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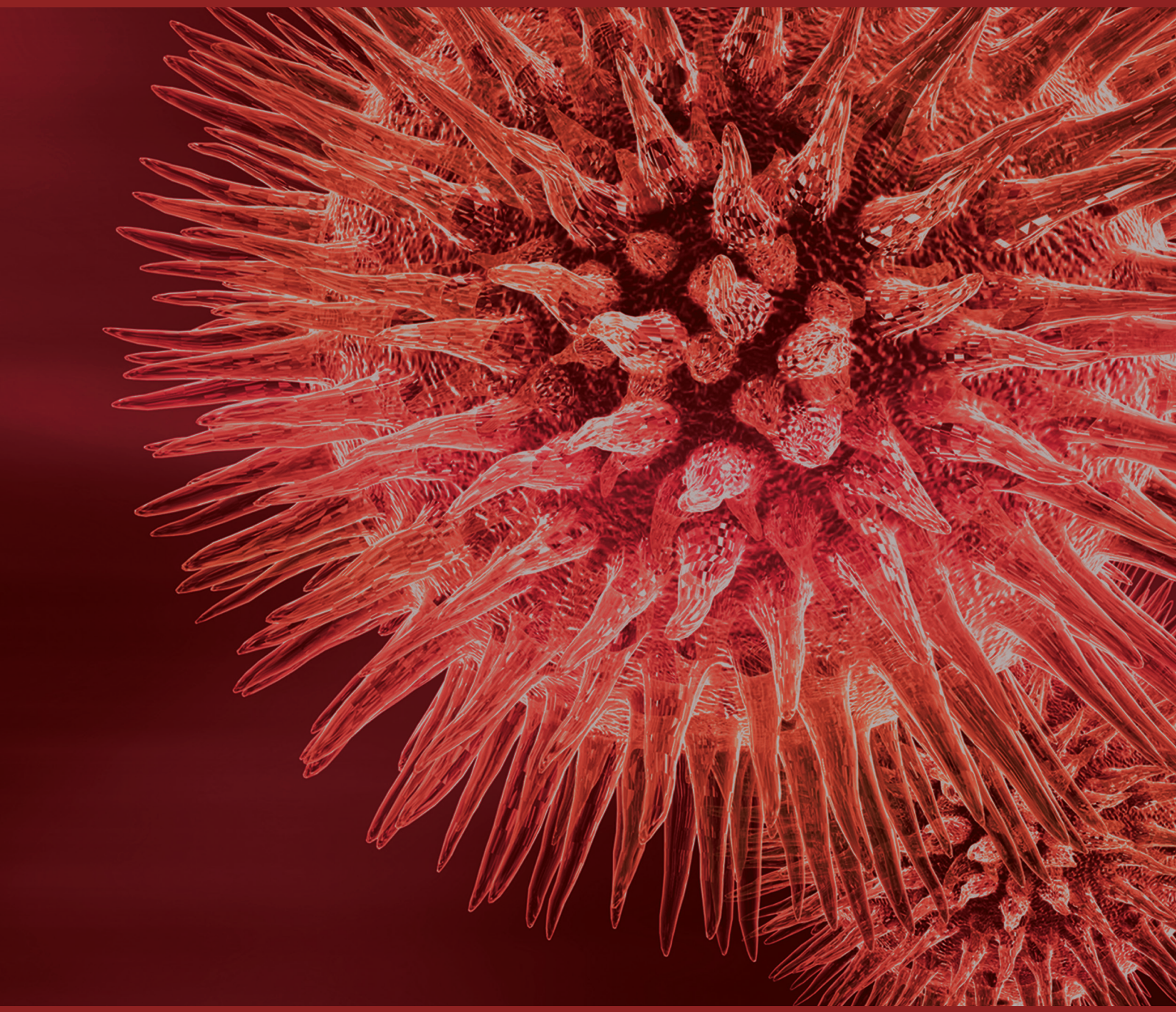
Guest Editors: Li-Jen Wang, Joëlle L. Nortier, Bin Tean Teh, Cheng-Keng Chuang, and Shen-Yang Lee



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Editorial

Chronic Kidney Disease and Upper Tract Urothelial Carcinomas

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Upper tract urothelial carcinomas (UTUC) have high prevalence rates in patients with chronic kidney disease (CKD), including dialysis patients and kidney transplant recipients. Analgesic nephropathy, Balkan endemic nephropathy, and aristolochic acid (AA) nephropathy share the common association with the development of CKD and UTUC. Genome studies allow identification of patients with genetic predisposition to Balkan endemic nephropathy and AA nephropathy. Furthermore, the identification of aristolactam deoxyribonucleic acid (DNA) adducts now provides robust evidence for AA exposure in UTUC patients, even in the absence of recallable exposure history. Diagnosis and treatment of UTUC in CKD patients are challenging. Clinical diagnosis of UTUCs in CKD patients with the available urological and imaging methods is much more difficult than in patients with normal renal function due to atrophic kidneys and poor excretory functions in these patients. High prevalence rates of contralateral upper tract and urinary bladder involvements of UC in CKD, either at presentation or recurrence, raise concerns of the necessity and techniques as well as perioperative risk of complete urinary tract exenteration. The standard treatment of radical nephroureterectomy with bladder cuff excision for UTUC patients may result in deterioration of renal function, rendering adjuvant chemotherapy unsuitable. This special issue collectively addresses these important topics regarding UTUC in CKD patients.

The authors in one paper nominate three mutant genes (CELA1, HSPG2, and KCNK5) in Balkan endemic nephropathy patients, which are associated with angiogenesis. This finding suggests that angiogenesis might play an important role in the development of Balkan endemic nephropathy. Another paper shows an elevated risk of urothelial carcinomas (UC) in ESRD patients with the analysis of a national wide cohort in Taiwan. Female patients have 9–18 folds of increased risk of UC as compared to 4–14 folds in male ESRD patients, which may reflect female predominance in AA consumption. One paper discusses the challenges of UTUC diagnosis in CKD patients. It reviews the detection rate of UTUCs in dialysis patients and kidney transplant recipients with the use of a variety of imaging and urological methods in the literature. Combination use of urological and imaging methods has been suggested for diagnosing UTUCs in symptomatic dialysis patients and kidney transplant recipients.

Another paper develops a predictive model for postoperative renal insufficiency in UTUC patients undergoing radical nephroureterectomy, which help in selecting eligible patients for cisplatin based adjuvant chemotherapy. Older age, lower estimated glomerular filtration rate before

surgery, smaller tumor size, renal pelvis tumor, and absence of hydronephrosis or multifocal tumors are predictors for ineligibility for adjuvant chemotherapy. One paper describes modified incision complete urinary tract exenteration for UC in dialysis patients, providing short operative time, early oral feeding, and no major perioperative complications of this modified surgical procedure. Another paper reviews epidemiological evidence of AA exposure associated with occurrence of nephropathy and UC, including AA exposure scenarios in Belgium, Taiwan, and the Danube River as well as occupational exposure in Chinese herbalists. The identification of aristolactam DNA adducts in these patients further corroborates biological plausibility of AA exposure contributing to the occurrence of UTUC.

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Review Article

Chinese Herbs Containing Aristolochic Acid Associated with Renal Failure and Urothelial Carcinoma: A Review from Epidemiologic Observations to Causal Inference

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Herbal remedies containing aristolochic acid (AA) have been designated to be a strong carcinogen. This review summarizes major epidemiologic evidence to argue for the causal association between AA exposure and urothelial carcinoma as well as nephropathy. The exposure scenarios include the following: Belgian women taking slimming pills containing single material Guang Fang Ji, consumptions of mixtures of Chinese herbal products in the general population and patients with chronic renal failure in Taiwan, occupational exposure in Chinese herbalists, and food contamination in farming villages in valleys of the Danube River. Such an association is corroborated by detecting specific DNA adducts in the tumor tissue removed from affected patients. Preventive actions of banning such use and education to the healthcare professionals and public are necessary for the safety of herbal remedies.

1. Introduction

Epidemiology usually starts from careful clinical observation. In 1993, Vanherweghem et al. first reported an unusual observation that many young Belgian women took slimming pills containing Chinese herb subsequently developing renal failure and upper tract urothelial carcinoma [1, 2]. The slimming pills contained a Chinese herb "Guang Fang Ji" that has an off-label usage as a diuretic or an immune modulator [3]. At that time, the public generally believed that herbal products are harmless. However, based on the special pathological characteristics of extensive interstitial fibrosis of renal cortex without glomerular lesions, overwhelming upper tract urothelial carcinomas (UTUC), and identification of aristolochic acid (AA) in Chinese herbs [4], Vanherweghem and his colleagues hypothesized that ingestion of the *Aristolochia* species herbs may be the culprit for the epidemic in Belgium. Subsequently, Chinese herbs associating renal failure and urothelial cancer were reported in many countries [5–8]. Exposure to *Aristolochia* herbs has raised global public health concerns.

2. The Epidemiologic Approach

Because AA is derived from an extract of plants of the *Aristolochia*, *Bragantia*, and *Asarum* species, the further step

is to determine whether an association between AA and the nephropathy exists in individuals who had similar exposures.

2.1. Exposures through Prescribed Chinese Herbal Products in Taiwan. AA is a common ingredient in many Chinese herbs, such as Ma Dou Ling (*Aristolochia debilis*), Tian Xian Teng (*Aristolochia contorta*), Qing Mu Xiang (*Aristolochia cucurbitifolia*), Guang Fang Ji (*Aristolochia fangchi*), Guan Mu Tong (*Aristolochia manshuriensis*), and Xixin (*Radix et Rhizoma Asari*) [9–11], and these herbal remedies are commonly prescribed for many different illnesses, including at least hepatitis, urinary tract infection, vaginitis, oral ulcer, upper respiratory tract infection, eczema, headache, dysmenorrhea, arthralgia, neuralgia, hypertension, cerebrovascular accident, bronchitis, pneumonia, heart failure, and edema [12]. In Taiwan, Chinese herbal remedies are regularly reimbursed through the national healthcare system, which covers more than 97% of the population [13]. By analyzing the National Health Insurance Reimbursement Database (NHIRD), Hsieh et al. found that herbal drugs containing AA had been prescribed to more than one-third of the population [14]. Besides, patients with a similar pathological finding of AAN in Belgium and history of taking Chinese herbal remedies containing AA were successively reported in Taiwan [5, 6, 15, 16]. As Taiwan was once reported to be the highest incidence of end-stage renal disease (ESRD) worldwide [17, 18], AA-associated nephropathy was therefore hypothesized to be one of the major risk factors for developing ESRD in Taiwan. Different from exposure to single Chinese herb in Belgium, Chinese medicine commonly applies mixtures of herbs to enhance efficacy and/or minimize toxicity [19, 20]. Lai et al. conducted a retrospective follow-up study using a systematic random sample from the NHIRD from 1997 to 2002 and found that the prescription of more than 30 g of Mu Tong or more than 60 g of Guang Fang Ji was associated with an increased risk of chronic kidney disease (CKD) in Taiwan [21]. This study explored the doses of prior exposure to AA containing Chinese herbal remedies and the occurrence of CKD and controlled confounding of use of nonsteroidal anti-inflammatory drugs and other risk factors to corroborate the causal hypothesis, indicating that mixtures of herbs did not reduce the nephrotoxicity of AA. In addition, Lai et al. also analyzed the NHIRD in Taiwan and found that people taking AA-containing herbal products had a dose-dependent increased risk of urinary tract cancer [22]. There is also an extremely high incidence of UTUC in Taiwan among hemodialysis patients [23], which was corroborated by cases from another hospital with added finding of high recurrence [24]. Although there was no statistical association between taking Chinese herbs and recurrence of cancer [24], it deserves further studies for effective prevention. Wang et al. followed the incidence of urothelial carcinoma among a national representative cohort of 58,739 patients with ESRD in Taiwan during 1997–2002 and reported significantly increased risks for UTUC (SIR = 11.6; 95% CI: 10.1–13.1) and bladder cancer (SIR = 13.9; 95% CI: 12.4–15.0) [25], which is later shown to be associated with the exposure to AA associated Chinese herbal products, especially Guan Mu Tong) [26]. Wang et al. further followed 90,477 newly diagnosed cases of ESRD in Taiwan between 1997 and 2008 covering the patients aged 40–85. Results showed that female patients had higher risk of UTUC than male after the diagnosis of ESRD; moreover, the cumulative incidence rates and the standardized incidence ratios of UTUC in females appear to decline after the calendar year of 2000 and the time trend is compatible with the decreased consumption of AA after 1998 [27]. In Taiwan, most AA-containing herbs have been prohibited since 2003. If AA is solely the causative agent for the epidemic in Taiwan, we expect that the incidence rate of UTUC in Taiwan will gradually decrease one decade after the cessation of exposure. Common Chinese herbs and formulas containing AA are summarized in Table 1.

2.2. Occupational Exposures of Chinese Herbalists in Taiwan. A different exposure setting is through the manufacturing of herbal products by Chinese herbalists. Chinese herbalist is a traditional occupation in Chinese society, and these individuals work in traditional herbal stores [28]. Yang et al. followed the mortality of urological cancers in 6,548 Chinese herbalists in Taiwan between 1985 and 2000, and the results showed that Chinese herbalists had significantly higher risks of mortality due to urological cancers (SMR = 3.10; 95% CI: 1.41–5.87) and due to chronic and unspecified nephritis, renal failure, and renal sclerosis (SMR = 2.40; 95% CI: 1.40–3.84) [29]. Yang et al. also followed the development of cancer until 2001; the SIRs for kidney and upper urinary tract cancers and bladder cancer were 4.24 (95% CI 2.47–6.80) and 2.86 (95% CI 1.52–4.89), respectively [30]. By assessing the herbalists' exposure at the individual level, Yang et al. found that manufacturing and selling Chinese herbal medicines as well as processing and dispensing herbal medicines containing AA increased the risk of renal failure and UTUC among herbalists due to the ingestion and inhalation of herbal powders at work [31, 32]. Because herbal particles are visible powders with large molecular weights, the exposure to AA was suspected to occur also via oral route, not only by ingesting herbal remedies containing AA but also by swallowing deposited powder particles in the oropharynx. This viewpoint seems to be supported by the highest risk of UTUC among workers who were exposed to grinding and packing procedures, which usually generates the most significant amount of airborne powders [32].

2.3. Exposure through Food Contamination in the Balkans. Balkan endemic nephropathy (BEN) is an environmental disease in farming villages in valleys of the Danube River. BEN and Chinese herbal AAN present similar clinical findings of nephropathy and urothelial carcinoma, which is characterized by slow, progressive renal failure with extensive hypocellular interstitial sclerosis, tubular atrophy, global sclerosis of the glomeruli, and cellular atypia and is also associated with UTUC [33]. These similarities with AAN led to the hypothesis that BEN was associated with dietary exposure to AA-contaminated bread when the seeds of *Aristolochia clematitis* are harvested along with the wheat used for bread making. In fact, *Aristolochia clematitis* is a plant that grows

Table 1: A list of Chinese herbs containing aristolochic acids.

Chinese herbal name	Botanical name	Chinese herbal formula containing this herb
Guang Fang Ji (Fangchi)	*Aristolochia fangchi*	Shu Jing Huo Xue Tang Shang Zhong Xia Tong Yong Tong Feng Fang Ji Huang Qi Tang Xiao Xu Ming Tang Jie Geng Tang Mu Fang Ji Tang
Xixin	*Radix et Rhizoma Asari*	Chuan Qiong Cha Diao San Xiao Qing Long Tang Du Huo Ji Sheng Tang
Guan Mu Tong	*Aristolochia manshuriensis*	Long Dan Xie Gan Tang Xin Yi San Ba Zheng San Gan Lou Xiao Du Dan Dao Chi San Dang Gui Si Ni Tang Mu Tong Guo Qi Yin Xiao Ji Yin Zi Ju He Wan Zheng Gu Zi Jin Dan
Qing Mu Xiang	*Aristolochia cucurbitifolia*	Xiang Sha Liu Jun Zi Tang Gui Pi Tang Zheng Gu Zi Jin Dan
Ma Dou Ling	*Aristolochia debilis*	
Tian Xian Teng	*Aristolochia contorta*	

as a weed in endemic areas and contains AA [34], and the etiological evidence of epidemic was also corroborated by Grollman and Jelaković [35].

2.4. Evidence of DNA Adducts Combined with Epidemiological Approaches. The suspicion that ingestion of the *Aristolochia* species herbs may be responsible for the epidemic in Belgium was further corroborated by detection of aristolactam-(AL-) DNA adducts formed by metabolites of aristolochic acid (aristolactams) in samples of kidneys removed from five patients with nephropathy [36]. Cosyns et al. subsequently analyzed the urothelial lesions of kidneys and ureters removed during renal transplantation from 10 patients, overexpressed TP53 was observed, which suggested that a TP53 gene mutation plays a role for the carcinogenic effect [37]. Jelaković et al. extracted DNA from the renal cortex and urothelial tumor tissue of 67 patients that underwent nephroureterectomy for carcinomas of the upper urinary tract and resided in regions of Balkan endemic nephropathy. AL-DNA adducts and TP53 mutations were verified in tumor tissues of most patients. In contrast, neither AL-DNA adducts nor specific mutations were detected in tissues of patients residing in nonendemic regions [38]. Wu et al. investigated the chromosomal aberrations of UTUC specimens from seven dialysis patients in Taiwan by conventional comparative genomic hybridization and results showed that gains at 5p, 7, and 19q and losses at 4q, 9p, and 15q are common in UTUC of ESRD patients. In addition, female ESRD patients with UTUC had more frequent chromosomal aberrations than their male counterparts [39]. By whole-genome and exome analysis of nine AA-associated UTUCs in Singapore, Poon et al. found a high somatic mutation rate and the AA-induced mutations were also significantly enriched at splice sites, suggesting a role for splice-site mutations in UTUC pathogenesis [40]. Chen et al. analyzed 151 UTUC patients in a medical center of Taiwan and found that AL-DNA adducts were present in the renal cortex of 83% of patients with A : T to T : A mutations in TP53, FGFR3, or HRAS [41]. Results of the molecular epidemiology study were in coherence with the preceded finding in Belgium and therefore provided evidence for the biologic plausibility that the exposure to AA contributes to the development of UTUC.

