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TERT 啟動子突變與 GATA3、CK20、CK5/6 及 p53 之表現於膀胱泌尿上皮腫瘤之生物學意義

Biological Significance of TERT Promoter Mutation and Expression of GATA3, CK20, CK5/6 and P53 in Urothelial Neoplasm of the Urinary Bladder

王中傑

Chung-Chieh Wang

指導教授：鄭永銘教授

Advisor: Yung-Ming Jeng, Professor

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本論文係王中傑君（學號 D00444003）在國立臺灣大學病理學研究所完成之博士學位論文，於民國 108 年 12 月 4 日承下列考試委員審查通過及口試及格，特此證明

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（指導教授）

潘競成

蕭永孝

黃昭淵　　成佳憲

系主任、所長 鄭永銘 （簽名）

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致謝

猶記八年前剛入學時，我才剛升任主治醫師不久。當時雖然已經習慣於病理診斷工作，對於研究領域卻仍相當陌生。經過張逸良前主任的鼓勵，我報考並就讀了病理學研究所；在鄭永銘教授不厭其煩的指導下，我從對於病理研究完全外行的門外漢，一點一滴地產生了心得與興趣。

這八年來，研究之路其實走得跌跌撞撞。在職場上，出國進修與分院支援讓我一度中斷學業；身體健康的問題，也讓我不得不二度休學。此外，不論是診斷工作還是病理研究，都需要投注相當大量的時間與精力；兩者之間的優先順序安排，常帶給我不小的考驗。我之能夠寫成論文，首先必須感謝鄭永銘教授；除了提供研究的方向與充沛的資源，在計畫撰寫、研究技術與論文寫作方面，鄭教授也給了我全面性的援助與教導。由於論文內容涉及臨床應用，黃昭淵醫師與蔡育傑醫師等臨床先進的指導，對於這篇論文的完成相當重要。此外，感謝賴寶蓮小姐協助切片與蠟塊借調；感謝莊郁琳小姐等研究助理協助進行 DNA 萃取、PCR 與定序工作；感謝切片室與免疫組織化學室同仁在免疫染色的支援；感謝張晉豪博士與統計諮詢團隊在生物統計上的指導。如果沒有各位的努力，這篇論文也不可能完成。

研究的路或許稱不上辛勞，但苦悶與挫折總是在所難免。感謝愛妻柏瑩支持著家庭，讓我能有溫暖而安穩的歸宿；感謝愛女恆音的笑容，在每天的開始帶給我奮鬥的動力。感謝父母的勸勉與鼓勵，幫助我面對各種人生的挑戰；感謝吾弟中瑋與吾友重佑，時時為我分憂解悶。最後，將我的感謝與讚美獻給天父上帝；願此研究成果能使醫學進步，造福人類，榮耀祢的名。

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中文摘要

膀胱泌尿腫瘤是泌尿科的常見疾病，而其治療主要依據為腫瘤分期與形態學上的分化程度。近期分子病理研究發現了不少重要的腫瘤生物標記，但它們尚未被整合到臨床的治療指引中。本篇論文包含兩部分，分別闡述相關標記於兩類膀胱泌尿腫瘤中的生物學意義：早期的低度非侵襲性泌尿上皮腫瘤，以及晚期的肌肉侵犯性膀胱癌(MIBC)。

在第一部分，我們在低度非侵襲性泌尿上皮腫瘤檢測 *TERT*、*FGFR3* 及 *HRAS* 三種基因的突變狀態，並分析其預後意義。*TERT* 基因之啟動子突變在膀胱癌頗為常見，但是在低惡性度乳突狀泌尿上皮腫瘤(PUNLMP)的相關資料並不多。本研究中，我們收集了 21 例良性之倒生性乳突瘤、30 例 PUNLMP，以及 34 例低度非侵襲性乳突狀泌尿上皮癌(NIPUC)。*TERT* 基因之啟動子突變出現於 10 (33%)例之 PUNLMP 與 17 (50%)例之低度 NIPUC，但未出現於倒生性乳突瘤。相對於倒生性乳突瘤，PUNLMP 與低度 NIPUC 較常出現 *FGFR3* 基因之突變($p = 0.009$)，*HRAS* 基因突變則較為少見($p < 0.001$)。在預後方面，PUNLMP 病例有 *TERT* 啟動子突變者較容易復發($p = 0.024$)，但是低度 NIPUC 並無此現象($p = 0.530$)。此外，PUNLMP 病例有 *TERT* 啟動子突變者，其復發率與低度 NIPUC 無顯著差異($p = 0.487$)。

在第二部分，我們分析了 GATA3、CK20、CK5/6 及 p53 在 MIBC 的表現與其生物學意義。近期基因研究將 MIBC 區分為數種子分類，而上述之免疫組織化學染色(IHC)標記與這些子分類有關。其中，GATA3 與 CK5/6 分別被視為管腔型與類基底-鱗狀型的代表性標記；p53 染色常被用於代替 *TP53* 基因突變檢測，而傳統上一般將細胞核染色比例高者視為異常。本研究共收集了 91 名 MIBC 病人的膀胱全切除術組織檢體。在這些病例中，GATA3 表現量較低及 CK20 陰性者，其 Ki-67 增殖指數較高(p 值分別為 0.006 與 0.002)。相對地，CK5/6 呈廣泛性表現者，Ki-67 增殖指數較高($p = 0.001$)。P53 染色則有三種類型與高 Ki-67 指數相關：完全陰性、廣泛的細胞核強染色，以及廣泛的細胞質強染色。此外，CK20 與 CK5/6 的染色結果通常呈現互補關係，但 91 例中的 13 例(14.29%)同時有廣泛的 GATA3 與 CK5/6 表現。在 78 名未接受術前化學治療的病人裡，GATA3 表現量較低者於單變項與多變項分析均有顯著較高的復發率(p 值分別為 0.008 與 0.002)。CK20、CK5/6 及 p53

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的表現則與預後無關。

總結來說，根據我們的研究結果，*TERT* 基因的啟動子突變可做為 PUNLMP 病人的預後標記。在 MIBC 病患，GATA3 的表現量則可做為膀胱切除術後的復發危險性指標。此外，由 Ki-67 增殖指數的關聯性來看，以前述三種染色類型做為判斷 p53 染色異常的標準，應比傳統上的核染色比例準則更佳。

關鍵詞：膀胱癌；泌尿上皮腫瘤；*TERT* 啟動子；GATA3；CK20；CK5/6；p53

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Abstract

Clinical management of bladder urothelial neoplasm depends mainly on the tumor stage and grade. Recent advances in molecular pathology discovered several essential biomarkers, and their value in clinical application warrants investigation. In our study, we focused on the relevant biomarkers in two separate fields of bladder tumors: the early low-grade noninvasive papillary urothelial neoplasm, and the advanced muscle-invasive bladder cancer (MIBC).

In the first part, we investigated the mutation status of the *TERT* promoter, *FGFR3* gene, and *HRAS* gene in low-grade papillary urothelial neoplasms and evaluated their prognostic significance. Mutations in the promoter region of the *TERT* gene have been frequently found in urothelial carcinoma of the urinary bladder, but related data for papillary urothelial neoplasm of low malignant potential (PUNLMP) are limited. In our study, we included 21 cases of inverted papillomas, 30 PUNLMPs, and 34 low-grade noninvasive papillary urothelial carcinomas (NIPUCs). *TERT* promoter mutations were observed in 10 (33%) PUNLMPs and 17 (50%) low-grade NIPUCs, but not in any inverted papilloma. *FGFR3* mutations were more frequently observed in PUNLMP and low-grade NIPUC than in inverted papillomas ($p = 0.009$), whereas the opposite trend was noted for *HRAS* mutations ($p < 0.001$). Regarding the clinical outcome, *TERT* promoter mutation was associated with a higher recurrence rate in PUNLMP ($p = 0.024$)

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but not in low-grade NIPUC (p = 0.530). Notably, PUNLMP cases with *TERT* promoter mutations had a similar recurrence rate to that in low-grade NIPUC cases (p = 0.487). Our results suggest that the status of the *TERT* promoter mutation may serve as a biomarker of prognostic stratification in patients with PUNLMP.

In the second part, we investigated the biological and prognostic significance of GATA3, cytokeratin (CK) 20, CK5/6 and p53 in MIBCs from 91 patients who underwent radical cystectomy. Genetic profiling studies on muscle-invasive bladder cancers (MIBCs) have discovered several subtypes with different biological characteristics, and these markers were found to be associated with the molecular subtypes. According to our results, high Ki-67 indices were associated with negative CK20 (p = 0.002) and diffuse CK5/6 (p = 0.001) staining. By contrast, tumors with diffuse GATA3 expression had low Ki-67 index (p = 0.006). Regarding p53, three staining patterns were associated with a high Ki-67 index: (1) complete absence, (2) diffusely strong nuclear reactivity, and (3) diffusely strong cytoplasmic staining (p < 0.001 compared with other patterns). CK5/6 and CK20 expression was typically present in a reciprocal fashion; however, diffuse GATA3 and CK5/6 coexpression was observed in 13 (14.29%) cases. Among 78 chemotherapy-naïve patients, low GATA3 staining was associated with worse recurrence-free survival in both univariate (p = 0.008) and multivariate analyses (p = 0.002). CK20, CK5/6, or p53 expressionwas not associated

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with clinical outcome. Based on our results, IHC staining for GATA3 may help risk stratification in patients with MIBC receiving radical cystectomy. In addition, the differences in Ki-67 indices suggested that aberrant p53 expression was better defined by the three aforementioned patterns, rather than percentage of nuclear staining alone.

