

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 F2-Isoprostane-III and Arachidonic Acid in Healthy Individuals,

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F_2-Isoprostanes ($iPF_{2\alpha}$) 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 $iPF_{2\alpha}$ isomers, $iPF_{2\alpha}$-III, is recognized as a sensitive and reliable index of lipid peroxidation in vivo. $iPF_{2\alpha}$-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 $iPF_{2\alpha}$-III. The aims of this study were (a) to investigate the biological variation of plasma total $iPF_{2\alpha}$-III in healthy individuals over a period of 4 months, and (b) to determine whether the use of the plasma $iPF_{2\alpha}$-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 μmol/L indomethacin as a cyclooxygenase inhibitor. Blood samples were centrifuged at 2400g for 10 min at 4 °C. Aliquots (1 mL) of plasma were transferred to Eppendorf tubes containing 20 μmol/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 $iPF_{2\alpha}$-III was isolated by immunoaffinity extraction. $iPF_{2\alpha}$-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-$iPF_{2\alpha}$-III antibody immobilized on cyanobromide-activated Sepharose 4B gel supplied by Assays Design Inc. $iPF_{2\alpha}$-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 μL of distilled water, 10 μL of concentrated hydrochloric acid, 400 μL of ice-cold Folch solution (chloroform–methanol, 2:1 by volume), and 50 ng of AA-d_8 (internal standard) to 10 μL of hydrolyzed sample. After thorough vortex-mixing, the sample was centrifuged at 2400g for 5 min. The lower organic layer was transferred to another Eppendorf tube and evaporated under nitrogen. The residue was then dissolved in 200 μL distilled water, and AA was extracted by the addition of 400 μL of hexane. The hexane extracts were dried under nitrogen. AA was analyzed as the pentafluorobenzyl ester by GC-NICI-MS, according to the modified method of Hadley et al. (4).

To evaluate the imprecision of $iPF_{2\alpha}$-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 $iPF_{2\alpha}$-III and AA and the $iPF_{2\alpha}$-III/AA ratio were gaussian, and the data are expressed as the mean (SD). Para-