2.5. Molecular Mechanisms of Renal Damage and Tumor Formation. AA-induced renal damage involves the tubular injury and the interstitium as well [34]. In acute phase, toxicity causes proximal tubule injury [42]. In chronic phase, the apoptosis of the tubular epithelial cells, defective activation of antioxidative enzymes, mitochondrial damage, impaired regeneration of proximal tubular epithelial cells, and interstitial cell proliferation lead to tubular atrophy and interstitial fibrosis [34, 43–45]. Because human urothelial tissue is rich in peroxidases, the aristolactams activated by peroxidase may result in the formation of the AL-DNA adducts in urothelial tissue [46, 47] and lead to A : T to T : A transverse mutations in the TP53 tumor suppression gene

[38, 41, 47, 48], as demonstrated by overexpression of TP53 protein in patients with AAN and urothelial carcinoma [37]. Since the TP53 can promote cell-cycle checkpoints, DNA repair, and apoptosis [49], its mutation may play an important role for the AA-induced carcinogenic effect in human studies [37, 38, 41]. In rat model, AL-DNA adducts were also detected in the renal cortex of AA-treated rats [42]. In vitro studies, AA can induce AL-DNA adducts in proximal tubular cell line [50].

2.6. From Epidemiologic Observations to Causal Inference. AA-induced kidney disease was once referred to as Chinese herbs nephropathy because of frequent occurrence in people taking Chinese herbal remedies, but it is now more accurately termed as aristolochic acid nephropathy (AAN) because the nephropathy is also observed in food contamination [51], occupational exposure [29, 30, 32], and so forth. Exposure to AA causes not only UTUC but also bladder cancer [52]. In determining whether an association is causal, the Hill criteria of causation are often applied [53, 54]. Clear temporal relationship between AA exposure and subsequently developed nephropathy and urological caner are observed in all epidemiological studies in Belgium and Taiwan. The association cannot be explained by alternative factors, including arsenic, cigarette smoking, and NSAID after appropriate control of confounding in these studies [22]. Moreover, a dose-response relationship was documented for AA exposure and UTUC based on the national-wide Chinese herbal pharmacoepidemiological studies in Taiwan, in which a threshold of herbal doses is also provided [22]. In different exposed populations in Belgium, Taiwan, and Balkan Peninsula, there are consistent and replicable findings that the AA exposures associate with increased risks for the occurrence of nephropathy and urothelial carcinoma. Moreover, a biologically plausible mechanism has been demonstrated by detection of gene mutations and DNA adducts, which appear to be specific in pathological finding and corroborates such a causal relationship (Figure 1). Thus, it is not a surprise that herbal remedies containing plant species of the genus Aristolochia have been designated to be a Group 1 carcinogen in humans [55].

3. Diagnosis of AA-Induced Nephropathy and Urothelial Carcinoma

3.1. Clinical Characteristics. AAN is characterized by anemia, mild tubular proteinuria, and initially normal blood pressure in approximately one-half of patients. Differences in disease progression may relate to the total dose of AA that was ingested [6]. Human subjects with high AA intake may progress to renal failure after 1 to 7 years, although subjects with a low cumulative AA intake can often maintain relatively normal kidney function over the course of 2–8 years of follow-up [56]. Based on a study including 39 patients with AAN who underwent prophylactic surgery, a cumulative dose of more than 200 g of Guang Fang Ji was associated with a higher risk of urothelial carcinoma [2]. Most of the cases of urothelial carcinoma were detected in AAN patients with ESRD. However, AA-induced urothelial carcinoma may occur without severe renal failure [57]. In contrast to the usual concept that urothelial carcinoma is more likely to develop in males than females [58, 59], the female gender has been shown to be associated with a higher risk of developing AA-related urothelial carcinoma [29, 60, 61].

3.2. Laboratory Findings. In AAN, microalbuminuria and the urinary excretion of beta 2-microglobulin appear to represent an early marker of tubular damage [62]. In such cases, there are also elevated levels of urine retinol-binding protein, urine N-acetyl-beta-glucosaminidase, anemia, and glucosuria [63].

3.3. Pathological Findings. Proximal renal tubular dysfunction and structural destruction would be the main positive findings in renal biopsy [63]. AAN has been classified as a separate entity of progressive tubulointerstitial nephropathy. The major pathological findings of AAN include hypocellular interstitial fibrosis, tubular atrophy, tubular brush border ablation, fibromyxoid or fibrous intimal thickening mainly of the interlobular arteries, mild to severe hyalinization, and sclerosis of the glomeruli decreasing from the outer to the inner cortex [63–67]. Urothelial malignancy is observed mainly in the upper urinary tract, such as the ureter and pelvis, during the first three years after exposure [37] but can also be observed in the bladder, with an approximately equal tendency, after longer follow-up periods [52]. The carcinogenesis of AA is associated with specific A : T to T : A mutations in the TP53 tumor-suppressor gene [37, 68–70].

3.4. AA Detection Method. To verify the exposure to AA, ultra-high-performance liquid chromatography-multistage fragmentation mass spectrometry can be applied to determine the presence of aristolochic acid I (AA I) and aristolochic acid II (AA II) in herbal dietary supplements [71]. Yang et al. reported a hollow fiber liquid-phase microextraction technique in conjunction with high-performance liquid chromatography for the extraction and quantitation of aristolochic acid I in human urine samples [72]. Following metabolic activation, AAs can form AL-DNA adducts in the renal cortex or ureteral tissues, which can serve as biomarkers of exposure to AA [2, 36, 38, 48, 73]. In addition, the ^{32}P-postlabelling method can be used to detect AL-DNA adducts [74], although new liquid chromatography-mass spectrometry (LC-MS) methods have been applied in human renal cortex tissues [73, 75]. A noninvasive and efficient method using ultraperformance liquid chromatography-triple quadrupole mass spectrometry has also been developed to detect AL-DNA adducts in exfoliated urothelial cells [73, 76].

3.5. Treatment and Follow-Up. Steroid therapy was shown to slow the progression of renal failure in AAN [77, 78]. Regular medical follow-up is necessary for Chinese herbalists and those who have taken AA-containing herbal remedies. Because the prevalence of urothelial carcinoma among patients with end-stage AAN is high [2], prophylactic surgery to remove the ureter and kidney has been performed in patients with end-stage AAN in Belgium [37]. Because

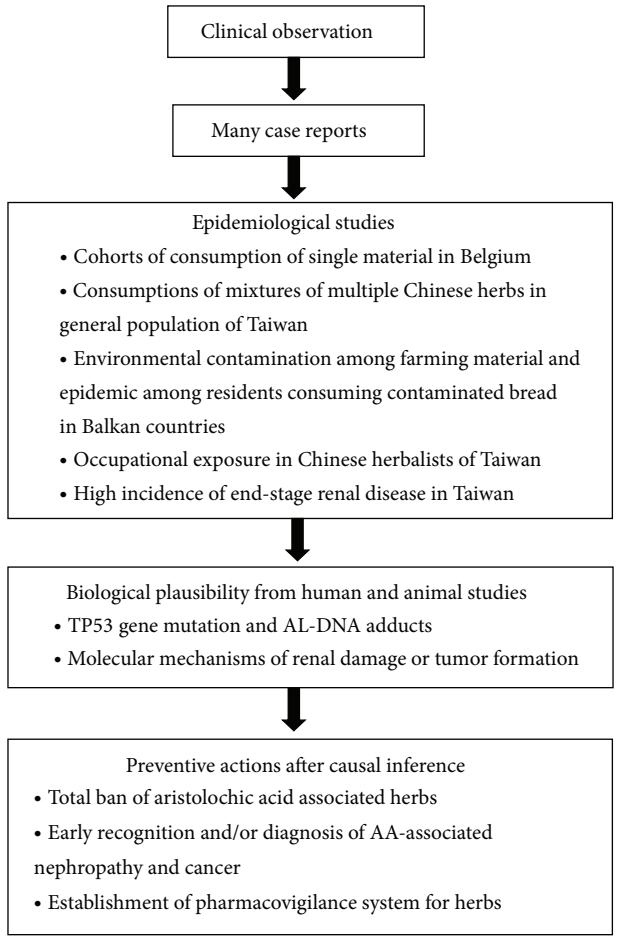

Figure 1: The sequence of establishing causality.

the exposure to AA increases the risk for bladder cancer [22, 27, 52], yearly screening by cystoscopy and computed tomography is recommended in patients with AAN [79, 80].

4. From Observational Studies to Preventive Actions

There has been an increase in the use of Chinese herbal remedies in Taiwan [14] and possibly worldwide [81]. Thus, AA epidemics have a significant implication for the safety of herbal remedies. Moreover, the misuse or substitution of Chinese herbs is common due to the inconsistent nomenclature system and the similarity of the names and appearance of many herbs [82]. For example, Han Fang Ji is often replaced by Guang Fang Ji, Mu Tong by Guan Mu Tong, and Mu Xiang by Qing Mu Xiang [82–85]. Although Chinese medicine is regarded as a formal medical treatment in the health care system of Taiwan [86] and Chinese herbal remedies are manufactured in certificated pharmaceutical factories, adulteration remains common due to the loose regulations and enforcement requiring factories to validate relatively few items. To proactively prevent the adverse reactions caused by Chinese herbal remedies and protect the public, Chinese herbal remedies and online herbal products must be regularly monitored by the Food and Drug Administration (FDA) to ensure that products are evaluated for their safety before marketing. Proper labeling and good surveillance systems will further protect consumers. Moreover, incorporating Chinese herbal drugs into the adverse drug reaction or poison surveillance system may be another effective way to achieve this goal [87]. In clinical practice, we recommend that physicians always keep in mind the possible consumption of herbs when treating a patient with renal disease or urological cancer. Although the US FDA bans all botanical remedies known or suspected to contain AA, AA I and AA II were detected in 20% and 7%, respectively, of tested samples in a survey of thirty herbal products marketed in the US via the Internet [71]. Xixin, which contains minute amount of AA [10, 88, 89], is still widely used in Asia, including Japan, Korea, China, and Taiwan, because Chinese medicine practitioners claim that its toxicity is negligible. However, AAN associated with Xixin was reported [15]. We suggest that the governments must adopt more restrictive regulations to eliminate all AA-containing herbs to protect public health.

In Chinese medicine, toxic herbs are seldom prescribed or used alone; in fact, traditional Chinese remedies are usually prescribed as complex mixtures of several different medicinal plants based on the ancient principle of "sovereign, minister,

assistant, and courier," which assigns each ingredient in a prescribed Chinese herbal formula a unique role so that the combination of them can enhance the main effect and reduce the toxicity of herbs [18, 19]. For example, minerals are commonly used in Chinese herbals remedies, and Chinese herbalists believe that the toxicity of minerals can be removed by traditional processing operations, including heating and quenching in vinegar, referred to as "pao zhi" [90]. However, no scientific evidence has shown that toxic contents, such as AA or heavy metals, can be eliminated by these methods. As the Chinese herbal products containing AA have been consistently shown to be associated with urothelial carcinoma, we must inform the general public, Chinese medicine practitioners, and healthcare professionals of proactively taking necessary precautions to avoid exposure to AA associated Chinese herbal products, establishing a pharmacovigilance system for traditional herbs, and continuing monitoring those who were exposed before. Because Taiwanese people may obtain herbs and herbal medicines from many different sources [3, 91], physicians shall still keep on watching potential nephrotoxicity of medicines even after AA-associated herbs were mostly removed from Chinese herbal products prescribed under the National Health Insurance system.

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

A Modified Single Mini-Incision Complete Urinary Tract Exenteration for Urothelial Carcinoma in Dialysis Patients

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Objective. To present our experience with single mini-incision complete urinary tract exenteration (CUTE) for female dialysis patients suffering from urothelial carcinoma (UC). *Patients and Methods*. Institutional review board approval was obtained. From 2005 through 2012, 14 female dialysis patients with UC underwent single mini-incision CUTE, in combination with radical hysterectomy and bilateral salpingo-oophorectomy. All were placed in the modified dorsal lithotomy position without repositioning. An infraumbilical midline mini-incision was made. Bilateral nephroureterectomy was first performed entirely extraperitoneally, followed by radical cystectomy with removal of the uterus and ovaries transperitoneally. *Results*. All procedures were done successfully without major complications. The median operative time was 242.5 minutes, and estimated blood loss was 500 mL. The median time to oral intake was 2 postoperative days; the median hospital stay was 11 days. Ten patients remained cancer-free at a median follow-up of 46.5 months; six patients were confirmed as having preoperatively undetectable UC or renal cell carcinoma, even after reviewing preoperative computed tomography. *Conclusions*. This modified technique provides a time-saving complete urinary tract extirpation to eliminate preoperatively undetectable malignancy, reduce metachronous recurrences, and avert perioperative complications associated with pneumoperitoneum and repositioning. Good cancer control and early convalescence can mutually be achieved in experienced hands.

1. Introduction

In Taiwan, there is an increased risk for urothelial carcinoma (UC) in patients of end-stage renal disease (ESRD), with the incidence ranging from 0.89% to 2.1%, especially women aged 50 years or younger [1]. On account of its high recurrence rate and rapidly progressive behavior among dialysis patients, total urinary tract exenteration is a recommended treatment modality to reduce the incidence of metachronous multicentric UC [2]. Besides, lack of suitable imaging studies for follow-up of the upper tract and the probability of morbidity related to stepwise urinary tract extirpation support the more aggressive surgical strategy for treating high-risk patients with ESRD.

Conventionally, complete urinary tract exenteration (CUTE) is performed via a long midline incision extending from the xiphoid process to the pubic symphysis or a bilateral flank approach followed by a midline infraumbilical laparotomy incision. Long operative time and high surgical risks were of great concern at that time. With the advent of laparoscopic techniques and the experience of open surgery, we develop a single mini-incision unilateral nephroureterectomy with bladder cuff excision, via an infraumbilical midline incision, for upper tract UC. By applying this method to synchronous bilateral nephroureterectomy, CUTE, through a single mini-incision approach, in single session can be the treatment option of choice as if there are indications for radical cystectomy in female patients receiving dialysis. Removal of gynecological organs may be undertaken simultaneously in peri- and postmenopausal patients.

In the present study we describe our modified technique in treating female dialysis patients with urothelial carcinoma.

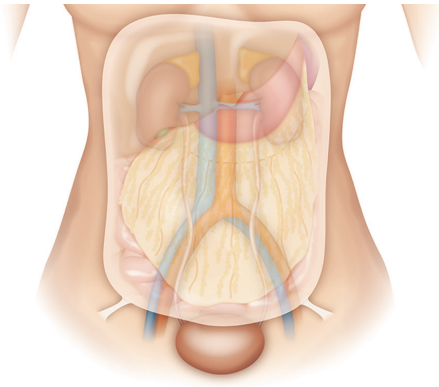

Figure 1: Illustration of two cord-like structures passing forwards in the peritoneal fold and entering the inguinal canal by the internal ring, considered to be the round ligament or spermatic cord in the female and the male, respectively.

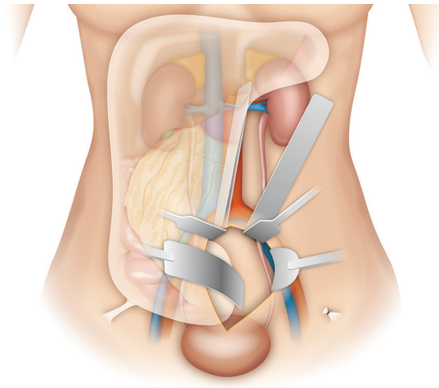

Figure 2: Illustration of *Peritoneal mobilization*. After division of the round ligament, two customized long right-angle retractors (upper 2 blades), as well as a Bookwalter retractor system (lower 2 blades), are used to push the peritoneum medially and anteriorly, thereby exposing the retroperitoneum.

Throughout the whole course, patient repositioning or rotating the operating table with cuff inflation is not needed; the specimens were extracted *en bloc* from the infraumbilical midline incision. We also compare our results with those of similar studies.

2. Patients and Methods

From 2005 through 2012, a total of 14 female dialysis patients underwent single mini-incision CUTE, radical hysterectomy, and bilateral salpingo-oophorectomy at our institution. Ten of them had multifocal UC (9 synchronous upper tract and bladder, 1 bilateral upper tract); 3 patients had organ-confined bladder UC (clinical stage T1, T2, or carcinoma in situ) with left-sided hydronephrosis; the remaining one only had urinary bladder UC. No lymph node or distant metastasis was noted preoperatively.

2.1. Extraperitoneal Preparation. All patients were placed in the modified dorsal lithotomy position with both legs supported in stirrups. An infraumbilical midline incision, about 10 cm in length, was made. The posterior rectus fascia was carefully dissected to gain access to the extraperitoneal space, which was then created from the pubic symphysis cephalad using blunt dissection and sweeping method. Once adequate working space was obtained with visualization of the psoas muscle, bilateral round ligaments attached to the peritoneum were identified, ligated, and divided (Figure 1). The retroperitoneal space could be widely explored using a Bookwalter retractor system (Codman, USA), in company with two customized long right-angle retractors (blade 15 or 23 cm). One was used to lift the abdominal wall up and the other sweeping the peritoneum and its contents medially (Figure 2).