Keywords: Bladder cancer; urothelial neoplasm; *TERT* promoter; GATA3; cytokeratin 20; cytokeratin 5/6; p53

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1. Introduction

1.1 Classification of urothelial neoplasm of the urinary bladder

Urothelial neoplasm of the urinary bladder, or simply bladder tumor, is a major disease entity commonly encountered by physicians in urology and genitourinary pathology. This entity encompasses a wide spectrum from the totally benign urothelial papilloma to advanced bladder cancer. According to the general concept of pathology, the current version of World Health Organization (WHO) Tumor Classification roughly divides urothelial neoplasms into the infiltrating urothelial carcinoma and noninvasive urothelial lesions [1, 2]. The noninvasive neoplasms can be further divided into flat and papillary lesions by their growth pattern, and the latter includes four categories by the ascending order of aggressiveness: (1) urothelial papilloma, (2) papillary urothelial neoplasm of low malignant potential (PUNLMP), (3) low-grade noninvasive papillary urothelial carcinoma (NIPUC), and (4) high-grade NIPUC [2, 3].

1.2 Clinical management on different categories of the bladder tumors

Clinical management on the urothelial neoplasms depends mainly on the tumor stage and the histologic grade for noninvasive lesions [4]. From the clinical point of view, invasion of the bladder tumor into or beyond muscularis propria (stage T2 or more) warrants aggressive treatment such as radical cystectomy. These advanced tumors are

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also known as muscle-invasive bladder cancers (MIBCs) in the clinical usage and treatment guidelines [4]. Systemic chemotherapy prior to operation, or neoadjuvant chemotherapy, may be required in some MIBC patients. By contrast, treatment of infiltrating urothelial carcinomas limited to the lamina propria (stage T1) is similar to that of high-grade NIPUC. Although these tumors have potential in progression to MIBC, most patients require only limited tumor resection, intravesical chemotherapy, and close clinical follow-up [4].

As their designation implies, patients with low-grade noninvasive papillary lesions usually follow an indolent clinical course. Urothelial papillomas are totally benign and do not recur after resection [2]. Low-grade NIPUC has potential of recurrence and even progression, but most patients require only surgical resection and follow-up [4]. Between them is the entity of PUNLMP, which is characterized by thickening of the urothelium with minimal cytological atypia [2, 3]. This entity is specific to the urinary tract and not equivalent to any certain premalignant lesion in other organs. Although it is associated with a better clinical outcome compared to low-grade NIPUC, it still has low potential of recurrence. In addition, the differential diagnosis between PUNLMP and low-grade NIPUC depends only on the degree (minimal versus mild) of nuclear atypia [2, 3], and this can be subjective in clinical practice. Therefore, WHO Tumor Classification recommends that patients with PUNLMP should be managed in the same

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manner as those with low-grade NIPUC [2].

1.3 Current problem in management of PUNLMP

Although we already have well-established treatment guidelines for the bladder urothelial neoplasms, there are still problems to be solved. Current recommendation for management of PUNLMP is resection and clinical follow-up, but most cases of PUNLMP never recur or progress (hence the name “low malignant potential”) [2, 3]. In my speculations, PUNLMP may be further divided into two groups: (1) benign papillomatous tumors with thickened urothelium, and (2) low-grade NIPUC with minimal nuclear atypia. Identification of the first group can avoid unnecessary clinical follow-up in these patients, but these two groups cannot be distinguished by morphology alone. Genetic studies may provide clues for classification, and knowledge about the common genetic changes in PUNLMP becomes important in design of our studies.

1.4 Common gene mutations in low-grade urothelial neoplasms

As the evidence from genetic studies suggests, urothelial neoplasms are likely to develop through two different routes: high-grade and low-grade pathways [1, 5]. Histologic characteristics of the tumors are often parallel to their genetic changes, as inactivating mutations of the *TP53* gene and subsequent genomic instability are more commonly seen in tumors with high-grade features [1, 5]. By contrast, mutations

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involving the tyrosine kinase receptor gene *FGFR3* and the oncogene *HRAS* are mainly associated with the more indolent low-grade papillary urothelial neoplasms [1, 2, 5]. In addition to these grade-specific alterations, *TERT* promoter mutations were found in a considerable percentage of tumor samples regardless of the tumor stage and histologic grade [6–9]. Details about the three frequently-mutated genes in low-grade urothelial neoplasms (*TERT*, *FGFR3*, and *HRAS*) are described in the following sections.

1.4.1 *TERT* promoter mutations

The *TERT* gene encodes the human telomere reverse transcriptase, which maintains the telomere length in tumor cells. Mutations in the promoter region of the *TERT* gene have been found in not only bladder cancer but also other types of human cancers, including malignant melanoma, thyroid carcinoma, and glioma [6]. In bladder cancer, C228T was reported to be the most common mutation pattern in the *TERT* promoter [7]. In the two series included by Allory *et al*., *TERT* promoter mutations were identified in 70% and 79% of the bladder cancer samples, respectively [7]. Vinagre *et al.* found *TERT* promoter mutations in 67% and 56% of low-grade and high-grade tumors, respectively [6]. Hosen *et al.* reported that the rate of the *TERT* promoter mutation was 66.5% in 158 NIPUC cases, which is similar to a mutation rate of 65.4% in 327 combined cases of all stages of bladder cancer [8]. Allory *et al*. also found that *TERT* promoter mutations were not significantly associated with the histologic grade, tumor stage, or clinical

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outcome [7].

The data of *TERT* promoter mutations described above included samples with different grades and stages, and studies focused on low-grade papillary urothelial neoplasms are limited. Cheng *et al.* reported the presence of *TERT* promoter mutations in PUNLMP (15 of 35 cases), benign urothelial papilloma (12 of 26 cases) [10], and inverted papilloma (4 of 26 cases) [9]. Rodriguez Pena *et al*. collected 30 *de novo* cases of PUNLMP and found *TERT* promoter mutations in 19 (63%) of them. In addition, there was no association between *TERT* promoter mutations and the recurrence rate [11]. Taken together, the biological significance of *TERT* promoter mutations in these low-grade urothelial lesions remains elusive according to current evidence.

1.4.2 *FGFR3* mutations

FGFR3 gene encodes the human fibroblast growth factor receptor 3, and mutations in this gene are found in both urothelial carcinoma [12, 13] and benign urothelial papillomas [14, 15]. *FGFR3* mutations occur mainly in the exons 7, 10, and 15, and the most common form is S249C (g. C746G) close to the 3' end of the exon 7 [12–17]. These mutations are associated with lower grade and lower stage bladder cancers and are thus considered a favorable prognostic marker [1, 16, 17]. Furthermore, a previous study demonstrated a correlation between *FGFR3* mutations and improved progression-free survival (PFS) [17]. In contrast to their well-established role in

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urothelial carcinoma, evidence about *FGFR3* mutations in PUNLMP is limited. One recent study showed 60% of cases with mutated *FGFR3* gene in a series of PUNLMP, and no significant association with clinical outcome was found [11]. The value of *FGFR3* mutations in stratification of PUNLMP may need further investigation.

1.4.3 *HRAS* mutations

Mutations in another mitogen-activated protein kinase pathway-related gene, *HRAS*, have been observed in some urothelial carcinomas [18, 19]. One large-scale study showed an overall *RAS* mutation rate of 4% to 15% cases in MIBC depending on the molecular subtype [20] (see section **1.5** for details). *HRAS* mutations are more common in the benign inverted papillomas, as the percentage reached 81% (13/16) in two separate studies [21, 22]. The most common form of *HRAS* mutations was Q61R, which occurred in exon 3 of this gene [18, 21, 22]. Evidence about *HRAS* mutations is other low-grade papillary urothelial neoplasms was more limited; the percentages of mutation were 18% (2/11) and 3% (1/30) in urothelial papilloma and PUNLMP, respectively, according to two separate studies [11, 22]. The association of *HRAS* mutations and malignant potential in urothelial neoplasms is uncertain under current evidence.