2.2. Bilateral Nephroureterectomy. The ureter on one side was first identified, and ureteral skeletonization was performed cephalad using electrocautery toward the renal pelvis. After exploring the lower pole of the ipsilateral kidney, a

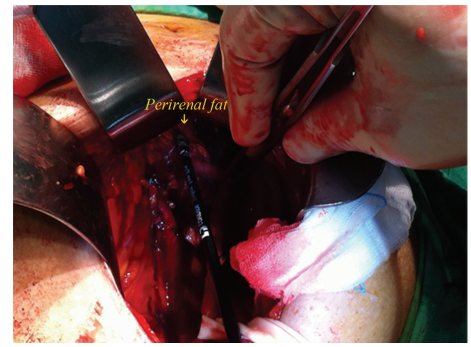

Figure 3: Intraoperative image of *Perirenal dissection*. A circumferential dissection of the kidney was performed along the plane between the renal capsule and the perinephric fat, with assistance of LigaSure.

circumferential dissection was performed manually along the plane between the renal capsule and the perinephric fat (Figure 3). Besides, bipolar electrocautery would be used to facilitate dissecting cephalad from the lower pole of the kidney toward the upper pole, by dividing any side-wall attachments. As the kidney was completely mobilized (Figure 4), the renal pedicle was isolated, ligated, and divided *en bloc* with Endo-GIA staplers (US Surgical Corporation, Norwalk, Connecticut), and the kidney was retrieved through the midline incision. The same procedure was repeated on the other side.

2.3. Radical Cystectomy. Radical cystectomy for women traditionally includes removal of the uterus, bilateral fallopian tubes, ovaries, and part of the vagina. After bilateral extraperitoneal nephroureterectomy, transperitoneal cystectomy, radical hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymph node dissection were accomplished using standard open surgical techniques. Frozen section pathology of the bladder neck was performed to ensure a safe margin. The entire specimens were *en bloc* retrieved from the

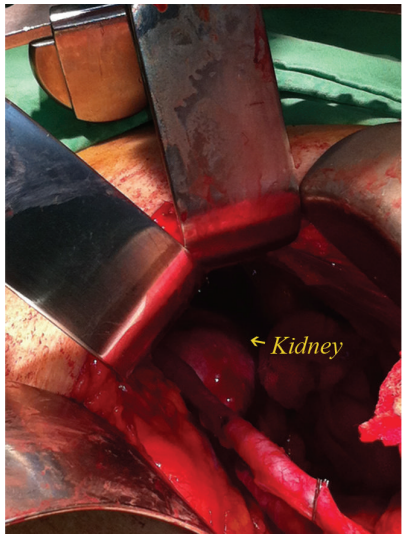

Figure 4: Intraoperative image showing good exposure of the retroperitoneum and complete mobilization of the kidney.

infraumbilical midline wound, and the urothelial continuity was maintained intact.

All surgeries were executed by one single surgeon (C. C. Yu). Follow-up abdominal computerized tomography (CT) was performed 3 months after surgery, every 6 months for the next 3 years, and then annually for life. With approval of the institutional review board, patient demographics and perioperative parameters, including operative time, blood loss, and convalescence and cancer control, were retrospectively reviewed and compared with peer-reviewed literature. Continuous variables were compared with the one sample t-test and categorical variables using the chi-square test. $P < 0.05$ was considered statistically significant.

3. Results

Patient demographics and perioperative outcomes were shown in Table 1. All patients were in good performance status (0-1), and all procedures were done successfully without major complications. The median duration of dialysis was 8.5 (6, 10.75) years. Ten patients remained cancer-free at a median follow-up of 46.5 (30.25, 87) months; four patients died of nonmalignant causes. Among ten patients with multifocal urothelial carcinoma, three (number 5, 8, 9) had incidental UC in the upper tract and one (number 1) was diagnosed as having a 1 mm Furhman grade I clear cell renal cell carcinoma (ccRCC) at the left kidney. Muscle-invasive bladder cancer with concomitant bilateral ureteral UC was incidentally found in one patient (number 11) presenting with recurrent bladder tumors and left hydronephrosis. Concurrent unilateral upper tract UC was also incidentally noted in the patient (number 13), who underwent CUTE for primary bladder cancer. All these preoperatively undetectable tumors were confirmed after carefully reviewing preoperative CT scans and postoperative histopathology. The median operative time was 242.5 (187.5, 268.75) minutes, and estimated blood loss was 500 (325, 750) mL. The median time to oral intake was 2 (1.75, 2) postoperative days, and the median hospital stay was 11 (9, 13.5) days. Postoperative complications included two cases of postoperatively prolonged ileus and one esophageal ulcer. No arteriovenous fistula formation was noted on follow-up CT scans after *en bloc* ligation of the renal pedicle. Statistical comparison of variables between different studies was shown in Table 2 [3–9]. In comparison with similar operations [7–9], our operative time and the interval to oral intake were significantly shorter; blood loss, hospital stay, and complications were insignificantly different. Except for more blood loss, our results were comparable to those of other smaller-scale surgeries in terms of operative time and convalescence [3–6].

4. Discussion

Urothelial carcinoma (UC) is the most common malignancy in dialysis patients of Taiwan [10]. On account of its high recurrence rate and rapidly progressive behavior, a more aggressive surgical strategy is recommended to improve the quality of life and prolong the survival of these patients [2, 11]. Complete urinary tract exenteration (CUTE) in single session is recommended for avoiding multistaged surgeries, associated with repeated analgesia, intraabdominal adhesions, delay in treatment, and higher morbidities and mortalities. Bothersome results, such as positive urine cytology and a suspicious filling defect within the urinary tract, and unpleasant follow-up procedures, like cystoscopy and retrograde pyelography, can be precluded and possible complications related to a contracted urinary bladder and nonfunctioning kidneys may be prevented as well. Traditional CUTE was performed through a long transperitoneal midline incision extending from the xiphoid process to the pubic symphysis. With the improvement of laparoscopic techniques and instrumentation, minimally invasive therapies may be offered [7–9].

Berglund et al. [12] first published the feasibility of laparoscopic radical cystoprostatectomy and bilateral nephroureterectomy for 2 male patients in 2005. Thereafter, Ou and Yang [7], Li et al. [8], and Lin et al. [9] successfully accomplished transperitoneoscopic or retroperitoneoscopic CUTE for dialysis patients. Pneumoperitoneum is the essential component for laparoscopy, but potential risks related to hypercapnia, cardiopulmonary compromise, hypothermia, subcutaneous emphysema, and air embolism exist. Besides, uremic patients on chronic dialysis often present with multiple comorbidities, including anemia, diabetes mellitus, cardiovascular disease, peptic ulcer disease and platelet dysfunction, and increasing perioperative morbidity and mortality. The extent of the surgery such as CUTE represents a considerable challenge to the patient, surgeon, and anesthesiologist. In order to reduce hemodynamic fluctuations related to this major high-risk surgery, pneumoperitoneum was replaced by a modified retractor system, and no 90-day postoperative mortality was reported in our series.

Tracing back to our history of evolution, unilateral hand-assisted retroperitoneoscopic nephroureterectomy (HARN)

Table 1: Patient demographics and perioperative parameters.

Pt	Age (y)	Duration of HD (yr)	BMI (kg/m^2)	ECOG	Clinically surgical indication	Hydronephrosis	Pathologic tumor location (T stage)[a]	Comorbidity
1	63	9	21.4	0	UB & LU	Left	UB(is) & LU(1) **LK ccRCC(1a)**	HTN
2	50	14	21.7	0	UB & RU	Right	UB(1) & RU(3)	HTN
3	61	8	25.9	1	UB & RU**	Right	UB(a) & RU(a)	HTN, HCC, cirrhosis, hepatitis B & C, SHPT
4	75	6	31.5	0	UB & LU LK	Left	UB(a) & LU(a) LK(a)	None
5	63	17	17.1	1	UB & LK	Bilateral	UB(1) & LK(3) **LU(a)**	HTN, hepatitis C, pulmonary TB
6	52	4	31.8	0	UB & RK	No	UB(1) & RK(1)	HTN, DM
7	67	1	20.4	1	BK & RU	Right	BK(a) & RU(1)	HTN, CHF
8	80	6	17.0	1	UB & LU**	Bilateral	UB(2a) & LU(a) **RK(a) & RU(a)**	HTN, DM, moderate MR
9	58	9	21.3	1	UB & RU	No	UB(is) & RU(1) **LU(is)**	SHPT
10	70	2	22.6	1	UB & RU**	Right	UB(1) & RU(1)	HTN, old CVA, parkinsonism
11	57	8	22.2	1	UB**	Left	UB(2a) **LU(is) & RU(3)**	SHPT
12	57	13	17.3	1	UB**	Left	UB(1)	Pulmonary TB
13	53	10	21.5	0	UB	No	UB(1) **LU(a)**	HTN, hepatitis B
14	48	11*	21.2	1	UB	Left	UB(1)	HTN, hepatitis C, SHPT, peritonitis***

HD, hemodialysis; BMI, body mass index; ECOG, eastern cooperative oncology group performance status; UB, urinary bladder; LU, left ureter; RU, right ureter; LK, left kidney; RK, right kidney; BK, bilateral kidney; ccRCC, clear cell renal cell carcinoma; HTN, hypertension; HCC, hepatocellular carcinoma; SHPT, secondary hyperparathyroidism; TB, tuberculosis; DM, diabetes mellitus; CHF, congestive heart failure; CVA, cardiovascular accident.
[a]All patients had high-grade urothelial carcinoma.
*Peritoneal dialysis.
**Surgical indication was recurrent urothelial cancer.
***Continuous ambulatory peritoneal dialysis (CAPD) related sclerosing peritonitis.

Table 2: Comparison of outcomes from concurrent upper and lower urinary tract surgery.

Author	Number	Extent of surgery	Age (y)	Operative time (min)[a]	Blood loss (mL)[a]	Hospital stay (d)[a]	Time to intake (hr)[a]	Complication (%)[b]
El-Galley et al., 2011 [3]	36	BN	N/A	222	175***	3.0***	N/A	22.2
Chueh et al., 2002 [4]	7	BNU	51.6	294**	218**	8.8*	39.0	14.3
Tai et al., 2009 [5]	33	BNU	52.4	309**	226**	10.2	58.0	12.1
Ou and Yang, 2011 [6]	13	BNU	60.0	215	216**	13.8	60.0	7.7
Ou and Yang, 2011 [7]	10	CUTE	57.6	328***	628	14.7*	62.4*	10.0
Li et al., 2009 [8]	5	CUTE	58.0	492***	378	12.2	72.0**	80.0
Lin et al., 2011 [9]	5	CUTE	66.6	397***	532	10.8	91.2***	20.0
Present study	14	CUTE	61.0	237.5	560.7	12.1	48.0	21.4

BN, bilateral nephrectomy; BNU, bilateral nephroureterectomy; CUTE, complete urinary tract exenteration.
[a]One sample t-test.
[b]Chi-square test.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for compared to present study.
N/A, not available.

was performed via a Gibson incision at our institution. Based on accumulated experiences and skills, unilateral extraperitoneal nephroureterectomy could be practiced through a paramedian incision [13]. During this period, a camera port was required to insert a laparoscope and ensure the security of *en bloc* ligation of renal vessels; an extended paramedian incision could immediately be made as if major bleeding needed to be treated. After becoming progressively proficient in surgical anatomy of the retroperitoneal space, better exposure could be attained and the renal hilum might be directly visualized without the need of laparoscopy. Additionally, palpitation of the renal pedicle, digital dissection, and retraction were utilized to collaborate with perirenal dissection. In consideration of postoperative analgesia and functional recovery, an infraumbilical midline incision was attempted to accomplish unilateral extraperitoneal nephroureterectomy, which had been done by one single surgeon in more than 200 patients.

For the purpose of good exposure of the retroperitoneum, two important surgical steps are addressed. First, division of the round ligament or spermatic cord facilitates mobilization of the peritoneum and its contents [14, 15]. Anatomically, there are two cord-like structures passing forwards in the peritoneal fold and entering the inguinal canal by the internal ring, considered to be the round ligament or spermatic cord in the female and the male, respectively. Mass ligation of these structures can separate the peritoneum off the iliac vessels and the psoas muscle is exposed laterally and posteriorly to help in identifying and skeletonizing the ureter cephalad. Second, along with a Bookwalter retractor system, two customized long right-angle retractors, of which the blade is 15 or 23 cm, are used to push the peritoneum medially and anteriorly. With traction on the peritoneum, the intraperitoneal contents may be naturally retracted and protected, lowering the risk of adjacent organ interference or injury, even in the peritoneal dialysis patient with a history of sclerosing peritonitis; a wide space may be provided for ureterolysis, perirenal dissection, and isolation of the renal pedicle.

On the other hand, in order to complete laparoscopic bilateral upper urinary tract surgery in single session, utilizing gravity to maneuver the bowel is of great concern. Some experts alternated inflatable air tourniquet cuffs or gel rolls on each side of the patient's back, or tilted the table to facilitate displacing the bowel by gravity [4, 9, 16, 17], thus saving the time of repositioning and redraping. All patients in our series were operated in the modified dorsal lithotomy position, with both arms outstretched, throughout the whole procedure. Plenty of time could be preserved, attributing to no change of patient's position. This could explain why our operative time was significantly shorter compared to those of published studies [7–9]. By virtue of minimally invasive surgical techniques with laparoscopic instruments, as well as tactile feedback and direct three-dimensional visualization, the method presented here permitted a faster upper urinary tract extirpation.

As for shorter interval to oral intake, it might be ascribed to shorter operative duration; other perioperative parameters appeared to be similar. Owing to very low premiums of the National Health Insurance in Taiwan, patients usually would not be discharged until being fully recovered. That is why our hospital stay could not be shortened. Comparing other studies with regard to synchronous bilateral renal surgery [3–6], there was significantly more blood loss in our series. The amount of bleeding might be correlated with the extent of surgery. Radical hysterectomy and bilateral salpingo-oophorectomy were simultaneously performed, partly explaining why the blood loss was significantly higher than that of other smaller-scale surgeries. Nevertheless, our surgical time and postoperative convalescence were still comparable.

With regard to oncologic control, 10 patients remained cancer-free at a follow-up of at least 19 months, the longest being 105 months. This might be attributed to the broadened indication for CUTE at our institute, including primary bladder cancer in uremic patients.

Preoperatively undetectable malignancy, such as small RCC or superficial UC, in the upper tract could be *en bloc* removed in single session. Six patients were postoperatively confirmed as having concurrent UC or ccRCC in our series, even after carefully reviewing preoperative CT scans. Although no other study is available to substantiate this finding, it is confirmed that an aggressive surgical approach should be executed to treat UC in uremic patients regarding its notorious behavior. However, there are still contraindications to our techniques, including advanced upper tract tumors and lymph node metastasis identified preoperatively, due to difficulty in attaining negative surgical margins and removing enough lymph nodes. In another aspect, it should be concerned that the risk of ruptured collecting system exists, especially in patients with moderate or severe hydronephrosis. There was no aforementioned complication encountered in our patients because most of them had localized upper tract tumors in atrophic kidneys.

The present study was limited by its retrospective nature and small case number. The amount of analgesia and quality of life were not evaluated as well postoperatively. Despite good exposure of the retroperitoneal space, it is still a confined space to perform perirenal dissection, even in hand-assisted retroperitoneoscopic surgery. Without proficient anatomical knowledge of the retroperitoneal space and sophisticated minimally invasive surgical skills, peritoneal violation might be encountered, thereby causing the bowel to protrude through the peritoneal defect and hinder access to the surgical field. Reproducibility may be an issue, but, in experienced hands, it can be done efficiently and safely with minimal morbidity. There were still two cases of postoperatively prolonged ileus, and it might be related to the transperitoneal approach for radical cystectomy. A prospective randomized study with a larger sample and long-term follow-up would be needed to demonstrate the possible benefit more conclusively.

The concept of peritoneal mobilization promotes our surgical evolution, leading to better exposure of the retroperitoneum. Even though peritoneal violation is present, the customized long right-angle retractors and moist laparotomy pads may be utilized to overcome this obstacle. Our modified technique provides an expanded indication for CUTE.

It is recommended that this method be implemented in dialysis patients with multifocal UC in which upper tract tumors are localized without lymph node metastasis or those with primary bladder cancer eligible for radical cystectomy, thus eliminating the preoperatively undetectable tumors and reducing the likelihood of metachronous malignancy. Gynecologic organs can simultaneously be removed to achieve better oncological control. Without pneumoperitoneum and repositioning, it can be performed via an infraumbilical midline mini-incision, and the entire specimen can be extracted *en bloc* with intact urothelial continuity.

5. Conclusions

This modified single mini-incision CUTE provides a time-saving method for dialysis patients with UC, eliminating preoperatively undetectable malignancy and reducing metachronous recurrences; perioperative complications associated with pneumoperitoneum and repositioning can be averted as well. Good cancer control and early convalescence may both be achieved in experienced hands.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Nomogram Predicting Renal Insufficiency after Nephroureterectomy for Upper Tract Urothelial Carcinoma in the Chinese Population: Exclusion of Ineligible Candidates for Adjuvant Chemotherapy

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Objectives. To report the decline of renal function after radical nephroureterectomy (RNU) in upper tract urothelial carcinoma (UTUC) patients and to develop a nomogram to predict ineligibility for cisplatin-based adjuvant chemotherapy (AC). *Methods.* We retrospectively analyzed 606 consecutive Chinese UTUC patients treated by RNU from 2000 to 2010. We chose an eGFR of 60 and 45 ml/min/1.73 m^2 as cut-offs for full-dose and reduced-dose AC eligibility. *Results.* Median eGFR for all patients before and after surgery was 64 and 49 ml/min/1.73 m^2 ($P < 0.001$). The proportion of patients ineligible to receive full-dose and reduced-dose AC changed from 42% to 74% and from 20% to 38.1%. Older age (OR = 1.007), preoperative eGFR (OR = 0.993), absence of hydronephrosis (OR = 0.801), smaller tumor size (OR = 0.962), and tumor without multifocality (OR = 0.876) were predictive for ineligibility for full-dose AC. Preoperative eGFR (OR = 0.991), absence of hydronephrosis (OR = 0.881), tumor located in renal pelvis (OR = 1.164), and smaller tumor size (OR = 0.969) could predict ineligibility for reduced-dose AC. The c-index of the two models was 0.757 and 0.836. Postoperative renal function was not associated with worse survival. *Conclusions.* Older age, lower preoperative eGFR, smaller tumor size, tumor located in renal pelvis, and absence of hydronephrosis or multifocality were predictors of postoperative renal insufficiency.

1. Introduction

Although radical nephroureterectomy (RNU) with excision of the bladder cuff is the gold-standard treatment for upper tract urothelial carcinomas (UTUC) [1], the oncologic outcomes for patients with high-grade or non-organ-confined disease remain poor, with 5-year cancer-specific survival rates less than 60% [2–4]. Multimodal approaches have been suggested and perioperative chemotherapy has been considered as an option to improve disease control [5–7].

The use of cisplatin-based chemotherapy has gained greater acceptance as evidenced by its use in neoadjuvant and adjuvant therapy, especially in patients with pT3-4 or pTxN+ [5–8]. But cisplatin-based therapy was associated with a higher risk of severe nephrotoxicity, and creatine clearance was important in determining whether patients should be treated with cisplatin [9]. However, the present limited ability to predict tumor stage and grade accurately before surgery makes it difficult to select proper candidates for neoadjuvant

therapy, while the loss of renal unit would limit the use of cisplatin-based chemotherapy in adjuvant therapy [10, 11].

Several reports have evaluated changes in renal function following RNU and demonstrated that the decline in renal function may render a substantial number of patients ineligible to receive adjuvant chemotherapy (AC) [10–15]. The ability to predict which patients would develop renal insufficiency following RNU would be extremely useful. Previous studies provided scarce information on risk stratification for worse chronic kidney disease (CKD) after RNU [13–15], and there are few published reports from research centers in China.

Therefore, in this large single-center cohort of patients, we sought to reveal the prevalence of CKD before and after RNU and to develop a nomogram to predict ineligibility for AC, which would help to accurately predict postoperative renal function and thus provide more optimal and personalized risk-based therapy options. Besides, we evaluated the association between postoperative renal function and survival.

2. Materials and Methods

2.1. Patient Selection. Following institutional review board approval and written informed consent from patients, we initially collected the clinicopathological data of 912 consecutive UTUC patients who received treatment in the Department of Urology, Peking University First Hospital, from 2000 to 2012. This was a large cohort drawing from 27 different provinces or autonomous regions of China. Patients underwent nephron-sparing surgery instead of RNU, with bilateral synchronous UTUC, and previous histories for UTUC, incomplete data on pre- or postoperative serum creatine (Scr), or no follow-up data were excluded. Six hundred and six patients were finally enrolled for evaluation.

All patients were diagnosed using computed tomography (CT) or magnetic resonance imaging (MRI), urologic ultrasound, and in some patients ureteroscopy with or without biopsy. All patients underwent surgery within two months after the occurrence of symptoms. Lower ureter and bladder cuff excision was performed through the Gibson incision in all cases. None of these patients received neoadjuvant chemotherapy (NC), while, for several patients, adjuvant chemotherapy or radiotherapy was administered when evidence of distant metastasis or retroperitoneal recurrence was documented on condition that patients had good general condition.

2.2. Patients Evaluation. The estimated glomerular filtration rate (eGFR) was calculated using the modified glomerular filtration rate estimating equation for Chinese patients: eGFR ($\mathrm{mL/min/1.73\,m^2}$) = $175 \times \mathrm{Scr}^{-1.234} \times \mathrm{age}^{-0.179}$ ($\times$ 0.79 if female) [16]. Comparison of eGFR before and after surgery was performed using the Scr drawn closest to 7 days after surgery (range: 3 days to 1 month after surgery). This timing was selected to best approximate the measured serum creatinine that would reflect the direct effect of RNU on renal function. And most patients could be included with available pre- and postoperative Scr data. We chose an eGFR of 60 and 45 $\mathrm{mL/min/1.73\,m^2}$ as possible cut-offs for full-dose and reduced-dose cisplatin-based AC. The eGFR cut-off of 45 $\mathrm{mL/min/1.73\,m^2}$ was defined for its compromise between lower limits for reduced-dose cisplatin in previous studies [17–19]; besides, it was cited as a more strict definition of CKD [12].

All pathological specimens were re-reviewed by a dedicated genitourinary pathologist to confirm the reproducibility of the diagnosis. Tumor stage was assessed according to the 2002 Union for International Cancer Control (UICC) TNM classification of malignant tumors. Tumor grading was assessed according to the World Health Organization (WHO) classification of 1973. Tumor architecture was defined as papillary or sessile by the examination of the final specimen. Tumor location was divided into 2 areas (renal pelvis and ureter) based on the site of the dominant lesion. Tumor multifocality was defined as the synchronous presence of two or more pathologically confirmed macroscopic tumors in any location. Ipsilateral hydronephrosis (HN) was determined by MRI or CT before operation.

2.3. Follow-Up Schedule. For patients who were followed up at our institute, the follow-up regimen of the affected patients included cystoscopy every 3 months for the first 3 years. The cystoscopy intervals were extended to 1 year thereafter. Chest X-ray, urine cytology, Scr, and abdominal ultrasound or CT/MRI were examined at the same time. The cause of death was determined by the patients' treating physicians or by death certificates. Follow-ups were censored until their last visit or death.

2.4. Statistical Analysis. Pearson's test and chi-square test were used to test the distribution of categorical variables, and the Mann-Whitney U test and paired-sample t-test were used for continuous variables. Multivariate logistic regression was used to calculate the predictive factors. Only variables that were identified as significant by the univariate analysis were considered for the multivariate analysis. Log-rank test was used in survival analysis. Multivariable logistic regression coefficients were used to generate a nomogram for impaired renal function [20]. Discrimination was measured using Harrell's concordance index (c-index), which is similar to the area under the receiver operating characteristic curve. Calibration was measured by calibration plots, which were generated to explore the nomograms performance using 200 bootstrap resamples.

The generation of the nomogram and calibration plots was performed with the R open-source statistical software, and other statistical tests were performed with SPSS 20.0 (IBM Corp., Armonk, NY, USA). All reported P values were single-sided with statistical significance considered at $P < 0.05$.

3. Results and Discussion

3.1. Patients Characteristics and Changes in Renal Function. The median patient age was 69 years (range: 25–94 years). The distribution of UTUC pathological stage in this cohort

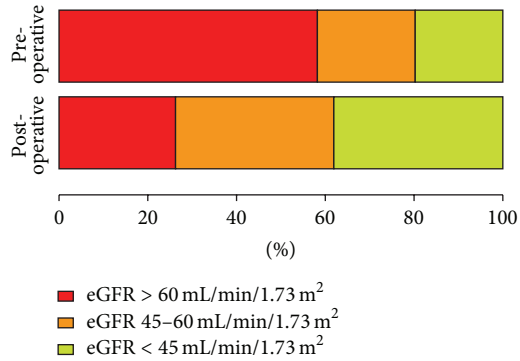


FIGURE 1: Percentage of patients with pre- and postoperative eGFR in selected ranges (over 60 mL/min/1.73 m^2, between 45 and 60 mL/min/1.73 m^2, and lower than 45 mL/min/1.73 m^2).

was pTa, pT1, pT2, pT3, and pT4 in 20 (3.3%) patients, 189 (31.2%) patients, 213 (35.1%) patients, 173 (28.5%) patients, and 11 (1.8%) patients, respectively. Pathological grades 1–3 tumors were present in 18 (3.0%) individuals, 333 (55.0%) individuals, and 255 (42.1%) individuals, respectively.

Median eGFR for all patients prior to surgery was 64 (interquartile range, IQR 49–81) mL/min/1.73 m^2, while after RNU it was 49 (IQR 38–60) mL/min/1.73 m^2. Comparison of pre- and postoperative eGFR revealed a mean decrease of 15 mL/min/1.73 m^2 and a median percentage loss of 24% after RNU ($P < 0.001$).

Using 60 mL/min/1.73 m^2 as the eligibility cut-off for full-dose AC, 58% of the study population was eligible before surgery, whereas only 26% remained eligible following RNU. Using a cut-off of 45 mL/min/1.73 m^2 for reduced-dose AC, 80% was eligible preoperatively, whereas only 61.9% remained above this cut-off after surgery (Figure 1).

Only 11 patients (1.8%) in this cohort actually received AC at our institute, with mean preoperative eGFR 71.5 mL/min/1.73 m^2 and postoperative eGFR 54.5 mL/min/1.73 m^2.

3.2. Risk Factors for Impaired Renal Function. Of the 353 possible candidates for NC, 193 (54.7%) were judged ineligible to undergo AC with their postoperative eGFR below 60 mL/min/1.73 m^2. On univariate analysis, older age ($P = 0.001$), lower preoperative eGFR ($P = 0.003$), tumor located in renal pelvis ($P = 0.007$), absence of preoperative HN ($P < 0.001$), tumor without multifocality ($P = 0.006$), tumor size ($P = 0.001$), lower tumor stage ($P = 0.001$), and papillary architecture ($P < 0.001$) were associated with postoperative eGFR lower than 60 mL/min/1.73 m^2. Multivariate analysis controlling for all preoperative factors demonstrated that older age (OR = 1.007 per year), lower preoperative eGFR (OR = 0.993 per mL/min/1.73 m^2), absence of preoperative HN (OR = 0.801), smaller tumor size (OR = 0.962 per centimeter), and tumor without multifocality (OR = 0.876) were independent risk factors predicting ineligibility for full-dose AC (Table 1). We used logistic regression coefficients to generate a corresponding nomogram (Figure 2(a)) and calibration plot (Figure 2(b)). The accuracy of the model nomogram measured by c-index was 0.757.

Similarly, when we defined cut-off of 45 mL/min/1.73 m^2, preoperative eGFR (OR = 0.991 per mL/min/1.73 m^2), absence of preoperative HN (OR = 0.881), tumor located in renal pelvis (OR = 1.164), and smaller tumor size (OR = 0.969) independently predicted impaired postoperative renal function in multivariate analysis (Table 2). The corresponding nomogram and calibration plot were shown in Figure 3. The accuracy of the model nomogram measured by c-index was 0.836.

3.3. Predictive Role on Survival. The median follow-up duration of this cohort of patients was 56 (IQR 24–72) months. One hundred and ninety-three patients (31.2%) died, and 166 patients (27.4%) died of urothelial cancer. The 5-year overall survival and cancer-specific survival were 69.8% and 72.6%, respectively. By log-rank test, postoperative renal function was not associated with worse OS ($P = 0.077$) or worse CSS ($P = 0.097$) (Figure 4). Besides, use of AC demonstrated no effect on survival (data not shown).

3.4. Discussion. The result of the present research confirmed that eGFR deteriorates significantly following RNU, and a substantial proportion (over 30%) of patients would miss the opportunity to undergo AC for impaired renal function, similar to previous reports [10–12]. Even when we set cut-off at 45 mL/min/1.73 m^2 for reduced dose of cisplatin-based chemotherapy, we could notice that although 80% of patients were qualified for NC, RNU rendered nearly 20% of patients ineligible for AC. Besides, we found no association between postoperative CKD and survival, which is in accordance with previous studies [12]. Though theoretically CKD is related to higher risk of cardiovascular events, the association of eGFR with survival in UTUC patients has not been determined and needs to be further clarified.

Due to the decline of eGFR, previous studies suggest strong consideration of neoadjuvant regimens when chemotherapy is indicated [10, 12]. But the current staging modalities hindered the extensive use of NC. As the only commonly accepted measure to get biopsy before RNU, ureteroscopy is associated with perioperative complications and higher risk of intravesical recurrence [21]. Without biopsy, we cannot firmly exclude the possibility of another pathological diagnosis instead of transitional cell carcinoma. Besides, predictive models for non-organ-confined or high-grade disease by preoperative factors in previous studies have not been testified in population-based study [22–25]. A casual NC might result in overtreatment in low-risk disease. On the other hand, AC could be carried after pathological examination of final specimen. Thus, prediction of postoperative renal function is important to detect patients not suitable for AC.

Older age and preoperative eGFR were proved to be predictive of decline of eGFR in previous reports [12, 15, 26]. There was no consensus about the predictive role of other clinical factors. Hoshino et al. [14] found the absence of higher grade HN was independent risk factor for patients ineligible for AC, while results were contrary in Rodriguez

Table 1: Predictive factors for identifying patients ineligible to receive full dose of cisplatin-based adjuvant chemotherapy[#].

	Postoperative eGFR (mL/min/1.73 m^2)			Univariate analysis		Multivariate analysis		
	All	≥60	<60	Chi-square	*P* value	OR	95% CI	*P* value
All, number (%)	353 (100)	160 (45.3)	193 (54.7)					
Gender, number (%)				0.004	0.516			
Male	167 (47.3)	76 (47.5)	91 (47.2)					
Female	186 (52.7)	84 (52.5)	102 (52.8)					
Age^, number (%)				10.966	**0.001***	1.007	1.002–1.012	**0.002***
<70	200 (56.7)	106 (66.3)	94 (48.7)					
≥70	153 (43.3)	54 (33.8)	99 (51.3)					
Preoperative eGFR (mL/min/1.73 m^2)^, number (%)				8.235	**0.003***	0.993	0.991–0.997	<**0.001***
<90	87 (24.6)	51 (31.9)	36 (18.7)					
≥90	266 (75.4)	109 (68.1)	157 (81.3)					
Previous or concomitant BT, number (%)				0.145	0.417			
No	313 (88.7)	143 (89.4)	170 (88.1)					
Yes	40 (11.3)	17 (10.6)	23 (11.9)					
Side, number (%)				0.533	0.267			
Left	173 (49.0)	75 (46.9)	98 (50.8)					
Right	180 (51.0)	85 (53.1)	95 (49.2)					
Location, number (%)				6.513	**0.007***	1.071	0.955–1.202	0.240
Ureter	144 (40.8)	77 (48.1)	67 (34.7)					
Pelvis	209 (59.2)	83 (51.9)	126 (65.3)					
Hydronephrosis, number (%)				22.657	<**0.001***	0.801	0.714–0.899	<**0.001***
No	188 (53.3)	63 (39.4)	125 (64.8)					
Yes	165 (46.7)	97 (60.6)	68 (35.2)					
Multifocality, number (%)				7.038	**0.006***	0.876	0.771–0.996	**0.044***
No	294 (83.3)	124 (77.5)	170 (88.1)					
Yes	59 (16.7)	36 (22.5)	23 (11.9)					
DM, number (%)				0.980	0.200			
No	297 (84.1)	138 (86.3)	159 (82.4)					
Yes	56 (15.9)	22 (13.8)	34 (17.6)					
Hypertension, number (%)				2.931	0.055			
No	228 (64.6)	111 (69.4)	117 (60.6)					
Yes	125 (35.4)	49 (30.6)	76 (39.4)					
Smoking, number (%)				2.853	0.060			
No	281 (79.6)	121 (75.6)	160 (82.9)					
Yes	72 (20.4)	39 (24.4)	33 (17.1)					
Tumor size (cm)^, number (%)				10.514	**0.001***	0.962	0.942–0.983	<**0.001***
<3	190 (53.8)	71 (44.4)	119 (61.7)					
≥3	163 (46.2)	89 (55.6)	74 (38.3)					
Architecture, number (%)				11.830	<**0.001***			
Papillary	268 (75.9)	109 (68.1)	159 (82.4)					
Sessile	80 (22.7)	50 (31.3)	30 (15.5)					
Stage, number (%)				10.873	**0.001***			
Ta/T1/T2	233 (66.0)	91 (56.9)	142 (73.6)					
T3/T4	120 (34.0)	69 (43.1)	51 (26.4)					

Table 1: Continued.

	Postoperative eGFR (mL/min/1.73 m^2)			Univariate analysis		Multivariate analysis		
	All	≥60	<60	Chi-square	*P* value	OR	95% CI	*P* value
Grade, number (%)				2.829	0.058			
G1-2	209 (59.2)	87 (54.4)	122 (63.2)					
G3	144 (40.8)	73 (45.6)	71 (36.8)					
Tumor necrosis, number (%)				2.766	0.069			
No	319 (30.4)	140 (87.5)	179 (92.7)					
Yes	34 (9.6)	20 (12.5)	14 (7.3)					
CIS, number (%)				0.389	0.374			
Absent	342 (96.9)	154 (96.3)	188 (97.4)					
Present	11 (3.1)	6 (3.8)	5 (2.6)					

*Statistically significant.
#Only patients with preoperative eGFR ≥ 60 mL/min/1.73 m^2 were included.
^Initially calculated as binary variables in univariate analysis and used as linear variable in multivariate analysis.
OR: odds ratio; CI: confidence interval; eGFR: estimated glomerular filtration rate; BT: bladder tumor; DM: diabetes mellitus; CIS: carcinoma in situ.