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1.5 Molecular classification of MIBC

While tumor recurrence is the main issue in management of low-grade papillary urothelial neoplasms, MIBC could result in systemic metastasis and mortality. The current treatment guidelines for MIBC are based on the tumor stage, overall clinical condition, and the patient's response to treatment [4]. In the recent decade, several independent studies have revealed distinct molecular subtypes by analyzing mRNA expression profiles in bladder UC specimens [23–26]. The concept of molecular classification in bladder UC is similar to that in breast cancers [27, 28], and immunohistochemical (IHC) staining for the breast cancer subtype-associated markers has been incorporated in the treatment guidelines [29, 30]. Based on the achievements in breast cancer studies, surrogate IHC markers for molecular subtypes of MIBC may aid clinical management of the disease.

1.5.1 Lund and MDA Classification Systems

Before proceeding, we first take a brief review of the evolution in the molecular classification systems. In 2012, a research group from Lund University (Sjödahl *et al.*) revealed five distinct subtypes by analyzing the mRNA expression profiles in bladder urothelial carcinomas. They named these subtypes as (1) urobasal A, (2) genomically unstable, (3) infiltrated, (4) urobasal B, and (5) squamous cell carcinoma (SCC)-like, according to their histologic or genetic characteristics [23]. Another group from MD

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Anderson Cancer Center (Choi *et al.*; abbreviated as MDA) described three subtypes including (1) luminal, (2) basal, and (3) p53-like [24]. Along with the study by Cancer Genome Atlas Research Network [25], each classification system could demonstrate the significant association with the clinical outcome [23–25]. For example, the MDA basal subtype is associated with a higher metastatic rate and shorter disease-specific survival (DSS) [24]. However, the subtypes among these classification systems are not interchangable because the gene sets for mRNA profiling are different. To be more confusing, the MDA "basal" subtype has a totally different meaning from the Lund "urobasal" subtypes [23, 24, 31].

1.5.2 Consensus Molecular Classification System

Because of the diversity among these classification systems, researchers from different groups held a consensus meeting and described a "basal/squamous-like (BASQ)" phenotype in the bladder urothelial carcinoma [31]. This BASQ phenotype is featured by (1) high levels of cytokeratin (CK) 5/6 and CK14 expression, (2) low levels of FOXA1 and GATA3 expression, (3) enrichment (higher percentage) of squamous differentiation, and (4) worse clinical outcome. Compared to other subtypes, the BASQ phenotype is more constantly defined among different classification systems; it is roughly equivalent to the MDA basal subtype, the Lund SCC-like subtype, and the TCGA cluster III and IV [31].

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After the consensus meeting, the researchers organized a multi-national study using 1750 MIBC transcriptomes and analysis of independent molecular classification systems [32]. The described a “consensus” set of six molecular classes: (1) luminal papillary, which is enriched in noninvasive papillary components, (2) luminal non-specified, (3) luminal unstable, (4) stroma-rich, (5) basal/squamous, which is similar to the concept of BASQ phenotype, and (6) neuroendocrine-like, in the descending order of overall survival (OS). These “consensus” subtypes differ in not only histologic and clinical characteristics but also possibly underlying oncogenic mechanisms [32].

1.5.3 Possible surrogate markers for molecular subtypes of MIBC

As stated in the previous section, four IHC markers are associated with the BASQ phenotype described by Lerner *et al.*: CK5/6, CK14, FOXA1, and GATA3 [31]. Choi *et al.* also reported consistency between the results of IHC staining and mRNA expression profiles in basal (CK5/6-positive) and luminal (CK20-positive) subtypes [24]. Dadhania et al. further proposed that IHC study on GATA3 and CK5/6 may sufficiently identify these two subtypes with more than 90% accuracy [33]. Among these IHC markers, GATA3, CK20, and CK5/6 are frequently used in the practice of diagnostic pathology. The prognostic significance of GATA3 has been investigated in previous studies, but the results were controversial. Miyamoto *et al.* revealed poor clinical outcome in MIBC

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with negative or decreased GATA3 expression [34], but three other studies demonstrated no prognostic significance of GATA3 staining in bladder UC [35–37]. Similarly, negative GATA3 staining in upper-tract UC (UTUC) was associated with poor clinical outcome in one study [38] but not in another [39]. The potential importance of these IHC markers in MIBC warrants further investigation.

1.6 *TP53* mutation and p53 IHC staining

In addition to subtype-specific genetic changes, *TP53* mutation is common in bladder urothelial carcinoma. In the practice of diagnostic pathology, IHC expression of p53 is commonly used as a surrogate marker for *TP53* mutations. This is based on the finding that missense mutations in *TP53* increase the half-life of p53, thereby increasing the percentage of positive IHC staining for p53 [40–42]. Traditionally, a positive p53 staining is defined as nuclear reactivity over a certain cut-off percentage, whereas the absence of p53 staining indicates a negative result [43, 44]. By using this criterion, the correlation of p53 IHC staining with clinical outcome may be controversial [43]. Studies on ovarian and endometrial carcinoma have reported two patterns related to *TP53* mutation–associated aberrant p53 staining: diffusely strong nuclear reactivity and complete absence of staining [45, 46]. The cut-offs for diffuse nuclear staining were 60% and 75% for ovarian [45] and endometrial [46] carcinoma, respectively. A bladder cancer study also demonstrated that abnormal (negative or ≥50%) p53 staining was

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correlated with significantly worse recurrence-free survival (RFS) [44]. Therefore, the clinical significance of p53 IHC staining in bladder cancers also requires further investigation.

1.7 Aims of our study

In order to improve clinical management of bladder tumors, we investigated the mutation status of the *TERT* promoter, *FGFR3* gene, and *HRAS* gene in cases of low-grade papillary urothelial neoplasms, including inverted papilloma, PUNLMP, and low-grade NIPUC. We then analyzed the association between the mutation status and the clinical outcome in these cases. In addition, we evaluated the IHC expression of GATA3, CK20, CK5/6 and p53 in a series of MIBC cases and correlated them with the associated clinical outcome, Ki-67 proliferative index, and other clinicopathological parameters to investigate their clinical significance.

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2. Materials and Methods

2.1 Patients and specimens

The patients included in our study were retrieved from the database of pathological diagnosis in the Department of Pathology, National Taiwan University Hospital (NTUH). For cases of low-grade noninvasive papillary urothelial neoplasm, we recruited all 66 patients diagnosed as PUNLMP of the urinary bladder from 2005 to 2014 and collected their tissue specimens of initial diagnosis. Four patients with the initial diagnosis of "transitional cell carcinoma, grade I" from 2000 to 2004 were also added to our study cohort. In addition, we randomly selected 26 patients with inverted papilloma from 2005 to 2014 for comparison. Hematoxylin and eosin (H&E)-stained sections of all cases were reviewed and reclassified according to the WHO 2016 tumour classification (**Figure 1**). Cases without adequate tumor contents for DNA extraction were excluded from the analysis. After confirming the histologic diagnosis of each case, the patients' age, sex, and follow-up data (status and periods of tumor recurrence and progression) were recorded. Tumor progression was defined as recurrence as high-grade NIPUC, urothelial carcinoma in situ, or invasive urothelial carcinoma. One representative section with an adequate tumor part and the corresponding formalin-fixed paraffin-embedded (FFPE) tissue block were selected for each case. This part of study was proved by the Research Ethics Committee, NTUH, on Oct 6th, 2015 (No.

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201508043RIND; revised on Dec 30th, 2016).

For MIBC cases, we included all 109 patients who underwent radical cystectomy from 2010 to 2016 and collected their clinical data. Patients with a history of UTUC were excluded to avoid confounding prognostic analysis. In each patient who received neoadjuvant chemotherapy, the latest resection specimen prior to cystectomy was selected for this study. In this group, those who did not have a preoperative specimen available at NTUH were excluded from this study. In patients without neoadjuvant chemotherapy, the cystectomy specimens were selected. Alternatively, the latest resection specimen before cystectomy was selected if no adequate tumor cells were present in the cystectomy specimen. H&E-stained sections of all cases were reviewed and re-staged by the TNM classification system defined in the AJCC Cancer Staging Manual (8th edition). Cases without definite muscularis propria invasion were excluded at this point. One representative section with an adequate tumor part and the corresponding FFPE tissue block were selected for each case. This part of study was approved by the Research Ethics Committee of NTUH on Oct 16th, 2017 (No. 201708055RIND; revised on Aug 17th, 2018).

2.2 DNA extraction and sequencing

For each case with low-grade noninvasive papillary urothelial neoplasm, five 10-μm unstained slides were sectioned from the representative tissue block. DNA was extracted

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using the QiaAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The mutation hotspots in the promoter region of the *TERT* gene, 3 exons of coding regions in the *FGFR3* gene, and 1 exon in the *HRAS* gene were amplified through polymerase chain reaction (PCR). The amplicons of the *FGFR3* gene included codons 248 and 249 in exon 7, codons 372 and 375 in exon 10, and codon 652 in exon 15. A similar assay in the *HRAS* gene focused on codon 61 in exon 3. We selected these exons of the target genes based on previous reports showing the frequent mutated genes in urothelial neoplasms and their mutational hot spots [6–19]. The primers for PCR are listed in **Table 1**, and the Sanger method was used to sequence the PCR products.