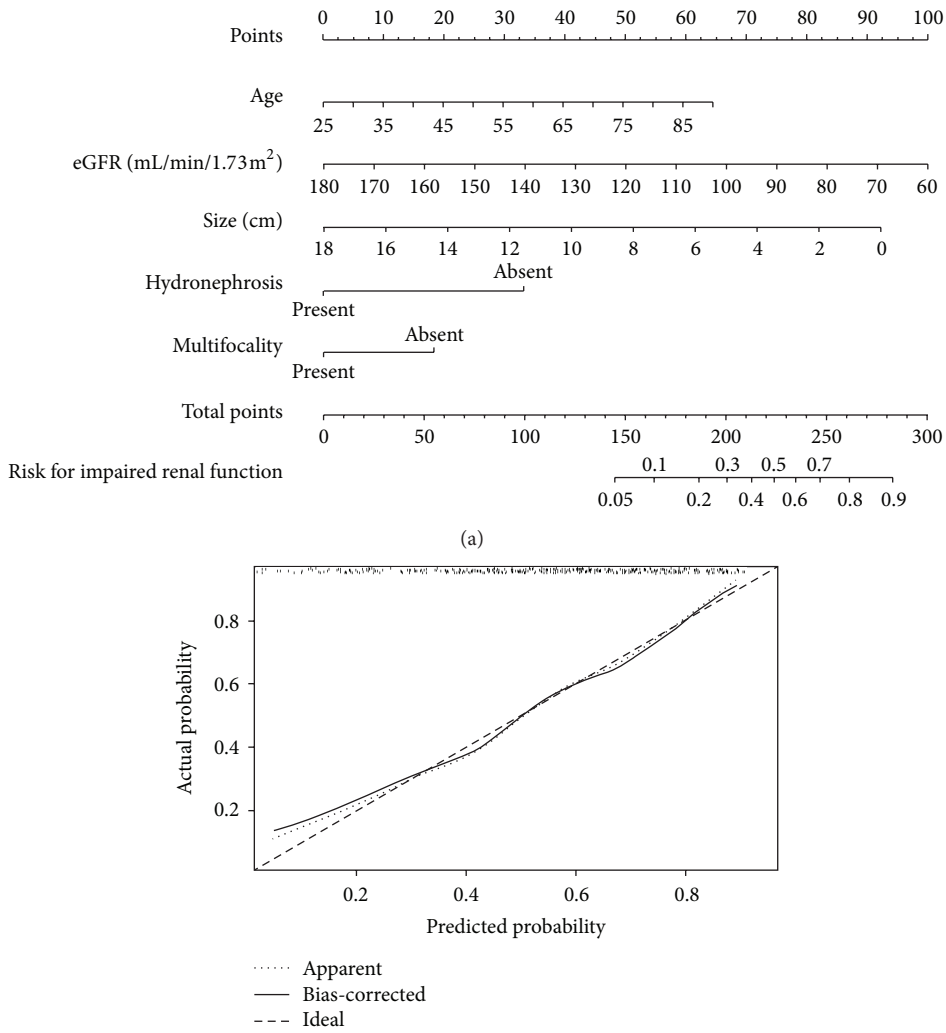


Figure 2: Nomogram (a) and calibration plot (b) for prediction of ineligibility to receive full-dose adjuvant chemotherapy with a c-index of 0.757.

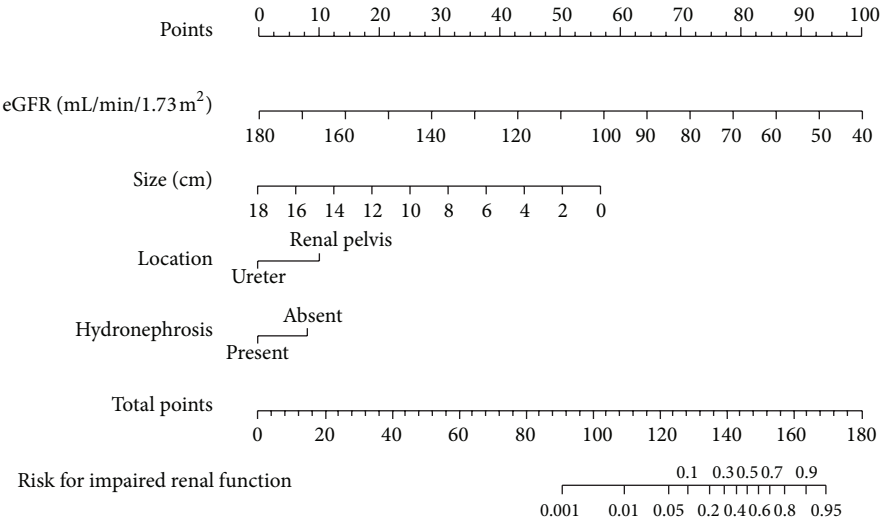
Table 2: Predictive factors for identifying patients ineligible to receive reduced dose of cisplatin-based adjuvant chemotherapy[#].

	Postoperative eGFR (mL/min/1.73 m^2)			Univariate analysis		Multivariate analysis		
	All	≥45	<45	Chi-square	*P* value	OR	95% CI	*P* value
All, number (%)	485 (100)	375 (77.3)	110 (22.3)					
Gender, number (%)				0.685	0.236			
Male	224 (46.2)	177 (47.2)	47 (42.7)					
Female	261 (53.8)	198 (52.8)	63 (57.3)					
Age^, number (%)				6.773	**0.006***	0.999	0.996–1.003	0.922
<70	260 (53.6)	213 (56.8)	47 (42.7)					
≥70	225 (46.4)	162 (43.2)	63 (57.3)					
Preoperative eGFR (mL/min/1.73 m^2)^, number (%)				34.365	<**0.001***	0.991	0.989–0.993	<**0.001***
<60	353 (72.8)	297 (79.2)	56 (50.9)					
≥60	132 (27.2)	78 (20.8)	54 (49.1)					
Previous or concomitant BT, number (%)				0.004	0.546			
No	418 (86.2)	323 (86.1)	95 (86.4)					
Yes	67 (13.8)	52 (13.9)	15 (13.6)					
Side, number (%)				0.645	0.244			
Left	235 (48.5)	178 (47.5)	57 (51.8)					
Right	250 (51.5)	197 (52.5)	53 (48.2)					
Location, number (%)				9.325	**0.002***	1.164	1.074–1.262	<**0.001***
Ureter	234 (48.2)	195 (52.0)	39 (35.5)					
Pelvis	251 (51.8)	180 (48.0)	71 (64.5)					
Hydronephrosis, number (%)				12.523	<**0.001***	0.881	0.813–0.956	**0.002***
No	228 (47.0)	160 (42.7)	68 (61.8)					
Yes	257 (53.0)	215 (57.3)	42 (38.2)					
Multifocality, number (%)				0.853	0.215			
No	398 (82.1)	311 (82.9)	87 (79.1)					
Yes	87 (17.9)	64 (17.1)	23 (20.9)					
DM, number (%)				0.091	0.444			
No	401 (92.7)	309 (82.4)	92 (83.6)					
Yes	84 (17.3)	66 (17.6)	18 (16.4)					
Hypertension, number (%)				0.877	0.204			
No	292 (60.2)	230 (61.3)	62 (56.4)					
Yes	193 (39.8)	145 (38.7)	48 (43.6)					
Smoking, number (%)				0.276	0.344			
No	392 (80.8)	305 (81.3)	87 (79.1)					
Yes	93 (19.2)	70 (18.7)	23 (20.9)					
Tumor size (cm)^, number (%)				16.163	<**0.001***	0.969	0.955–0.983	**0.001***
<3	267 (55.1)	188 (50.1)	79 (71.8)					
≥3	218 (44.9)	187 (49.9)	31 (28.2)					
Architecture, number (%)				13.392	<**0.001***			
Papillary	362 (74.6)	266 (70.9)	96 (87.3)					
Sessile	117 (24.1)	105 (28.0)	12 (10.9)					
Stage, number (%)				19.838	<**0.001***			
Ta/T1/T2	330 (68.0)	236 (62.9)	94 (85.5)					
T3/T4	155 (32.0)	139 (37.1)	16 (14.5)					

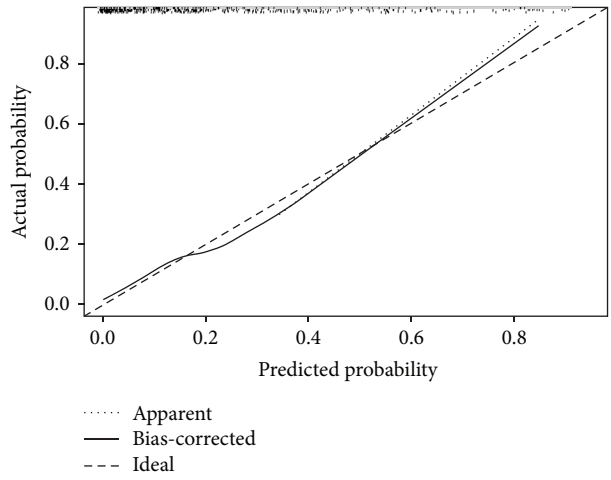
Table 2: Continued.

	Postoperative eGFR (mL/min/1.73 m^2)			Univariate analysis		Multivariate analysis		
	All	≥45	<45	Chi-square	*P* value	OR	95% CI	*P* value
Grade, number (%)				9.525	**0.001***			
G1-2	282 (58.1)	204 (54.4)	78 (70.9)					
G3	203 (41.9)	171 (45.6)	32 (29.1)					
Tumor necrosis, number (%)				3.385	**0.043***			
No	436 (89.9)	332 (88.5)	104 (94.5)					
Yes	49 (10.1)	43 (11.5)	6 (5.5)					
CIS, number (%)				0.579	0.350			
Absent	471 (97.1)	363 (96.8)	108 (98.2)					
Present	14 (2.9)	12 (3.2)	2 (1.8)					

*Statistically significant.
$^\#$Only patients with preoperative eGFR ≥ 45 mL/min/1.73 m^2 were included.
$^\wedge$Initially calculated as binary variables in univariate analysis and used as linear variable in multivariate analysis.
OR: odds ratio; CI: confidence interval; eGFR: estimated glomerular filtration rate; BT: bladder tumor; DM: diabetes mellitus; CIS: carcinoma in situ.



(a)



(b)

Figure 3: Nomogram (a) and calibration plot (b) for prediction of ineligibility to receive reduced-dose adjuvant chemotherapy with a c-index of 0.836.

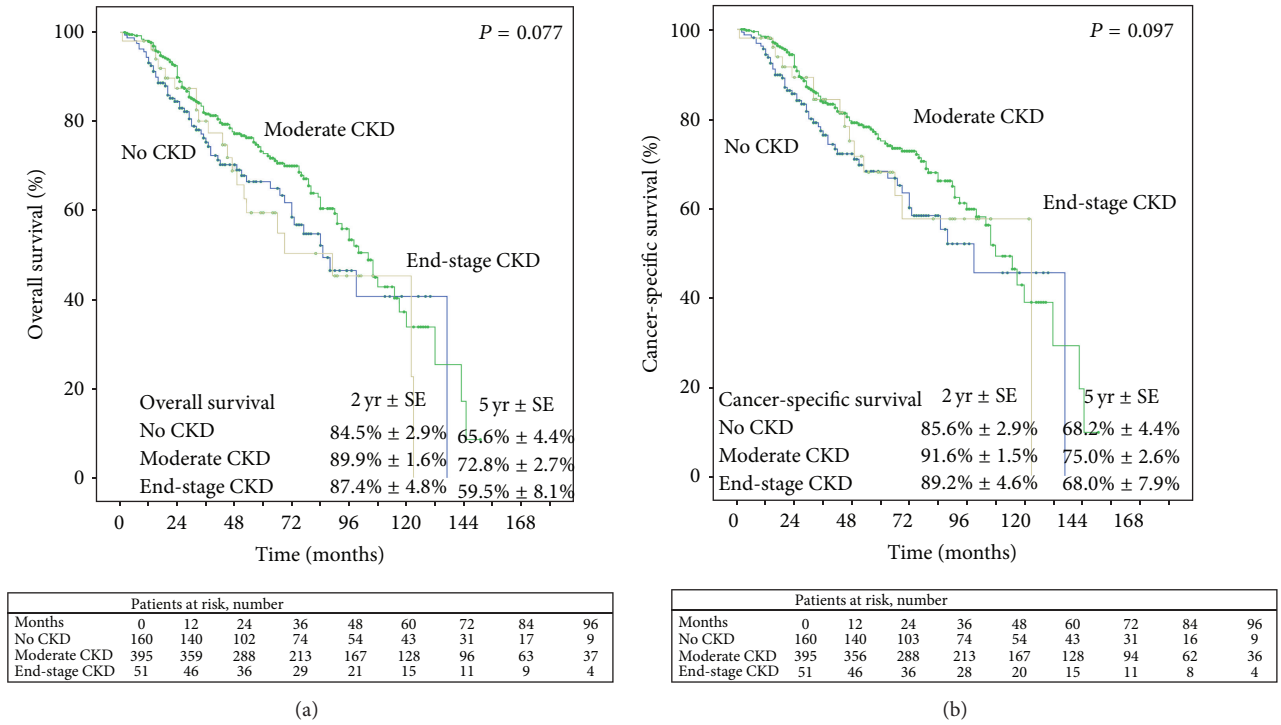


FIGURE 4: Estimated Kaplan-Meier overall survival curves (a) ($P = 0.077$) and cancer-specific survival curves (b) ($P = 0.097$) stratified by postoperative renal function.

Faba's research [15]. No explanation for these results was provided in these studies. A probable hypothesis for our results is that the presence of HN was always accompanied by thinner renal cortex and reduced eGFR. Total renal function would be compensated by the contralateral kidney. Thus, the resection of the impaired kidney would not result in significant decline of total eGFR as it only takes a small proportion. For patients with same preoperative eGFR, those with ipsilateral HN would probably have a better contralateral kidney and, as a result, a probable higher postoperative eGFR. Similarly, although not evidenced in previous clinical trials, it is easy to deduce that large tumor size, multifocality, and ureteral location would be associated with impaired function of the kidney with tumor (the kidney that would be removed); thus, they were demonstrated to be "protective" factor for postoperative eGFR after adjusting for preoperative renal function.

It is a pity that in most patients split renal function studies by nuclear renal scans were not routinely obtained before surgery. Evidence is scarce but, considering its function in evaluating the preoperative eGFR of the contralateral kidney directly, it might play an important role in predictive postoperative renal function after RNU.

Our model could help clinicians in optimizing timing of chemotherapy regimens and providing personalized therapy options based on preoperative factors. For patients evaluated as high risk for postoperative renal insufficiency, if systematic chemotherapy is considered, NC is recommended before their renal function is impaired, while, for patients less likely to suffer from decreased eGFR lower than 60 mL/min/1.73 m^2, clinicians could carefully evaluate the necessity of NC. Unless strongly indicated (e.g., high grade in biopsy, suspicious of lymph node metastasis, and/or adjacent organs invaded), clinicians could perform RNU without delay and carry AC if required based on final pathology.

NC has been demonstrated to decrease tumor burden and improve patients' survival [8, 27], while the effect of AC on prognosis was unsatisfied [5–7, 28, 29]. However, many of these trials were retrospective, single-center study with small sample size, and the regimen as well as doses was limited by poor postoperative renal function and was not standardized. Although the previous results of NC are promising, we should not neglect the possible benefits of AC which clearly needs more research.

There are some limitations in our study, especially the limitation of retrospective itself, and our study cohort might have been subject to selection and recall bias. Additionally, since we focused on early time-points in postoperative Scr, we could not exclude the possibility of spuriously low Scr measurements due to perioperative intravenous hydration or spuriously high Scr measurements as it is too early for the contralateral kidney to fully compensate. But our result could be interpreted as evaluation of acute renal disease short-term after RNU, and Kaag et al. [13] demonstrated that the decline of renal function after RNU showed no evidence of recovery over time. Another limitation is that, due to various reasons, there were extremely few patients that actually received AC.

Despite these limitations, to our knowledge, it is the first research that provides an applicable tool to predict postoperative renal insufficiency that could help risk stratification and

treatment strategies selection. Future studies on long-term monitoring of renal function and prospective clinical trials on AC would be required.

4. Conclusions

In the Chinese patients with UTUC, older age, lower preoperative eGFR, smaller tumor size, tumor located in renal pelvis, and absence of HN or tumor multifocality were demonstrated to be significant predictors of impaired renal function following RNU. The nomogram accurately predicts ineligibility for AC. Postoperative renal function did not correlate with patients' survival. The clinical significance of those results needs to be further assessed in external multi-institutional validation cohorts.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Dong Fang and Qifu Zhang contributed equally to this paper.

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Research Article

Increased Upper and Lower Tract Urothelial Carcinoma in Patients with End-Stage Renal Disease: A Nationwide Cohort Study in Taiwan during 1997–2008

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Background. Urothelial cancer (UC) is the leading cancer of patients with end-stage renal disease (ESRD) in Taiwan. The aims of this study were to explore the time trends of UC incidences and propose possible etiologic factors. *Methods*. Abstracting from the National Health Insurance Research Database (NHIRD), there were 90,477 newly diagnosed cases of ESRD between 1997 and 2008 covering the patients aged 40–85. Among them, 2,708 had developed UC after diagnosis of ESRD. The CIR_{40-85} (cumulative incidence rate) of upper tract UC (UTUC) and lower tract UC (LTUC) were calculated for ESRD patients and general population, as well as SIR_{40-85} (standardized incidence ratio) for comparison. *Results*. Female ESRD patients were found to have 9–18 times of elevated risks of UC, while those of males were increased up to 4–14 times. The time trends of CIR_{40-84} and SIR_{40-84} of UTUC in females appear to decline after calendar year 2000. These trends may be related to AA associated herbal products after 1998. *Conclusions*. Patients with ESRD are at increased risks for both LTUC and UTUC in Taiwan. We hypothesize that the time trends associate with the consumption of aristolochic acid in Chinese herbal products (female predominant).

1. Introduction

Various studies carried out in different regions around the world have found that the incidence of malignancy is generally higher in patients suffering from end-stage renal disease (ESRD) [1–4]. Compared with other countries, Taiwan has a remarkably high incidence and prevalence of patients with ESRD [5, 6]. In Taiwan, bladder, liver, and kidney cancers are leading malignancies in patients with chronic dialysis in a 12-year cross-sectional study between 1997 and 2008. Standardized incidence ratios (SIR) of upper tract urinary cancer (UTUC) decline yearly after first dialysis. However, lower urinary tract urothelial cancer (LTUC) persists high in SIR years after first dialysis [7]. However, this study did not explore the possible etiology for the increased risks nor did it stratify for UTUC and renal cell carcinoma.

Among the possible causes for the urothelial cancer, cigarette smoking is among the top candidates associated with increased bladder [8]. Since aristolochic acid (AA) has been documented to be both nephrotoxic and carcinogenic [9, 10], AA-containing CHPs (Chinese herbal products) were banned in November 2003 in Taiwan. However, the effect

of banning licensed AA-containing CHPs has never been systematically evaluated.

This study is conducted to determine the cumulative incidence rates (CIR) and SIR in different calendar years for LTUC and UTUC to elucidate the time trends and, furthermore, to preliminarily explore their possible etiologies including tobacco smoking and prescription frequency of AA-containing CHPs. The registry of catastrophic illnesses in Taiwan National Health Insurance and the reimbursement database provides us an excellent opportunity to explore the hypothesis.

2. Methods

This study was approved by the Ethics Review Board of the National Taiwan University Hospital before commencement. In addition, the study complies with personal data protection regulations (Data Protection Act).

2.1. Study Population. In this study, the selected subjects registered under catastrophic illnesses from the NHIRD cover the period of 1997–2008. Data on patient demographics (gender and date of birth), diagnoses and treatment (the date treatment began and the date of death or transplantation), and follow-up duration between January 1997 and December 2008 were retrieved. We allowed two months (up to the end of February of 1997) to exclude prevalent cases of ESRD from the above retrieved NHIRD data. Enrolled incident cases included those coded with the following international classification of disease 9th revision (ICD-9) codes: bladder cancer (ICD-9 code 188), renal pelvis and ureter (ICD-9 codes 189.1 and 189.2, resp.), and ESRD (ICD code 585). NHI database enrollees, namely, general population of Taiwan, were taken as the reference group after excluding all the patients with ESRD. Since the age band of urothelial caner (UC) developed cases falls between 40 and 84, we enrolled patients within this category to calculate SIR_{40-84} and CIR_{40-84}. The entire procedure for the inclusion of the subjects (aged between 40 and 84) is illustrated in Figure 1.

2.2. Statistical Analysis. The study interval is defined from January 1997 to December 31, 2008 since registration system is more comprehensive after early 1997. Follow-up time is calculated as person-year at risk beginning with the date of registered ESRD and ending at situation of death, transplantation, or diagnosis of urinary tract cancer depending on which one comes first. Since all ESRD patients are waived from any copayment in our National Health Insurance (NHI), none (or, extremely rare) would abandon such an important benefit except the deceased. Incidence rates were calculated per 100,000 person-years at risk of UC stratified by age and sex. The age stratified incidence rates for UC in the reference population were used to calculate the number of expected cases under the assumption that the reference population would share the same cancer experience as patients with ESRD for each age stratum. The total number of observed UC cases summed up across all age strata divided by that of expected cases was then defined as the standardized incidence ratio (SIR). We then calculated the 95% confidence intervals (95% CIs) under the assumption of Poisson distribution. The cumulative incidence rate (CIR) formula was calculated as follows: $CIR = 1 - \exp[-\Sigma i(IR_i)(\Delta t_i)]$ where IR_i represents the age-specific incidence rate and Δt_i indicates the range of each age stratum. We calculated the CIR_{40-84}, which could be interpreted as the cumulative risk of developing UC for an average ESRD patient, if he or she had lived from the age of 40 to 84. All of the above analyses were carried out using the SAS software package, Version 9.1 (SAS Institute Inc., Cary, NC).

We examine the trend of CIR and SIR in every 6-year period from 1998 to 2008 by fitting a simple linear regression model: $\log(Y_t) = \alpha + \beta \times t + \varepsilon_t$, where $t = 1, 2, \ldots, 6$ and Y_t is the tth CIR or SIR of the examined period [11]. The coefficient β determines strength of the trend and the direction of increase or decrease in that period.

3. Results

A total of 103,527 newly diagnosed ESRD patients were identified between March 1997 and December 2008. After excluding those who accepted kidney transplantation and those with incomplete patient information, we obtained a total of 90,447 ESRD cases and 419,884 person-years at risk. Within the age group of 40–84, there were approximately 7 million people registered in the NHI system during the period of 1997–2008 (as summarized in Figure 1). After 12 years of follow-up, there were 41,115 UC developed from the general population with an approximate 100 million person-years at risk accumulated. A total of 2,214 cases were excluded from the ESRD cohort because the diagnosis of UC was ahead of that of ESRD, leaving 2,708 new UC cases developed after the diagnosis of ESRD. Development of UTUC and LTUC is persistent after first dialysis. And age is a specific factor in both genders and UTUC and LTUC. About 93.4% of upper tract and 97.4% of lower tract patients were provided with histopathologic reports, which are taken from either biopsy or surgical pathology specimen.

According to the demographic characteristics of ESRD patients, which are summarized in Table 1, there are 1,481 cases of LTUC and 1,227 cases of UTUC enrolled. The SIR_{40-84} of urothelial cancer is summarized in Table 2, which shows an increased trend for all types of UC after 1997 and becomes stabilized after 2004 for both male and female. The female SIRs of different calendar years are generally higher than those of males, of which the SIRs of UTUC are the highest among all UC.

The age-sex specific incidence rates and CIR_{40-84} for all types of UC are summarized in Table 3, which shows that both upper and lower UC occurred in both genders and such trends begin with young age groups. It also shows that the CIR_{40-84} of females are generally higher than those of males in all types of UC for the same time periods with an exception of LTUC in 1997–99. Similar to the SIR_{40-84}, CIR_{40-84} of both genders seems to show an increased trend during the 12 years of follow-up and slightly decreased in the last 3 years, especially for UTUC. As the CIR_{40-84} of

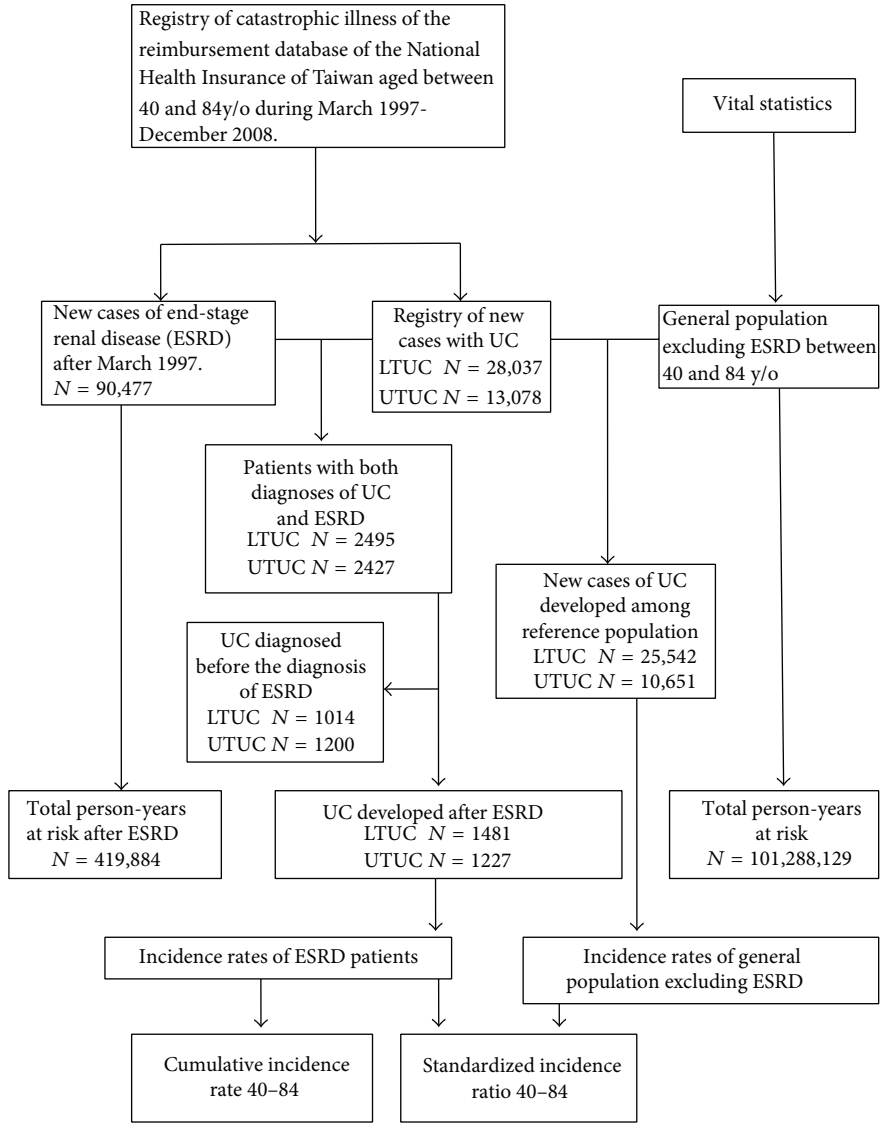

Figure 1: Flowchart of recruitment of subjects in this study. LTUC, lower urinary tract urothelial cancer; UTUC, upper tract urinary tract urothelial cancer; ESRD, end-stage renal disease; CIR, cumulative incidence rate; SIR, standardized incidence ratio.

the general population (excluding ESRD) during the same period have been very stable around 0.021–0.027 in males and 0.007–0.009 in females for LTUC and those of UTUC were between 0.006 and 0.010 in both genders, the epidemic of UC in patients with ESRD (between 0.1 and 0.4 in Table 3) seemed not yet over. Moreover, following inference could be drawn from the SIR_{40-84} in Figure 3: (1) for female UTUC, the increasing trends were found in the first two periods but were not significant. Significant decreasing trend was detected at the last period of 2003–08; (2) female LTUC had significant increasing trends in the first four time periods, but the strength is also weakening. At the last period of 2003–08, the trend disappeared; (3) for male LTUC, significant decreasing trends were found in the periods of 2000–05 and 2001–06; (4) patterns of time trend for male UTUC are similar to those of male LTUC and female UTUC, but no significant trend was found.

4. Discussion

Quite different from studies of western countries, we have found that females seem more vulnerable to develop upper or lower location of urothelial cancer after diagnosis of end-stage renal disease, especially in UTUC (Table 1). In fact, female ESRD patients were found to have 9–16 and 11–18 times of elevated risks of LTUC and UTUC, while those of males were increased up to 4–8 and 7–14 times, as summarized in Table 2. The trend of female majority is further corroborated by the estimation of CIR_{40-84} (Table 3). The CIR_{40-84} estimates the probability that in Taiwan an average person with ESRD would develop the event (namely, UTUC or LTUC) if he/she lived up to the age of 85, while the SIR_{40-84} is the total number of observed UC cases summed up across all age strata divided by the total number of expected cases. Moreover, we have found a consistently increasing

Table 1: Characteristics of patients with end-stage renal disease between 40 and 84 years old under maintenance dialysis in the registry of catastrophic illnesses between March 1997 and December 2008.

	Male	Female
Number of the subjects with ESRD	43,937	46,496
Total person-years at risk	197,753	222,131
Age at start of dialysis (total person-years)		
<40 years	35,307	26,653
40–49 years	35,821	41,327
50–59 years	50,873	50,373
60–69 years	56,140	66,570
70–84 years	54,919	63,861
Duration of follow-up years: number (%)		
<1 year	10,575 (24.1)	10,313 (22.1)
≥1 and <5 years	19,426 (44.2)	19,701 (42.4)
≥5 and <10 years	11,543 (26.3)	13,415 (28.9)
≥10 years	2393 (5.4)	3067 (6.6)
Urothelial cancer cases		
Lower tract urothelial cancer: number (%)	634 (42.8)	847 (57.2)
Upper tract urothelial cancer: number (%)	417 (34.0)	810 (66.0)

Table 2: Age-standardized incidence rates (ages between 40 and 84) for patients under maintenance dialysis stratified by sex and calendar year.

	LTUC		UTUC	
	O/E	SIR_{40-84} (95 %CI)	O/E	SIR_{40-84} (95 %CI)
Male				
1998	16/2.7	5.9 (3.6–9.7)	7/0.5	13.0 (6.2–27.3)
1999	27/6.0	4.5 (3.0–6.6)	18/1.3	13.9 (8.8–22.1)
2000	48/5.7	8.4 (6.4–11.2)	19/1.6	12.2 (7.8–19.1)
2001	43/6.8	6.3 (4.7–8.5)	28/2.2	12.9 (8.9–18.7)
2002	68/8.4	8.1 (6.4–10.3)	34/2.9	11.9 (8.5–16.6)
2003	65/10.5	6.2 (4.8–7.9)	49/3.5	13.9 (10.5–18.4)
2004	63/12.5	5.1 (4.0–6.5)	53/4.1	12.8 (9.8–16.8)
2005	61/13.7	4.5 (3.5–5.8)	35/4.9	7.2 (5.2–10.0)
2006	74/14.0	5.3 (4.2–6.6)	52/5.5	9.4 (7.2–12.3)
2007	79/14.7	5.4 (4.3–6.7)	58/6.1	9.5 (7.3–12.3)
2008	83/16.6	5.0 (4.0–6.2)	65/5.8	11.2 (8.8–14.2)
Female				
1998	12/1.3	9.0 (5.1–15.8)	11/0.8	14.3 (7.9–25.8)
1999	29/3.1	9.5 (6.6–13.7)	20/1.8	10.9 (7.1–16.9)
2000	36/3.3	10.8 (7.8–14.9)	47/2.7	17.7 (13.3–23.6)
2001	49/3.9	12.6 (9.5–16.6)	56/3.1	18.4 (14.1–23.9)
2002	76/5.1	14.8 (11.8–18.5)	57/4.1	13.9 (10.7–18.0)
2003	83/5.5	15.1 (12.1–18.7)	83/5.0	16.5 (13.3–20.4)
2004	89/6.7	13.3 (10.8–16.4)	94/5.7	16.6 (13.5–20.3)
2005	118/7.3	16.1 (13.5–19.3)	93/6.8	13.7 (11.2–16.8)
2006	136/8.3	16.4 (13.9–19.5)	113/7.8	14.5 (12.1–17.4)
2007	121/7.4	16.3 (13.7–19.5)	107/7.9	13.5 (11.2–16.3)
2008	100/8.3	12.0 (9.9–14.6)	116/8.5	13.6 (11.4–16.4)

LTUC: lower urinary tract urothelial cancer; UTUC: upper urinary tract urothelial cancer; and SIR: standard incidence rate.

Table 3: Sex- and age-specific rates (per 100,000 person-years) and cumulative incidence rates up to 84 years old ($CIR_{40–84}$) of urothelial cancer calculated for every 3-year interval between 1997 and 2008.

	Age category	Male		Female	
		LTUC	UTUC	LTUC	UTUC
1997–99	40–49	372.0	148.8	361.9	180.9
	50–59	491.3	109.2	600.0	250.0
	60–69	774.9	122.3	771.1	175.3
	70–84	952.8	129.9	504.5	232.8
	$CIR_{40–84}$	0.3	0.1	0.2	0.1
2000–02	40–49	400.1	100.0	700.1	140.0
	50–59	1013.8	362.1	999.5	309.4
	60–69	1027.3	296.3	1059.3	554.1
	70–84	1103.5	120.4	948.5	210.8
	$CIR_{40–84}$	0.3	0.1	0.3	0.1
2003–05	40–49	327.9	131.2	526.6	292.6
	50–59	832.1	282.3	1125.7	616.8
	60–69	701.8	259.9	1158.0	496.3
	70–84	1035.8	172.6	1140.5	245.2
	$CIR_{40–84}$	0.3	0.1	0.4	0.2
2006–08	40–49	274.7	85.9	511.0	148.4
	50–59	639.4	108.4	1366.9	431.7
	60–69	810.8	170.7	1018.6	392.5
	70–84	840.4	166.1	992.6	215.1
	$CIR_{40–84}$	0.3	0.1	0.4	0.1

LTUC: lower urinary tract urothelial cancer; UTUC: upper urinary tract urothelial cancer; and CIR: cumulative incidence rate.

trend of $SIR_{40–84}$ (Table 2) and $CIR_{40–84}$ (Table 3) of UTUC in ESRD before 2003 and it stabilized or slightly dropped after 2003–05 based on a nationwide data for both males and females. The $CIR_{40–84}$ of LTUC among females were initially lower than those of males before 2000, and they became higher than those of males after 2003 (Table 3). And the $SIR_{40–84}$ of LTUC in males seems to decrease after 2000 but that of females appears elevated throughout the observation period. The $CIR_{40–84}$ of UTUC among females increased about 1.5 times that of males before 2000, which further climbed up to about 2 times.

It is interesting to know when the UC develops after initiation of dialysis. SIR of UTUC increased up to about more than 20–30 times in the first year after dialysis and then decreased slowly during the 12 years of observation [7]. The decreasing trend along calendar period could be expressed in the regression coefficient of Figure 3, especially among the females. However, while the male LTUC seems to show a decreasing trend expressed by the consistent negative signs of linear regression coefficients, those of females remain elevated in SIR until the period of 2003–8 to show a negative sign. We have also summarized the cumulative incidence rates of developing UC along time after dialysis in Figure 4.