2.3 IHC staining

For each case with MIBC, 5-μm sections were taken from the representative tissue block for IHC staining. The staining procedures were conducted with a Ventana Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. Primary antibodies against p53 (clone DO-7, dilution 1:1000, Dako Denmark A/S, Glostrup, Denmark), GATA3 (clone L50-823), CK20 (clone SP33), CK5/6 (clone D5/16B4), and Ki-67 (clone 30-9) were included. All antibodies other than anti-p53 antibody were purchased from Ventana Medical Systems and were ready to use. The antibody reactivity was visualized with a Ventana OptiView

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DAB IHC Detection Kit. Finally, the slides were counterstained with hematoxylin.

All IHC results were examined under a light microscope and scored under the following criteria. The percentage and intensity of tumor cells stained with GATA3 (nuclear staining), CK20, and CK5/6 (membranous-type or cytoplasmic staining) were recorded for each case and their immunoreactive scores (IRS) were calculated using Remmele and Stegner's criteria [47, 48] (**Table 2**). According to the cut-offs used in IRS, their staining percentage was also classified as negative (<10%), partial (10%–80%), and diffuse (>80%). The standards for p53 scores are demonstrated in **Figure 2** by combining the criteria used in prior studies of bladder [44] and ovarian [45, 49] cancers. The Ki-67 indices were evaluated by following the recommendations from the International Ki67 in Breast Cancer Working Group [50]. In brief, at least three 400× fields containing 500 or more invasive tumor cells were selected for each case. Tumor cells with nuclear staining were considered positive regardless of the staining intensity. The Ki-67 index was calculated as the percentage of the positive cells among the total number of tumor cells in the scored area.

2.4 Clinicopathological correlation and survival analysis

Student *t* test and one-way analysis of variance (ANOVA) were used to evaluate differences in the continuous parameters between or among comparable groups. Chi-square test and Spearman rank correlation test were applied to analyze the

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association among categorical and continuous parameters, respectively. For cases of PUNLMP and low-grade NIPUC, we calculated the cumulative recurrence-free survival (RFS) and PFS from the point of initial diagnosis by using the Kaplan–Meier method. In chemotherapy-naïve MIBC patients, we calculated OS and DSS after radical cystectomy in addition to RFS. The differences in survival time were determined using log-rank tests. For MIBC patients, Cox regression was used to determine the association between continuous parameters and clinical outcomes. Multivaraite analyses were also performed using Cox regression. $P < 0.05$ was considered statistically significant. Cox regression was performed with SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) with the assistance of the Department of Medical Research, NTUH, and the other statistical analyses were conducted using Microsoft Excel 2007 and Prism (version 7.03; GraphPad Software, Inc., La Jolla, CA, USA).

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3. Results

3.1 Mutation status in each histological entity of low-grade noninvasive papillary urothelial neoplasms

After review of all 96 cases with low-grade noninvasive papillary urothelial neoplasms, 4 were reclassified as high-grade NIPUC and excluded from our study. Four additional cases were excluded because the tissue specimens of initial diagnosis were not available or limited in amount. In the mutation analyses, PCR for *TERT* promoter sequences were unsuccessful in 3 cases after two repeated experiments. These 3 cases were also excluded. The 85 cases finally included in this study comprised 21 inverted papillomas, 30 PUNLMPs, and 34 low-grade NIPUCs. The clinical characteristics of patients and the results of the mutation status in each entity were summarized in **Table 3**.

TERT promoter mutations were found in 33% (10 of 30) of PUNLMP, 50% (17 of 34) of low-grade NIPUC and none of the 21 inverted papillomas. The rates of *FGFR3* mutations were also much higher in PUNLMP (30%, 9 of 30) or low-grade NIPUC (50%, 17 of 34) than in inverted papilloma (10%, 2 of 21) ($p = .009$). Conversely, *HRAS* mutations were more frequently observed in inverted papilloma (76%, 16 of 21) than in PUNLMP (10%, 3 of 30) or low-grade NIPUC (15%, 5 of 34) ($p < .001$). We compared the mutation frequency between PUNLMP and low-grade NIPUC groups and found no significant difference in the mutation frequency of the 3 genes.

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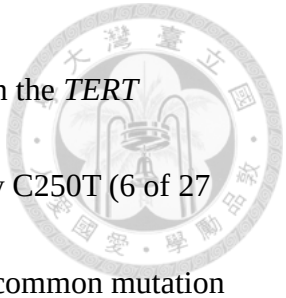

Regarding mutation patterns, the most frequent point mutation in the *TERT* promoter region was C228T (19 of all 27 mutated cases), followed by C250T (6 of 27 cases) and C228A (2 of 27 cases) (**Figure 3**, upper panel). The most common mutation in *FGFR3* was S249C (16 of all 28 mutated cases), followed by Y375C (7 of 28 cases) (**Figure 3**, middle panel). Other mutations in *FGFR3* included G372C (1 case), S373C (1 case), K652E (1 case), and K652T (2 cases). Mutations in the *HRAS* gene included Q61R (14 of all 24 mutated cases), Q61K (7 cases), and Q61L (3 cases) (**Figure 3**, lower panel) (**Table 4**).

3.2 Prognostic significance of the mutation status in PUNLMP and low-grade NIPUC

The PUNLMP and low-grade NIPUC patients were followed at our outpatient clinics with a median follow-up period of 5.7 years, and most (61 of 64, 95.3%) cases have been followed for more than 2 years. We first analyzed the clinical outcomes of all patients with PUNLMP or low-grade NIPUC. Patients with low-grade NIPUC had shorter RFS than those with PUNLMP (p = 0.002, **Figure 4A**), and the *TERT* promoter mutation status had borderline significance regarding RFS in these patients (p = 0.052, **Figure 4B**). We then separately analyzed the survival data of 30 patients with PUNLMP and 34 patients with low-grade NIPUC. In the PUNLMP group, patients with *TERT* promoter mutations had shorter RFS than those without mutations (p = 0.024, **Figure**

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4C). In contrast, the *TERT* promoter mutation status was not related to RFS in the low-grade NIPUC group (p = 0.530, **Figure 4D**).

Tumor progressions occurred in only three patients. One patient progressed from PUNLMP to high-grade NIPUC and then high-grade invasive urothelial carcinoma. The other patient initially diagnosed as PUNLMP progressed to high-grade invasive urothelial carcinoma directly. The third patient progressed from low-grade NIPUC to high-grade NIPUC and then high-grade invasive urothelial carcinoma. Neither the histologic entity nor the *TERT* promoter mutation status had significant impact on PFS. In addition, mutation status of the *FGFR3* gene has no prognostic association in PUNLMP, low-grade NIPUC, or combined. Analysis of the association between survival and the mutation status of *HRAS* was not performed due to the limited number of mutated cases in each group.

3.3 Prognostic grouping by combination of histological classification and mutation status of *TERT* promoter

Because of the prognostic significance of the *TERT* promoter mutation in PUNLMP, we further categorized PUNLMP and low-grade NIPUC cases into 4 groups based on the mutation status of the *TERT* promoter. Group 1 was defined as PUNLMP cases without *TERT* promoter mutations, and group 2 represented those with the mutations. Low-grade NIPUC cases with wild-type and mutated *TERT* promoter regions were

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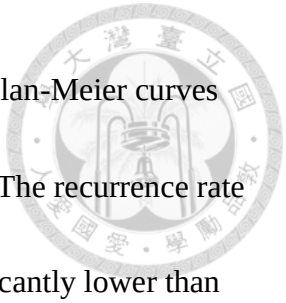

referred to as groups 3 and 4 respectively. We then compared the Kaplan-Meier curves of RFS among these groups, and the result was shown in **Figure 4E**. The recurrence rate of PUNLMP without *TERT* promoter mutations (group 1) was significantly lower than other groups (overall $p = 0.007$). Moreover, there was no significant difference in RFS among PUNLMP with *TERT* promoter mutations (group 2) and low-grade NIPUC cases (groups 3 and 4; $p = 0.487$).

3.4 Demographic and clinicopathological data regarding patients with MIBC

Among the 109 patients recruited for our MIBC study, definite muscularis propria invasion (i.e. stage pT2 or higher) was not identified in 9, the microscopic slides were unavailable in 7, a history of UTUC was noted in 1, and loss to follow-up after radical cystectomy was noted in 1; these 18 cases were all excluded. Finally, we included 91 patients (median age: 67 years [range: 39–89 years]; male-to-female ratio: 2.37:1). Of them, 13 (14.3%) received neoadjuvant chemotherapy. In patients not receiving neoadjuvant chemotherapy, the median follow-up time was 2.46 years. The demographic and clinicopathological data of these patients are summarized in **Table 5**.