Given such increased risks, we must consider the potential etiological factors. In general, risk factors that are reported to be associated with urothelial cancer include analgesics (phenacetin), herbal usage (aristolochic acid), heavy metals (arsenic), and tobacco smoking reported in international agency in research on cancer (IARC) [12]. Long-term use of phenacetin or NSAID is a potential risk factor for LTUC [13, 14]. Since IARC classified phenacetin as category 1 carcinogen for urothelial cancer in 1987, its compounds have been banned from Taiwan market since July of 1985 [15]. Moreover, patients with ESRD under dialysis are instructed to avoid taking NSAID (nonsteroidal anti-inflammatory drugs), which were illustrated by our data showing 99% of ESRD patients were prescribed NSAID for less than 500 pills within the two years before and after dialysis in both cancer and reference groups. Thus, phenacetin and/or NSAIDs did not seem to explain the above increased trend for both UTUC and LTUC. Since all patients with ESRD are recommended to avoid any food or medication suspected to contain metals that might accumulate in our body and only one out of 2708 cases of urothelial cancer after ESRD resided in the area of endemic arsenic poisoning (blackfoot disease), the etiological connection with arsenic seems unlikely. A preliminary study determining both arsenic and cadmium content in renal parenchyma tissue of three UTC cancers among patients with ESRD did not find any increase of these two metals in comparison with 26 samples without such cancers.

In several studies smoking is strongly related to LTUC and possibly UTUC in occurrence, recurrence, and progression [8, 12, 16]. Prevalence rate of tobacco consumption appears decreased from 60% down to below 40% in males, while that in females has remained about 5% or lower during the last 20 years according to data from the Department of

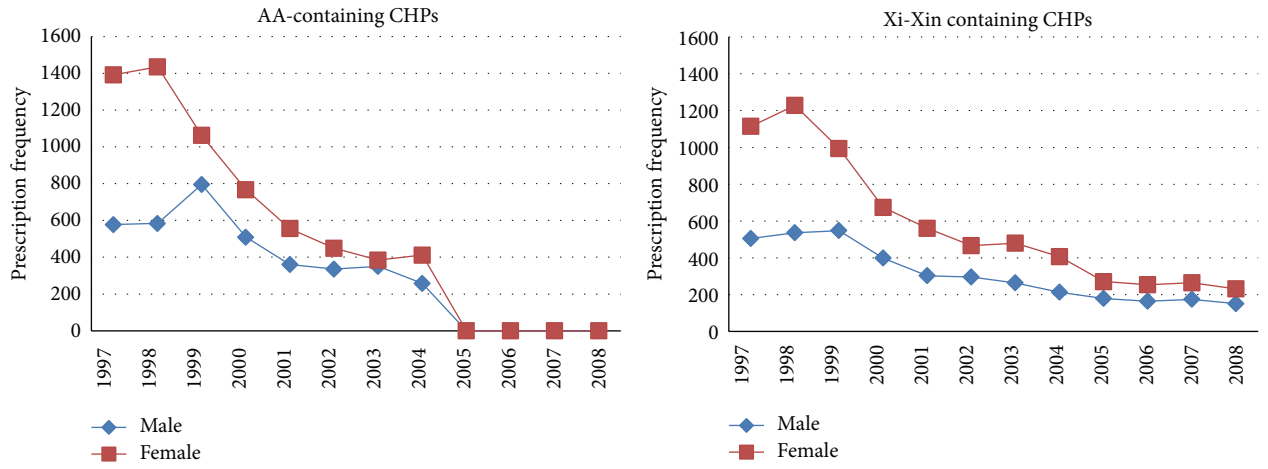

Figure 2: Prescription frequencies of aristolochic acid (AA) related and Xi-Xin Chinese herbal products (CHPs) in 90,477 patients with ESRD (end-stage renal disease), stratified by sex.

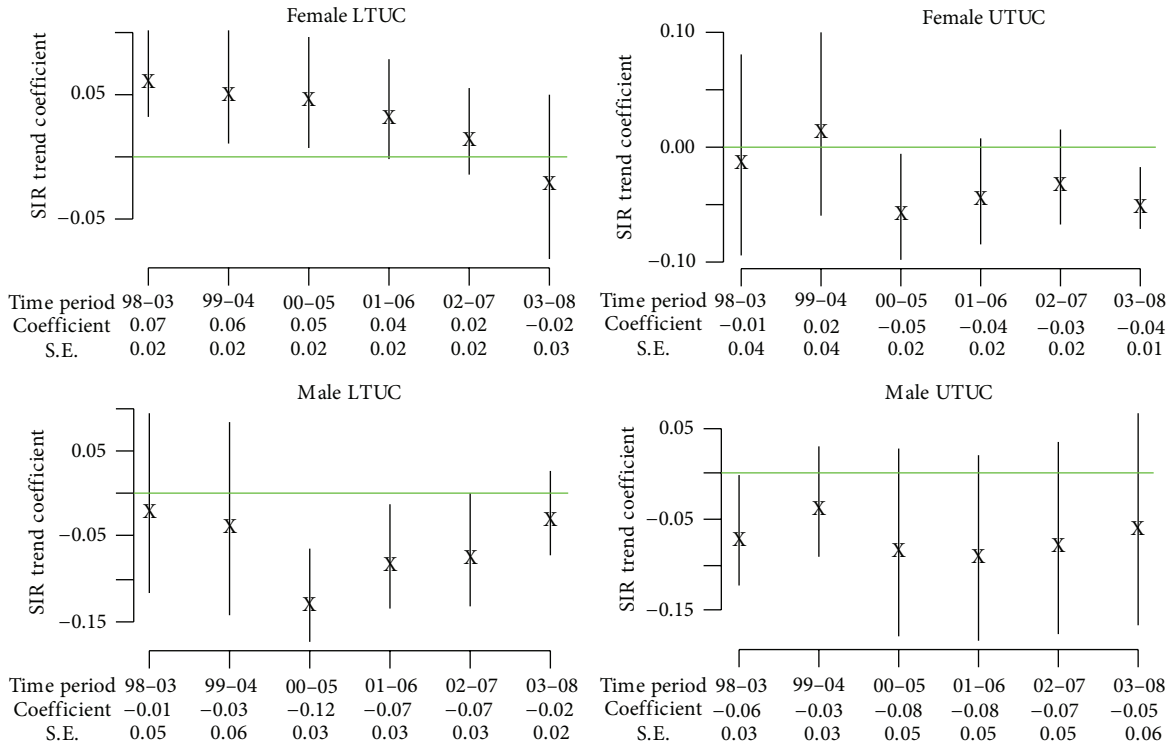

Figure 3: Estimated coefficients with 95% confidence intervals and standard errors (S.E.) for SIRs (standardized incidence ratios) linear trend during specified periods.

Health, Executive Yuan in a survey of general population [17]. Although the whole picture appears compatible with the trend of decreased LTUC in males (Figure 3), it cannot explain the increased SIR_{40-84} in females after age standardization. Moreover, almost all patients with ESRD were usually advised to quit or stay away from smoking by physicians. Since the time trends of UTUC for both genders in Table 2 were unrelated to the trends of cigarette consumption in Taiwan, we thus concluded that the increased UTUC in ESRD patients is probably not associated with smoking.

Aristolochic acid has been documented as nephrotoxic and carcinogenic and its related products or remedies are banned in many countries, including USA, UK, and Canada [18]. The above trends appear to correspond with the decreased consumption of several AA-containing products beginning in 1999 (Figure 2) and the ban on AA-containing CHPs in Taiwan on November 3 of 2003 (Figure 2) [8, 18]. The time trends of CIR_{40-84} of LTUC and UTUC in females appear to decline after calendar year 2000. This trend seems compatible with reduced consumption after 1998 and the

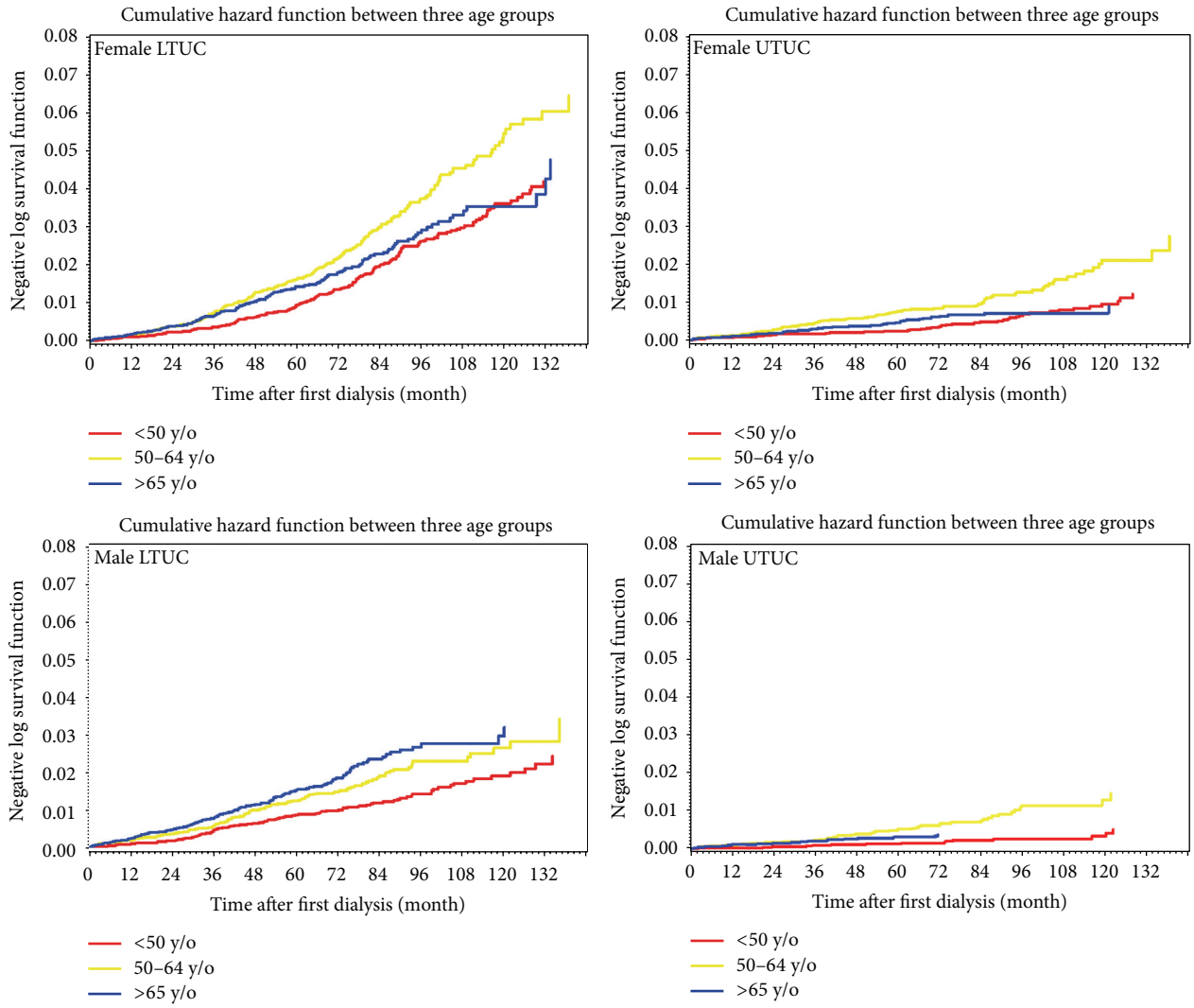

FIGURE 4: Accumulative hazard function to demonstrate the incidence rates of developing UC (urothelial carcinoma) along time after first dialysis stratified by sex, age (<50, 50–64. >65 years old), and upper tract (UTUC) and lower tract (LTUC).

ban of aristolochic acid (AA) associated herbal products in 2003. After the year of 2000, the SIR_{40-84} of UTUC appears to drop in both genders and the SIR_{40-84} of LTUC declines in male significantly. These trends may be related to decreased consumption of cigarettes in males after 1990 and AA associated herbal products after 1998. Similar to what was reported in the AA related cancer in Belgium, the peak of LTUC usually appears as early as 3-4 years later than that of UTUC [19, 20]. Unfortunately, CHP that contains Xi-Xin is still available in market of Taiwan [21, 22], which might contain minute amount of AA after 25 times of concentration of the products and might still produce carcinogenic effect for patients with ESRD whose kidney is unable to excrete toxics [23]. As Xi-Xin is totally banned in America and Europe, AA related DNA adduct has been documented in cases of UTUC in Taiwan [24, 25]. We are currently conducting another study to estimate the doses of AA from prescribed Chinese herbal products and the association with occurrence of UC in dialysis patients, which may provide a more direct evidence for Taiwan to consider a similar action.

Limitations of this study include possible ecological fallacy and the lack of personal genetic and life style information from the NHRI database, and details of tumor staging are not available in the NHIRD. We are also unable to quantify the exact consumption of analgesics and CHPs, as many of them are sold without prescription. Since the results of this study provide a preliminary inspection of the trend, they must be interpreted cautiously, and more studies are needed in the future to corroborate the proposed hypothesis.

5. Conclusions

Patients with ESRD requiring dialysis have increased after beginning of NHI system in Taiwan since 1995. Female patients diagnosed of ESRD were found to have 9–16 and 11–18 times of elevated risks of LTUC and UTUC, while those

of males were increased up to 4–8 and 7–14 times. The time trends of SIR_{40-84} and CIR_{40-84} of UTUC seem to synchronize with the increased prescription and consumption of AA associated herbs until 2000 [8, 26]. Further corroboration of this hypothesis may uncover the actual etiology and prevent more victims from urothelial cancer in Taiwan.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

NGS Nominated *CELA1*, *HSPG2*, and *KCNK5* as Candidate Genes for Predisposition to Balkan Endemic Nephropathy

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Balkan endemic nephropathy (BEN) is a familial chronic tubulointerstitial disease with insidious onset and slow progression leading to terminal renal failure. The results of molecular biological investigations propose that BEN is a multifactorial disease with genetic predisposition to environmental risk agents. Exome sequencing of 22 000 genes with Illumina Nextera Exome Enrichment Kit was performed on 22 DNA samples (11 Bulgarian patients and 11 Serbian patients). Software analysis was performed via NextGene, Provean, and PolyPhen. The frequency of all annotated genetic variants with deleterious/damaging effect was compared with those of European populations. Then we focused on nonannotated variants (with no data available about them and not found in healthy Bulgarian controls). There is no statistically significant difference between annotated variants in BEN patients and European populations. From nonannotated variants with more than 40% frequency in both patients' groups, we nominated 3 genes with possible deleterious/damaging variants—*CELA1, HSPG2*, and *KCNK5*. Mutant genes (*CELA1, HSPG2*, and *KCNK5*) in BEN patients encode proteins involved in basement membrane/extracellular matrix and vascular tone, tightly connected to process of angiogenesis. We suggest that an abnormal process of angiogenesis plays a key role in the molecular pathogenesis of BEN.

1. Introduction

Balkan endemic nephropathy (BEN) is a familial chronic tubulointerstitial disease with insidious onset and slow progression to terminal renal failure. It was first described in Serbia and in Bulgaria. The disease affects people living in the alluvial plains along the tributaries of the Danube River in Serbia, Bosnia, Croatia, Bulgaria, and Rumania [1]. Investigations were directed to the epidemiology, etiology, morphology, and treatment of BEN. Results of these studies were reviewed elsewhere [2, 3]. BEN has an onset of between the 40s and 60s, with a long preclinical period. The disease affects both genders with slight female predominance and often leads to terminal kidney failure. About 30–40% of the affected individuals develop uroepithelial tumours of the upper urinary tract [4]. These are mostly papillar carcinomas and are the most common causes of death in BEN patients.

Different chemical elements, organic and nonorganic compounds, viruses, and microorganisms are implicated in

BEN development. Heavy metals such as Mg, Mo, Cd, Pb As, and Se can be important for the development of the disease, but there is no clear evidence of their direct toxic effect on the development of the disease. Pathomorphologically BEN has similarities with Chinese herbal nephropathy, which is probably caused by the toxic effect of aristolochic acid, but there is no evidence supporting this theory for BEN. Other possible agents involved in the etiology of BEN are ochratoxin A and some viruses such as picornavirus, polyomavirus, herpes simplex 1 and 2, adenovirus, hepatitis B, cytomegalovirus, and Epstein-Barr virus [5, 6]. For now there is no unchallenged evidence supporting a viral etiopathogenesis of BEN.

BEN is a multifactorial disease with genetic predisposition to environmental risk agents. Familial manifestation of BEN implies a polygenic genetic predisposition [7, 8].

Previous studies have suggested genes located in chromosome band 3q25-3q26, genes for xenobiotic metabolizing enzymes, tumour-suppressor genes, and protooncogenes. The incidence of rapid debrisoquine metabolizers is higher in BEN patients than in healthy controls [9]. *CYP2D6* polymorphic variants predisposing to toxic effect of various chemical agents are suspected in BEN pathogenesis. *LCAT*-deficient individuals have evidence of renaltubular injury and this defect can be involved in BEN [10]. Cytogenetic research on lymphocyte cultures from BEN patients showed that *in vitro* higher folic acid inducedchromosomal fragility and more frequent spontaneous chromosomal aberrations. Some of the unstable regions contain oncogenes—1p36-*C-SRC*, 3p25-*RAF1,* 3q27-*FIM3*, 6q23-*MYB*, 1p13-*NRAS,* and 6p11-*KRAS1P* [11, 12].

Other studies show that environmental factors are very important and can influence genome function without changing the DNA sequence itself. The concept of epigenetics was suggested. The major epigenetic modifications include DNA methylation, histone modifications, and miRNA interference [13]. Epigenetic changes over time display familial clustering [14] and could be implicated in transmitting a "predisposition" over generations. Epigenetic modifications being heritable and adaptable at the same time may prove to make a significant contribution to BEN development and may be the link between the effect of environmental factors and genetic composition in BEN progression. In a previous study we have investigated the methylation status across the whole genome in different patient groups, based on gender and endemic region, in comparison to healthy controls from nonendemic regions. Differentially methylated regions (DMRs) were determined in BEN patient and controls and the commonly presented DMRs were determined to be the most promising methylation alterations in BEN. *SEC61G, IL17RA,* and *HDAC11* proved to be differently methylated throughout all patient-control pairs [15].