3.5 Association among GATA3, CK20 and CK5/6 staining in MIBC

After completion of IHC, we evaluated the correlation of the staining results among each marker. The results are summarized in **Table 6**. First, CK20 and GATA3 demonstrated a positive correlation in terms of both percentage and IRS score. The

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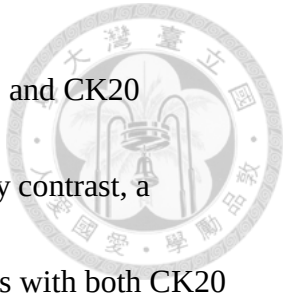

percentage of GATA3 staining tended to be higher than that of CK20, and CK20 staining was stained on GATA3-positive tumor cells in most cases. By contrast, a negative correlation was observed between CK20 and CK5/6. In cases with both CK20 and CK5/6 reactivity, the staining patterns of these two markers were generally reciprocal. Although they were not completely mutually-exclusive, CK20 tended to be stained on CK5/6-negative tumor cells and vice versa. **Figure 5A** illustrates two examples of such a reciprocal pattern. Of the 21 (23.08%) tumors with minimal (1%–9%) CK5/6 staining, 5 (5.49%) demonstrated basal alignment in the aggregates of tumor cells. Because the case number was limited, analyzing the biological significance of such a pattern is unfeasible.

Similar to CK20, GATA3 showed negative correlation with CK5/6 expression. However, coexpression of GATA3 and CK5/6 was observed in 44 (48.35%) cases; of them 13 (14.29%) had diffuse coexpression (staining in >80% of tumor cells for both markers). Two examples of GATA3/CK5/6 coexpression are illustrated in **Figure 5B**. In the 13 double-positive cases, 11 (84.62%) showed completely absent or minimal staining for CK20. Meanwhile, double-negative tumors for GATA3 and CK5/6 accounted for 3 (3.30%) of the 91 cases, and only one of them showed complete absence for each marker.

3.6 Association of Ki-67 index with GATA3, CK20, CK5/6 and p53 expression in

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MIBC

As presented in **Table 6**, Ki-67 indices were significantly correlated with staining percentages of GATA3, CK20, and CK5/6. We further categorized each of the latter three markers into three groups (negative, partial, and diffuse) and compared their difference through Ki-67 indices. Differences in Ki-67 indices was also compared among each group of the p53 score. The results are summarized in **Table 7**. In brief, tumors with diffuse GATA3 staining had significantly lower Ki-67 indices. By contrast, high Ki-67 indices were observed in negative CK20 and diffuse CK5/6 cases. The difference between negative and partial staining groups of GATA3 ($p = 0.616$) or CK5/6 ($p = 0.565$) was nonsignificant. Similarly, no difference was observed between cases with partial and diffuse CK20 reactivity ($p = 0.986$).

The association between p53 score and the Ki-67 index was more complex. Absence of p53 staining (score 0) was associated with a significantly higher Ki-67 index compared with the partial staining group (score 1, $p = 0.004$). In addition, the score 2 group had a significantly lower Ki-67 index than the score 3 group ($p < 0.001$), but no difference was found between the score 1 and 2 groups ($p = 0.079$). In addition, no difference was shown between the p53-absent (score 0) and score 3 groups ($p = 0.164$). These findings were consistent with the definition of aberrant p53 expression in ovarian carcinoma [41]. Notably, 1 (1.1%) case in our cohort showed diffuse

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cytoplasmic staining with variable nuclear intensity (score 4). This pattern was found to be associated with *TP53* mutation in a previous study on ovarian carcinoma [49] and considered p53-aberrant. Based on this definition, tumors with aberrant p53 staining had significantly higher Ki-67 indices ($p< 0.001$). No difference in GATA3, CK20, or CK5/6 expression was noted between p53-aberrant and non-p53-aberrant tumors.

3.7 Intratumoral heterogeneity in MIBC

In case RC01-22, a minor component (2% of total tumor area) with significantly different morphology was observed in the tumor. Although the major part showed considerable squamous differentiation, this minor component had usual histology of UC with heavy lymphocytic infiltration. As for the IHC markers, the major part expressed a typical basal/squamous profile of diffuse CK5/6 staining, minimal GATA3 reactivity, and a non-aberrant pattern of p53. By contrast, the minor component was partially positive for both GATA3 and CK5/6 with diffusely strong reactivity to p53 (**Figure 6**). This patient demonstrated tumor recurrence after radical cystectomy, but the diagnosis of recurrence was based on radiologic images without acquisition of tissue specimens. For analytical purposes, the IHC profile of the major part was used for this case. No other tumor with apparent intratumoral heterogeneity was noted in our MIBC study cohort.

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3.8 Prognostic significance of the IHC markers in MIBC

Among the IHC markers (GATA3, CK20, CK5/6, p53 and Ki-67), only GATA3 demonstrated significant correlation with clinical outcomes. In the 78 patients without neoadjuvant chemotherapy, higher percentage of GATA3 staining was associated with a significantly better RFS in both univariate ($p = 0.008$) and multivariate ($p = 0.002$) analysis by using Cox regression (**Table 8**). Analysis by IRS revealed similar results (**Table 9**). In the Kaplan-Meier plot, gradual difference in RFS was present among cases with diffuse, partial, and negative GATA3 staining ($p = 0.002$). The other significant prognostic parameters included the T stage (for RFS) and the presence of nodal metastasis (for DSS and RFS). The Kaplan–Meier plots for GATA3, T stage, and nodal metastasis are depicted in **Figure 7**.

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4. Discussion

4.1 Biological significance of *TERT* promoter mutation in papillary urothelial neoplasm of low malignant potential

In human oncology, urothelial neoplasm is one of the neoplasms with well-established pathogenetic models. At the genetic level, noninvasive papillary urothelial neoplasms can be roughly divided into 2 groups: a genetically stable low-grade group and a genetically unstable high-grade group. High-grade urothelial neoplasms are characterized by *TP53* mutations and exhibit aggressive biological behavior [2, 51–53]. By contrast, low-grade urothelial neoplasms are less likely to result in mortality. According to the current WHO tumor classification, low-grade neoplasms include totally benign urothelial papillomas (including exophytic papillomas and more common inverted papillomas), PUNLMP, and low-grade NIPUC [2]. Although PUNLMP and low-grade NIPUC are considered indolent tumors, they are not clinically insignificant due to their frequent recurrence and the potential of progression to high-grade NIPUC or invasive carcinoma. Between them, patients with low-grade NIPUC are more likely to suffer from tumor recurrence than those with PUNLMP [3], and our results are consistent with this general finding.

According to the current WHO criteria, classification of low-grade urothelial neoplasms depends only on histological characteristics without the requirement of

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genetic studies [2]. Some researchers have proposed the concept of "genetic grading" for urothelial neoplasms [17, 54], but the complexity and cost of genetic studies may hinder the feasibility of genetic grading in clinical practice. The results of our study indicated that PUNLMP with wild-type *TERT* promoter had a lower recurrence rate. In contrast, PUNLMP cases with *TERT* promoter mutations had significantly higher risks of recurrence, which were similar to those in the low-grade NIPUC group. These results suggest that PUNLMP cases can be stratified by *TERT* promoter mutation status for prognostic purposes, and investigation of this mutation status may be of value for clinical decision when treating patients with PUNLMP.

Compared with PUNLMP, our study did not show prognostic significance for *TERT* promoter mutations in patients with low-grade NIPUC. Similarly, a study of urothelial bladder cancers by Allory *et al.* found no association of the *TERT* promoter mutation status with the clinical outcome [7]. Rachakonda *et al.* reported that *TERT* promoter mutations are associated with poor survival and a higher recurrence rate in bladder cancer patients without the common polymorphism rs2853669, which is located in a preexisting Ets2 binding site in the *TERT* promoter. Such an association was not found in patients with that polymorphism. The possible mechanism underlying the association is that *TERT* promoter mutations affect the clinical outcome by creating de novo Ets/TCF binding sites, and that common polymorphism may modify such effects

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[55]. However, Rachakonda *et al.* did not include cases of PUNLMP, and further investigation is necessary to explain the possible mechanisms of the effects of *TERT* promoter mutations in PUNLMP.

Our results showed a high prevalence (76%) of *HRAS* mutation in inverted papillomas. A previous study also showed *HRAS* mutations in 10 (91%) of 11 inverted papillomas, and the authors postulated that isolated *RAS* mutations might induce cellular senescence and result in a benign clinical course [22]. In our cohort, the three cases of PUNLMP with *HRAS* mutations did not show coexistent *TERT* promoter or *FGFR3* gene mutations. Two of them showed partially inverted growth patterns, and none of these three tumors recurred after resection. Although these results were inconclusive due to the limited case number, PUNLMPs with *HRAS* mutations might have similar characteristics to the benign inverted papillomas. By contrast, two of the five low-grade NIPUCs with mutated *HRAS* also showed *TERT* promoter mutation, and one additional case had coexistent *HRAS* and *FGFR3* mutations in our study. None of these tumors had inverted growth in histology. The different biologic significance of *HRAS* mutations in these low-grade urothelial neoplasms may worth further investigation.