In the present study we aimed to perform exome sequencing of 22 000 genes with the Illumina Nextera Exome Enrichment Kit using NGS technology in order to find specific mutations for BEN.

2. Materials and Methods

Twenty-two BEN patients were selected for NGS exome analysis. Informed consent was received from all participants enrolled in the study. We obtained peripheral blood samples from 2 series of patients—11 Bulgarian and 11 Serbian. Clinical assessment was performed according to unified criteria and was applied to all sample cohorts. Genealogical analysis was performed to exclude relatives among all study subjects.

Bulgarian samples were collected by preliminary clinical screening in Vratza endemic regions in Bulgaria in 2003 [16]. All subjects were born of Bulgarian ancestry, born and living in the endemic region. DNA was extracted by standard phenol-chloroform extraction procedure and stored at −80°C. All samples were checked for DNA consistency by 1% gel electrophoresis.

Serbian samples were collected from Serbian endemic regions. DNA was extracted by DNA extraction kit and stored at −80°C. All samples were checked for DNA consistency by 1% gel electrophoresis.

The study was approved by the Serbian Ethics Committee of the University of Nis, School of Medicine, Nis, Serbia, and the Bulgarian Commission of Medical Ethics at the National Center of Hygiene, Medical ecology and Nutrition, Sofia, Bulgaria.

2.1. Library Preparation and Enrichment. The workflow in the Nextera Enrichment Sample Preparation Guide (Revision B) by Illumina was followed to prepare the libraries for whole-exome sequencing. A Nextera Exome Enrichment Kit was used. The 22 libraries were distributed in 10 enrichment reactions ("pools")—in 6 of them DNA from 3 libraries was mixed together, while the other 4 contained DNA from only one. The latter were prepared in order to obtain higher mean coverage for these samples. A different DNA quantity (500 ÷ 1000 ng) was added to form each pool depending on the quantity of the least concentrated sample in the reaction.

2.2. Library Quantification. After completing the enrichment procedure, DNA concentration of the 10 sequencing-ready pools was measured using a KAPA Library Quantification Kit. The reactions were run in triplicate on an Illumina qPCR Eco system. The Eco Study software was used to calculate the concentrations of the dilutions and then of the stocks.

2.3. Denaturation and Dilution. Pools 5–10 were denatured and diluted according to the Illumina guidelines in Preparing DNA Libraries for Sequencing on the MiSeq (Illumina, San Diego, USA). Due to lower concentrations Pools 1–4 were prepared for sequencing following the corresponding chapter in the TruSeq Custom Amplicon Library Preparation Guide, which uses heat denaturation instead of denaturation with 0.1 N NaOH.

2.4. Sequencing. The pools were sequenced on an Illumina MiSeq System using the MiSeq Reagent Kit v2 and a 500-cycle 14-tile flow cell. Only Pool 9 was sequenced using a 300-cycle MiSeq Reagent Micro Kit v2 and a micro 4-tile flow

Table 1: Nonannotated variants with frequency of more than 40% in BEN patients, discovered in our study.

Number of patients	Gene	Chr	Position	Exon	Mutation	Aminoacid change	Protein
11	*HSPG2*	1	22186113	43	c.5239A>C	p.Thr1747Pro	Heparan sulfate proteoglycan 2
10	*CELA1*	12	51740414	2	c.9_10delC	p.Leu4Phefs	Chymotrypsin-like elastase family, member 1
9	*KCNK5*	6	39162513	4	c.1397A>C	p.Thr108Pro	Potassium channel, subfamily K, member 5

cell. We experimented loading the flow cell at different pool concentrations (10 ÷ 30 pM) to establish what the optimal range is for the most efficient cluster density and hence yield.

2.5. Analysis. Analysis of the sequencing data was performed using the Softgenetics NextGene Software (version 2.3.3). The variants found were further analyzed by mutation prediction software Provean and PolyPhen-2 to filter the mutations, which are predicted to be deleterious/damaging. We searched for repeated mutations that are unique or that have significantly higher frequencies in BEN patients as compared to individuals from European populations. We focused on nonannotated variants, predicted as possibly deleterious/damaging by software analysis. We selected mutations based on the following criteria: (i) no data about variants; (ii) no incidence in healthy Bulgarian controls and in European populations; (iii) similar incidence in both Bulgarian and Serbian patients' groups; (iv) mutation frequency of more than 40% in BEN patients.

3. Results

Twenty-two Bulgarian and Serbian BEN patients were analyzed by NGS exome sequencing for 22 000 genes. Using the Softgenetics NextGene Software (version 2.3.3) the sequencing data were analyzed. Mutation prediction was performed by software Provean and PolyPhen-2.

We discovered in total 3666 missense variants with possible deleterious/damaging effect in our patients' groups. Among them, 1849 (50%) were not annotated.

In total, 980 nonsense and frameshift variants were detected in our study. Among them, 541 (55%) variants have not been annotated in human genome data base of genetic variations or no information about their frequency was available.

Among the annotated variants with possible deleterious/damaging effect we did not find statistically significant difference in the frequency between BEN patients and European populations. From nonannotated variants, we selected the variants with frequency of more than 40% in BEN patients—*HSPG2*, *CELA1,* and *KCNK5* (Table 1). These mutations, alone or in combination, occur in 77% of BEN patients.

The probable contribution of each of the nominated genes to the pathogenesis of BEN (according to their mutation frequency) is represented in Figure 1. The frequencies of each of the nominated mutant genes for Bulgarian and Serbian BEN patients are given in Figure 2. All selected variants occur with similar frequency in the Bulgarian and

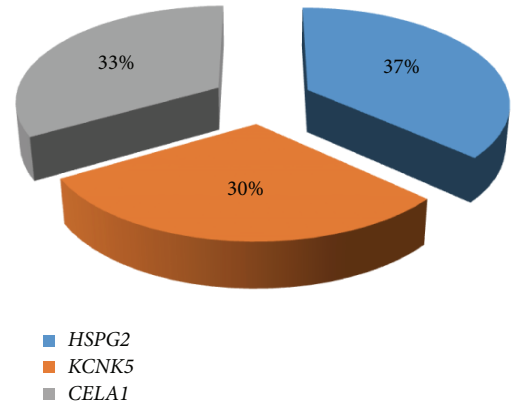

Figure 1: Nominated candidate genes, associated with BEN.

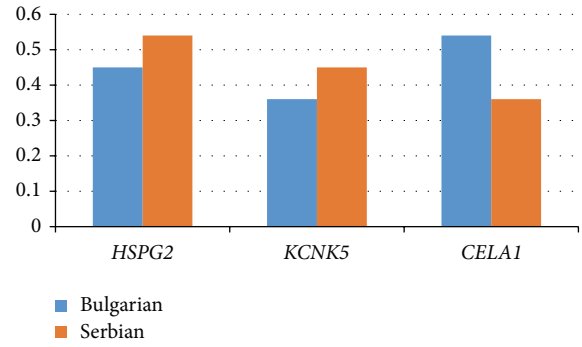

Figure 2: Frequency of nominated mutant genes in Bulgarian and Serbian BEN patients.

Serbian groups of BEN patients. The nominated variants are classified according to their function—genes involved in the constitution of basement membrane/extracellular matrix (ECM) (*HSPG2* and *CELA1*) and gene for renal potassium transport and membrane potential (*KCNK5*).

Figure 3 represents the distribution of the three mutant genes in all BEN patients. *KCNK5* variant c.1397A>C occurs only in combination with one or two of the other variants (*HSPG2* c.5239A>C and *CELA1* c.9_10delC), while *CELA1* variant is present as a single aberration in 6 out of 10 positive cases. In 5 of BEN patients none of these variants were found. This is probably due to the presence of other rare mutations, which did not pass the threshold criteria in our study (presented in more than 40% of the patients).

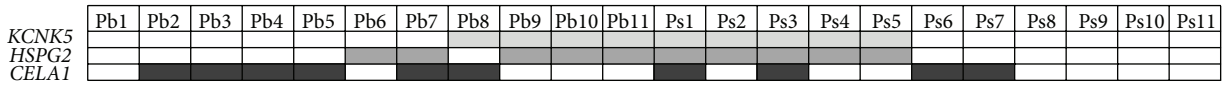

Figure 3: Combination of the three mutant genes found in all BEN patients. Pb: Bulgarian patients; Ps: Serbian patients.

4. Discussion

Balkan endemic nephropathy has been traditionally described as an end-stage kidney disease, characterized by bilaterally and symmetrically contracted kidneys of a very small size and reduced weight, coinciding with multiple upper urinary tract tumors in 8–48% of cases [17]. Basic histomorphological changes include tubular atrophy and interstitial fibrosis with sclerosed glomeruli usually of collapsing obsolescence and sclerotic changes in blood vessels. The hypothesis for BEN multifactorial pathogenesis [8] proposes that inherited genetic defects predispose the kidney to damage after exposure to different agents—toxic, immune, or infective. The familial clustering of the disease has prompted genetic investigations [12, 18, 19].

This is the first analysis by NGS sequencing in BEN patients aimed at discovering mutations associated with the disease. Despite intensive molecular studies in BEN, the etiopathogenesis of the disease is still not elucidated and there is no biomarker for disease predisposition. Most of the molecular studies so far have focused on single genes/polymorphisms and very limited information has been provided. Because gene-by-gene analysis by Sanger sequencing is too laborious and expensive, genetic testing has been the exception until recently. Now, next-generation sequencing (NGS) allows for simultaneous and efficient analysis of all known genes for a given trait. Here we applied exome sequencing (comprised of 22 000 genes) in 22 BEN patients in order to detect the most prominent genetic variants with highly probable pathological effect. Recently Poon et al. have applied whole-genome and exome sequencing for analysis of Aristolochic acid- (AA-) associated upper urinary tract urothelial cell carcinoma (UTUC) [20]. AA is a carcinogen that can cause nephrotoxicity as well. Authors observed a high frequency of somatic mutations in chromatin modifiers, particularly KDM6A, in AA-UTUC, demonstrated the sufficiency of AA to induce renal dysplasia in mice, and reproduced the AA mutational signature in experimentally treated human renal tubular cells. Our study was looking for germ-line mutations predisposing to another specific nephropathy-Balkan endemic nephropathy.

Three genes (*HSPG2, CELA1,* and *KCNK5*) were nominated as related to the pathogenesis of BEN based on their mutation frequency, their similar incidence in both Bulgarian and Serbian patients' groups, lack of information about the established variants in European population, and nonincidence in healthy Bulgarians. Analysis of their function sheds light on the possible pathophysiology in BEN, which we discuss here.

The first two genes *HSPG2* and *CELA1* are evidently involved in the process of angiogenesis. The gene *KCNK5* encodes a protein for potassium channel, which could also be involved in vascular disease and complications.

The number of patients requiring renal replacement therapy due to end-stage renal disease (ESRD) is increasing worldwide [21]. The prevalence of chronic kidney disease (CKD) and the importance of CKD as a risk factor in development of ESRD have been confirmed. In recent years, the involvement of angiogenesis-related factors in the progression of CKD has been studied, and the potential therapeutic effects on CKD of modulating these factors have been identified [22]. A number of angiogenic growth factors are involved in the development of the kidney and in the maintenance of glomerular structures and the glomerular filtration barrier function in adults.

Our study revealed significant candidate gene—*HSPG2*, which encodes perlecan protein, a major component of basement membranes, where it is involved in the stabilization of other molecules important for glomerular permeability to macromolecules and for cell adhesion [23, 24]. It binds to and crosslinks many extracellular matrix components and cell-surface molecules [25–29]. It has been shown that this protein interacts with laminin, prolargin, collagen type IV, tenascin-C, FGFBP1, FBLN2, FGF7, transthyretin, and so forth, and plays essential roles in multiple biological activities. Cukuranovic et al. have intensively studied the pathological changes in the kidneys of BEN patients and presented evidence that renal vascular changes occur early in Balkan nephropathy [30]. They detected by IHC a marked overexpression of laminin in renal interstitial capillaries. The pattern of laminin staining in glomeruli corresponded to focal and segmental glomerular sclerosis present in the advanced stages of Balkan nephropathy. Later stages were characterized by an intensive expression of laminin in atrophic tubules, much more in proximal than in distal ones. The coexpression of vimentin and cytokeratin in proximal tubular cells was also demonstrated. The changes described, particularly those taking place at the level of interstitium, bear the key responsibility for BEN progression.

CELA1, the second nominated gene, is also involved in the process of angiogenesis [31]. The gene encodes elastase-1, which degrades elastin in the vascular matrix. Tumor angiogenesis, chicken angiogenesis, and mesenteric angiogenesis data suggest that elastin and elastin degradation products play a key role in vascular morphogenesis. CELA1 was expressed in vascular cells in the embryonic lung and in a fetal mesenchymal cell line with angiogenic properties [32]. Degraded elastin causes deposition of hydroxyapatite-like mineral and osteogenic transformation of vascular smooth muscle cells (as they lose the specific α-SMA), resulting in vascular calcification [33]. In contrast, collagen-1 levels in areas of calcification are increasing [34]. Changes in both α-SMA and elastin inversely correlate with the hemodynamic

parameters such as pulse wave velocity (PWV) and lead to media remodeling. This was associated with the increased arterial stiffness observed in CKD rats with vascular calcification. Dysregulation of normal anticalcification factors and elastin degradation represent a pattern of vascular injury existing in patients with end-stage renal diseases [35].

The third gene nominated in our study was the potassium channel gene *KCNK5*. Potassium channels in the kidney play an essential role in controlling and maintaining plasma potassium levels in the normal range, as well as exerting very different functions such as cell volume control, membrane potential stabilization and excitability, or regulation of hormone or ion secretion [36–39]. In addition, potassium ion (K^+) channel activity is a major regulator of vascular muscle cell membrane potential and is therefore an important determinant of vascular tone. There is growing evidence that the function of several types of vascular K^+ channels is altered during major cardiovascular diseases, such as chronic hypertension, diabetes, and atherosclerosis [40]. Defects in potassium channels cause abnormal vasodilation responses reflecting a gradual deterioration of vascular mechanisms during the progression of diabetic nephropathy [41]. Enhanced dilator responses and basal activation of K^+ channels may occur in the renal circulation early during diabetes. An increased K^+ channel activity may therefore reflect a very high metabolic state of vascular smooth muscle cells [42].

5. Conclusion Hypothesis for the Pathogenic Mechanism in BEN

The molecular mechanisms leading to interstitial fibrosis and chronic kidney disease are complex and are probably related to the primary processes leading to renal injury. As blood vessels nourish all the tissues and organs in the body, abnormal formation and remodeling of blood vessels probably contribute to the pathogenesis of renal fibrosis. Neoangiogenesis is a complex process of recruitment, migration, proliferation, and apoptosis of stem/progenitor cells, endothelial cells, vascular smooth muscle cells, and other mural cells. The extracellular matrix plays important roles in vessel development via providing a supportive matrix scaffold and growth factors for cells. Close interactions between vascular cells and their ECM is crucial in blood vessel formation and remodeling. Our results suggest three new genes for predisposition to BEN pathology, related to angiogenic alterations. We hypothesize that mutations in *HSPG2*, *CELA1,* and *KCNK5* participate in extracellular matrix modifications, arterial media remodeling, and regulation of vascular tone, all these events leading to interstitial vessel remodeling, connected to renal interstitial fibrosis in BEN (Figure 4). Further elucidation of molecular pathogenesis of kidney fibrosis could lead to the development of a new target therapy of BEN by targeting specific angiogenic factors. Our results provide a basis for further investigations of the role of the nominated genes in BEN kidney pathology, including screening for variants in the nominated genes in a larger cohort of patients. Herein we suggest a possible mechanism between the three candidate genes and BEN.

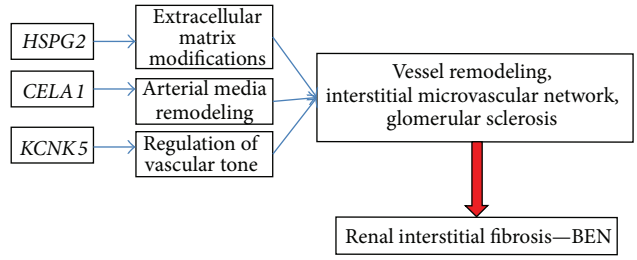

Figure 4: Proposed model for molecular pathogenesis of BEN kidney pathology.

The main pathological characteristic of BEN kidney is interstitial fibrosis. Abnormal angiogenesis and vascular remodeling probably contribute to pathogenesis of renal fibrosis. Interactions between endothelial cells, vascular smooth muscle cells and progenitor cells with an extracellular matrix (ECM) play an important role in these processes. Scattered glomeruli showing an obvious segmental or global thickening of the capillary walls with a double outline of the glomerular basement membrane were found in early BEN patients.

Interstitial sclerosis could result from the overproduction of extracellular matrix by injured proximal tubular epithelium and interstitial capillary endothelial cells—this could be the pathogenic role of mutated *HSPG2* gene. The increase of the cortical interstitial volume results in resistance of the postglomerular capillary network with impairment of the glomerular flow [43]. This impairment leads to chronic rise in hydrostatic pressure. The increase of the cortical interstitium additionally leads to an increase in the length of diffusion between the tubules and the intertubular and peritubular capillaries. This increase in the length of diffusion subsequently results in the atrophy of the tubules, reduction of reabsorbtion, and therefore impairment of the effective filtration pressure. The haemodynamic changes inversely correlate with α-SMA and elastin in vessels and here could be the additional pathogenic role of mutated *CELA1* gene. The mutated *KCNK5* gene is probably a factor stimulating the haemodynamics-driven vascular remodeling.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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