Back to the issue of *TERT* promoter mutation, comparing our results with previous results is potentially problematic. For survival analysis, many studies evaluating *TERT* promoter mutations in bladder tumors have included cases with different grades or

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stages [7, 8, 55]. In our study, the prognostic value of *TERT* promoter mutations fell to an equivocal level if PUNLMP and low-grade NIPUC cases were mixed (p = .052). Moreover, and probably more important, the actual cut-off points of histological classification are subject to interobserver variation [56, 57]. In fact, all of the low-grade NIPUC cases in our study were originally diagnosed as PUNLMP or even inverted papilloma. Although our study found no *TERT* promoter mutation in any case of inverted papilloma, Cheng *et al.* reported *TERT* promoter mutations in 15% (4/26) cases of inverted papillomas [8]. Such discrepancy might result from the ethnic factor or other causes; however, the potential interobserver variation may influence the accuracy of metanalysis among these studies.

There are three potential limitations in our study. First, tumor progression was uncommon in our study cohort and thereby limiting the analysis of PFS. Secondly, we met technical problems about *TERT* promoter mutation analysis in three cases, including one PUNLMP. This incident did not affect the statistical significance in our study, but it might hamper the clinical application of *TERT* promoter mutation analysis. Finally, to minimize the potential heterogeneity in our study cohort, we only selected the primary (initial) specimen for each patient with tumor recurrence(s). The recurrent tumors might exhibit different mutation patterns from their primary counterparts, but investigations of such difference and possible clinical importance were beyond our

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scope in this study.

4.2 Biological significance of GATA3, cytokeratin 20, cytokeratin 5/6 and p53 expression in MIBC

GATA3 is a transcription factor useful in histological diagnosis for UC [35, 58, 59]. It is also recognized as a marker of luminal subtype(s) in the bladder cancer according to recent research [20, 24, 33]. In this study, we noted that tumors with decreased GATA3 staining had significantly higher Ki-67 proliferative indices. In addition, IHC staining for GATA3 was correlated with a clinical outcome in chemotherapy-naïve patients with MIBC. Cases with diffuse GATA3 staining had the best outcome, and a minor proportion (12.1%) of GATA3-negative tumors were prone to early recurrence with a borderline trend of worse DSS. The prognostic significance of GATA3 was independent to stage and nodal metastasis in RFS. These findings are compatible with the relatively aggressive behavior observed in the BASQ tumors [24, 26, 32, 33].

The BASQ phenotype described by Lerner *et al*. included positive CK5/6 and negative GATA3 in IHC staining [31]. However, we found it difficult to define the actual BASQ subgroup in our study cohort. In this study, diffusely strong CK5/6 staining was not necessarily associated with negative GATA3. The association of these two markers with the Ki-67 index might aid in understinaing this problem. Difference in the Ki-67 index was significant at a 80% cut-off for GATA3, but the tumors with

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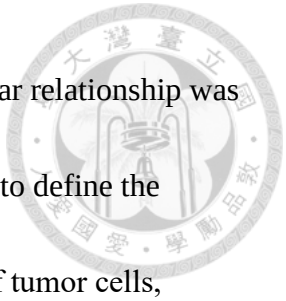

negative and partial GATA3 staining had similar Ki-67 indices. Similar relationship was observed between CK5/6 and Ki-67 index. If we use the 80% cut-off to define the BASQ phenotype (CK5/6 and GATA3 staining in >80% and ≤80% of tumor cells, respectively), this subgroup would account for 20 (22.0%) cases in our MIBC cohort. Similar to GATA3 alone, the BASQ cases had a worse RFS ($p = 0.027$) compared with others in the chemotherapy-naïve group. Moreover, CK5/6 alone was not prognostically significant regarding RFS. From the view of prognostication, decrease in GATA3 expression may be a more important component than diffuse CK5/6 staining in the BASQ phenotype.

Although GATA3 and CK5/6 demosntrated significantly negative correlation in our study, staining for these two markers showed certain overlap in 44 (48.4%) MIBC cases. Furthermore, diffuse coexpression was not rarely encountered (14.29%) in our study. Such a diffuse coexpression phenomenon has not been well-described in previous studies, and simultaneously high GATA3 and CK5/6 expression on protein level were uncommon or even absent in the studies by Sjödahl [60] and Hodgson [36]. Dadhania *et al.* showed some cases with overlap in GATA3 and CK5/6 staining; however, tumors with >80% positivity for both markers were absent in their data [33]. A possible explanation to this phenomenon is the ethnic difference. Further research based on Asian population is warranted to confirm these findings.

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The prognostic significance of GATA3 in bladder UC was controversial in previous studies [34–37]. Miyamoto *et al.* reported that loss of GATA3 expression predicted poor prognosis for patients with MIBC [34], but three other studies showed that GATA3 expression had no significant influence on either DSS or RFS [35–37]. In addition to ethnicity, three possible reasons can explain this discrepancy. First, we used whole slides of the tumor specimens for IHC staining instead of tissue microarrays (TMAs), which was the case in previous studies [35–37]. Partial staining might lead to false-negative results in TMA, and this could potentially affect the significance in survival analyses. Second, Kollberg *et al.* included 66 (17%) stage T1 tumors along with MIBC [37], which may influence the results. Finally, in contrast to previous studies [20, 32], the molecular subtypes in their cohort was not associated with clinical outcomes [37]. The potential difference in the underlying population might result in such discrepancy.

As CK20 and CK5/6 are well-established markers related to molecular subtypes [24, 31, 33, 60], it may appear unreasonable that these markers did not have prognostic significance. However, the molecular subtypes were defined through hierarchical analysis with a large panel of markers. Previous studies revealed that the molecular subtypes were associated with clinical outcomes [24, 31, 33, 60], but each marker related to the subtypes was not necessarily significant in clinical outcomes. Kollberg *et*

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al. also showed no prognostic significance in any single subtype-associated marker [37]. Therefore, the association of GATA3 expression and clinical outcome merits further investigation to prove its value in clinical management.

The interpretation of p53 IHC staining is also noteworthy. Although we could not find the prognostic significance of p53 staining, the differences in the Ki-67 index suggested that using the same criteria as those used for ovarian carcinoma would be more suitable when interpreting p53 staining in bladder cancer. Hodgson *et al.* discovered that null staining of p53 should be considered an aberrant staining pattern [44]. Our study further revealed the potential importance of intensity in the diffuse nuclear staining group. Tumors with diffuse nulcear staining but variable intensity for p53 (score 2) did not have higher Ki-67 indices than did the partial staining (score 1) group. However, the differences in the variable-intensity (score 2) and strong-intensity (score 3) groups were significant. Based on our findings, we plan to correlate these criteria with the *TP53* gene status and verify their superiority over the traditional overexpression criteria for bladder cancer in the future.

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5. Conclusion

In conclusion, the first part of our study provided the evidence that the *TERT* promoter mutation is related to a higher risk of recurrence in PUNLMP cases. Such an association was not observed in low-grade NIPUC, although the rates of the *TERT* promoter mutation were similar between these two entities. Furthermore, the risk of recurrence in PUNLMP cases with *TERT* promoter mutations was similar to that of low-grade NIPUC cases. Mutation study of the *TERT* promoter may facilitate risk stratification and assist the clinical decision when treating patients with PUNLMP of the urinary bladder.

In the second part of our study, decrease in GATA3 staining was significantly associated with high proliferative activity and poor clinical outcome in MIBC. IHC staining for GATA3 might facilitate in risk stratification in patients with MIBC receiving radical cystectomy. Combining CK5/6 and GATA3 for prognostic stratification has potential problems because coexpression is common. Defining the "aberrant" p53 staining by using the criteria for ovarian carcinoma (complete absence,strong nuclear reactivity in ≥60% of tumor cells, or diffuse cytoplasmic staining) may be more suitable than the traditional overexpressionconcept; however, our results did not demonstrate the direct association of p53 expression with clinical outcome.

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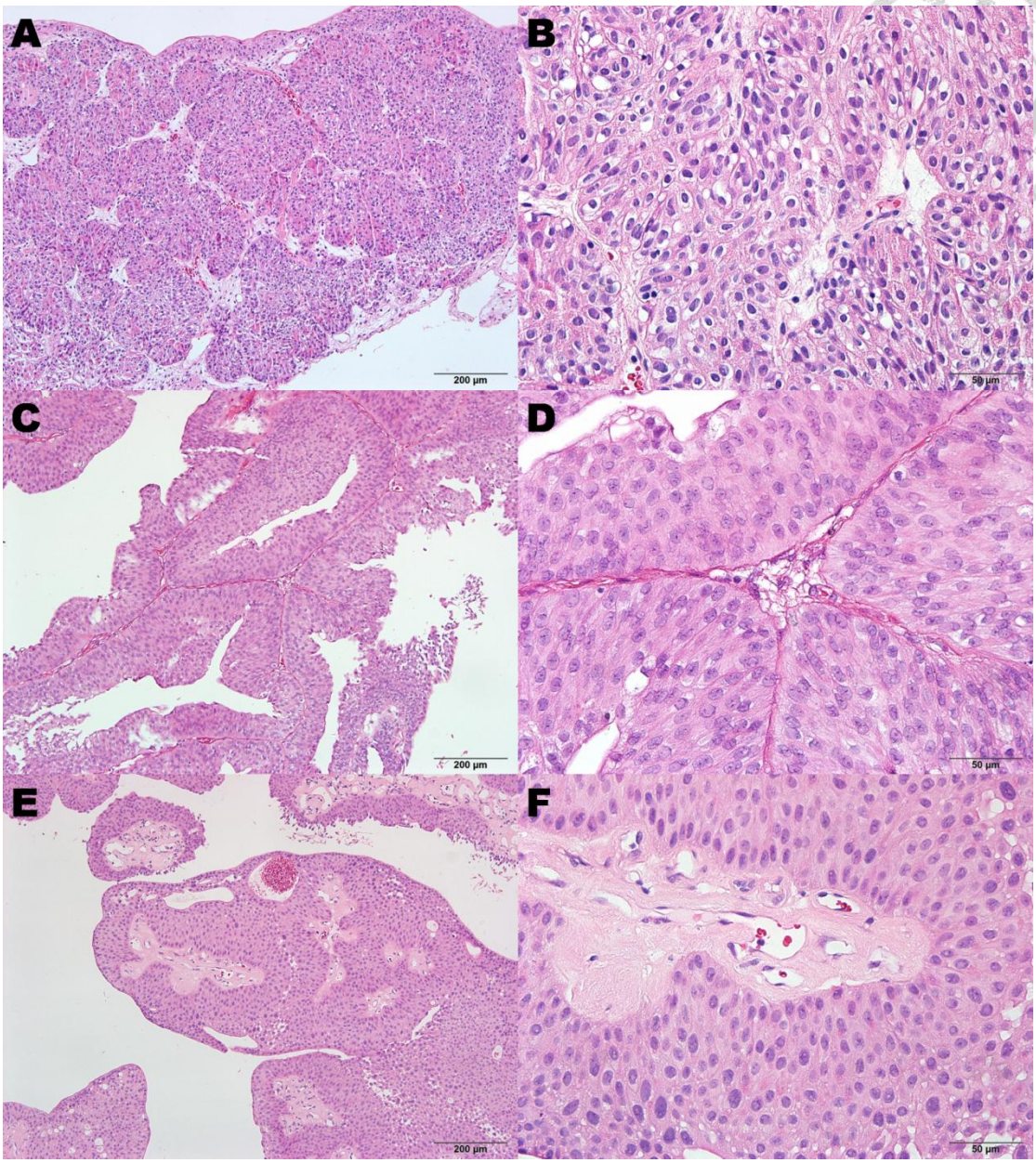

Figure 1. Representative histological images of inverted papilloma (**A** and **B**), PUNLMP (**C** and **D**), and low-grade NIPUC (**E** and **F**) (Original magnification: **A**, **C**, and **E**: 100×; **B**, **D,** and **F**: 400×).

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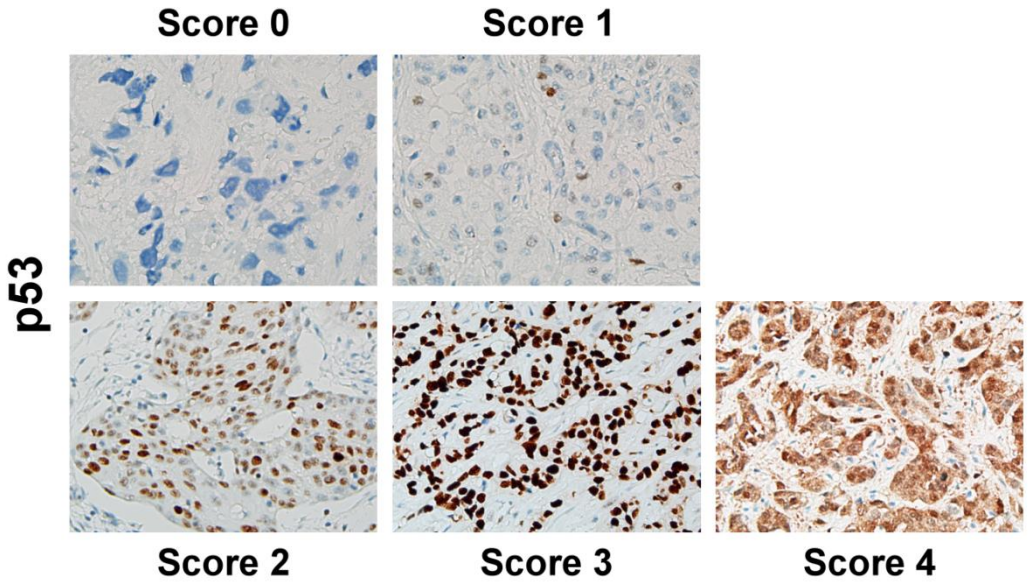

Figure 2. Scoring criteria for p53 IHC staining. **Score 0**: no nuclear staining in tumor cells; **score 1**: nuclear staining in 0%–50% of tumor cells; **score 2**: nuclear staining in ≥50% of tumor cells with strong nuclear reactivity in <60% of tumor cells; **score 3**: strong nuclear staining in ≥60% of tumor cells; **score 4**: diffuse and strong cytoplasmic staining without nuclear pattern of score 3 (original magnification: 200×).

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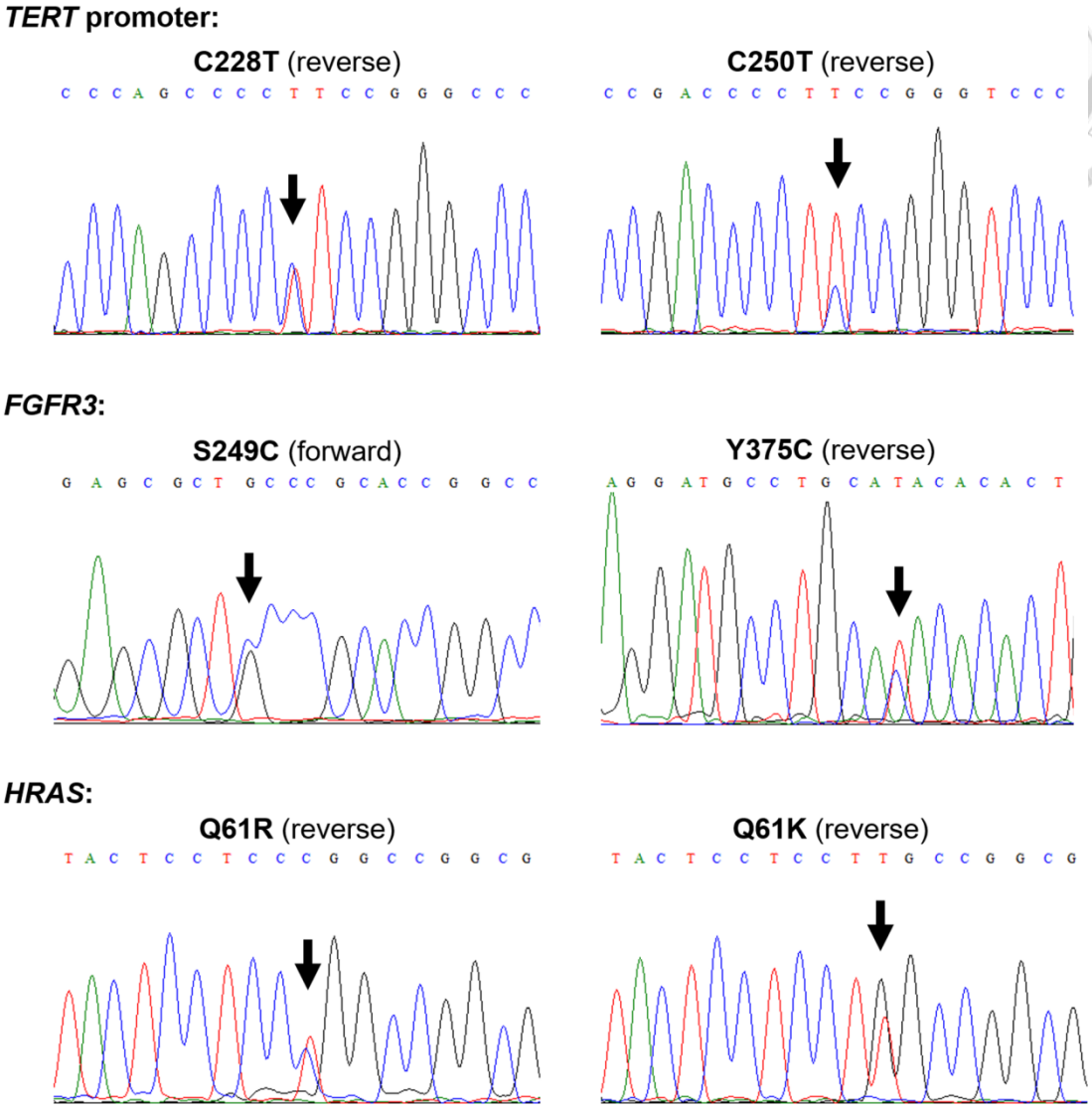

Figure 3. Common point mutations in the *TERT* promoter region (upper panel), *FGFR3* gene (middle panel), and *HRAS* gene (lower panel).

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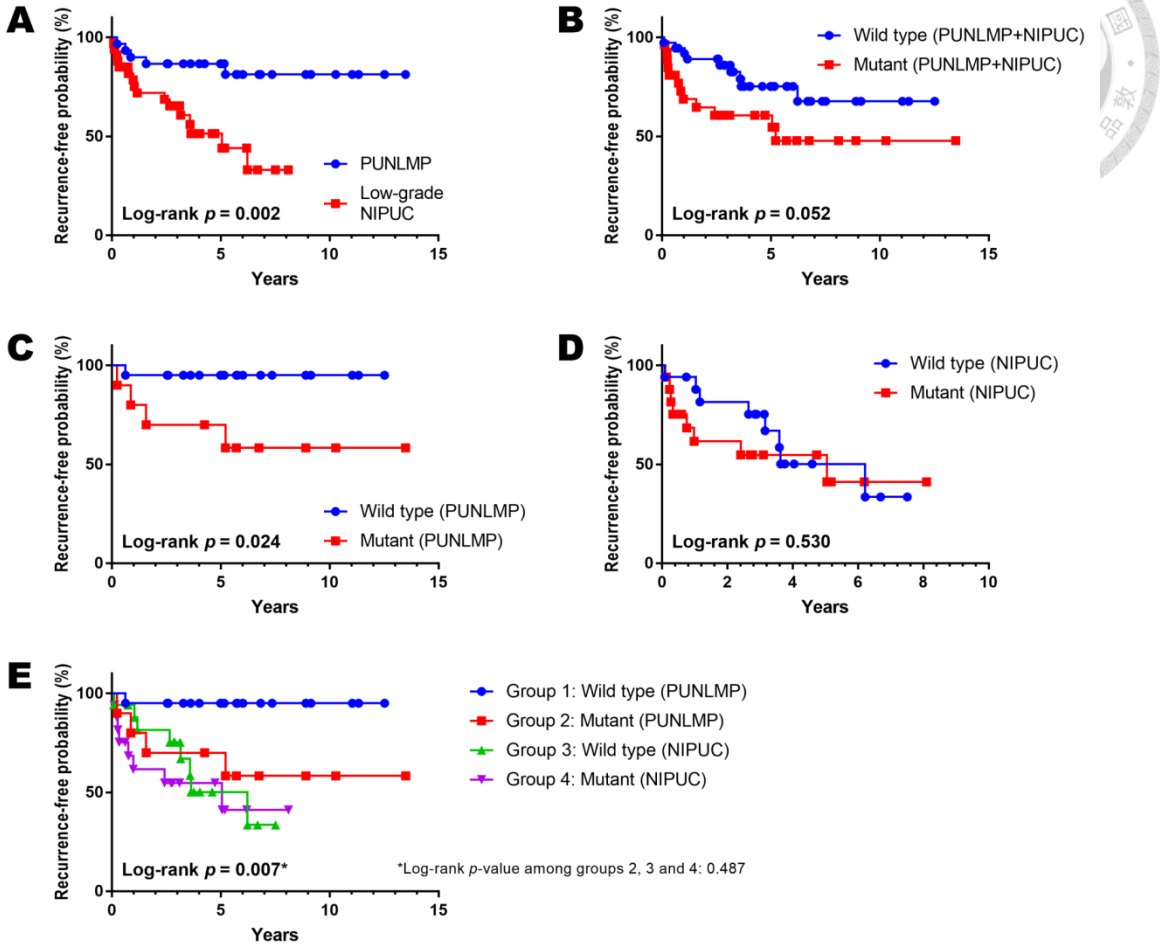

Figure 4. Kaplan–Meier curves of RFS in patients with low-grade noninvasive papillary urothelial neoplasms. **A**, PUNLMP versus low-grade NIPUC; **B** to **D**, non-mutants versus mutants of *TERT* promoter region in all cases of PUNLMP and low-grade NIPUC (**B**), PUNLMP only (**C**) and low-grade NIPUC only (**D**); **E**, comparison among four groups determined by histological entity and *TERT* promoter mutation status.

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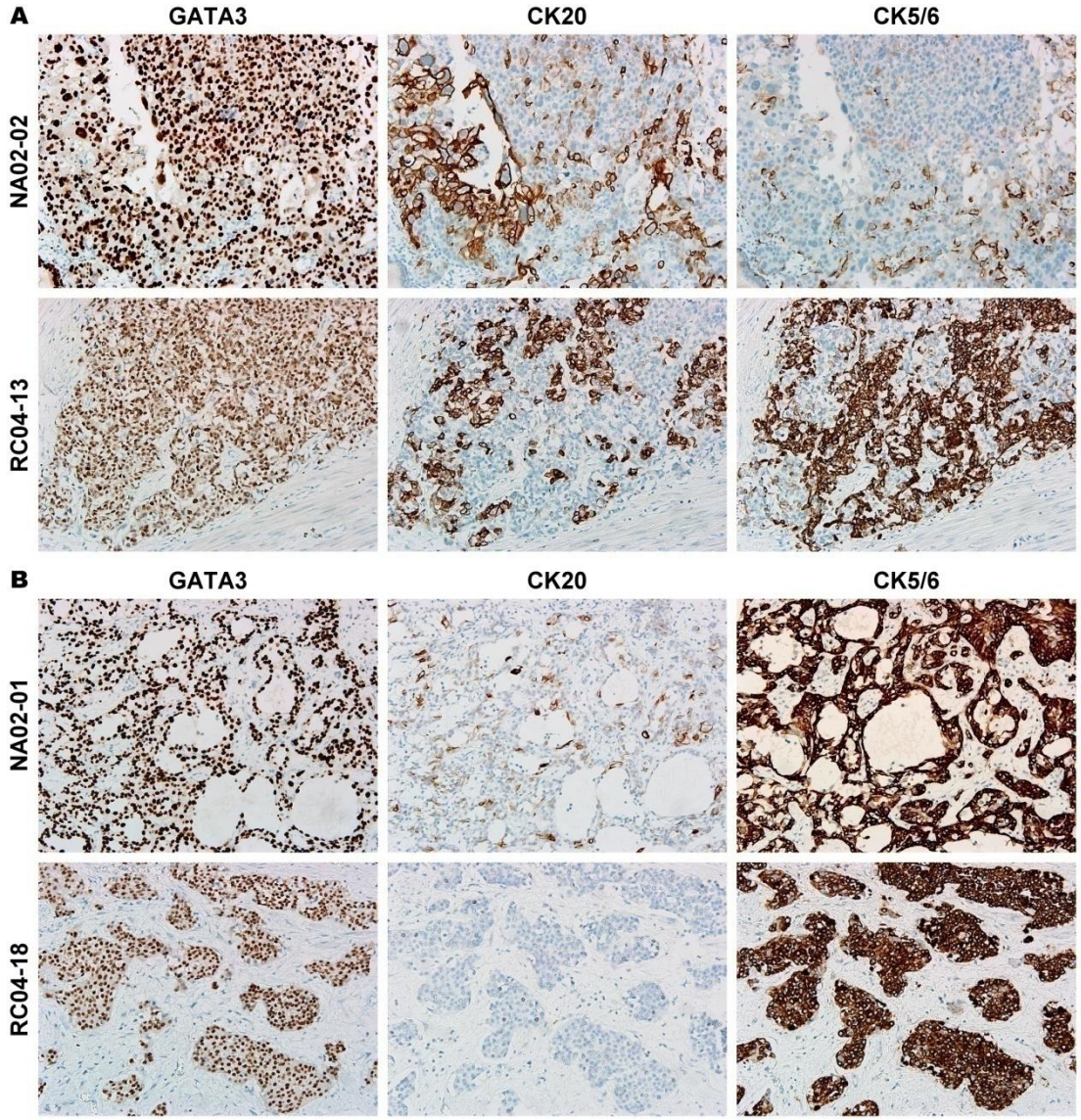

Figure 5. (A) Two examples showing reciprocal staining patterns of CK20 and CK5/6 in MIBC. (B) Two examples showing diffuse coexpression of GATA3 and CK5/6 (original magnification: 200×).

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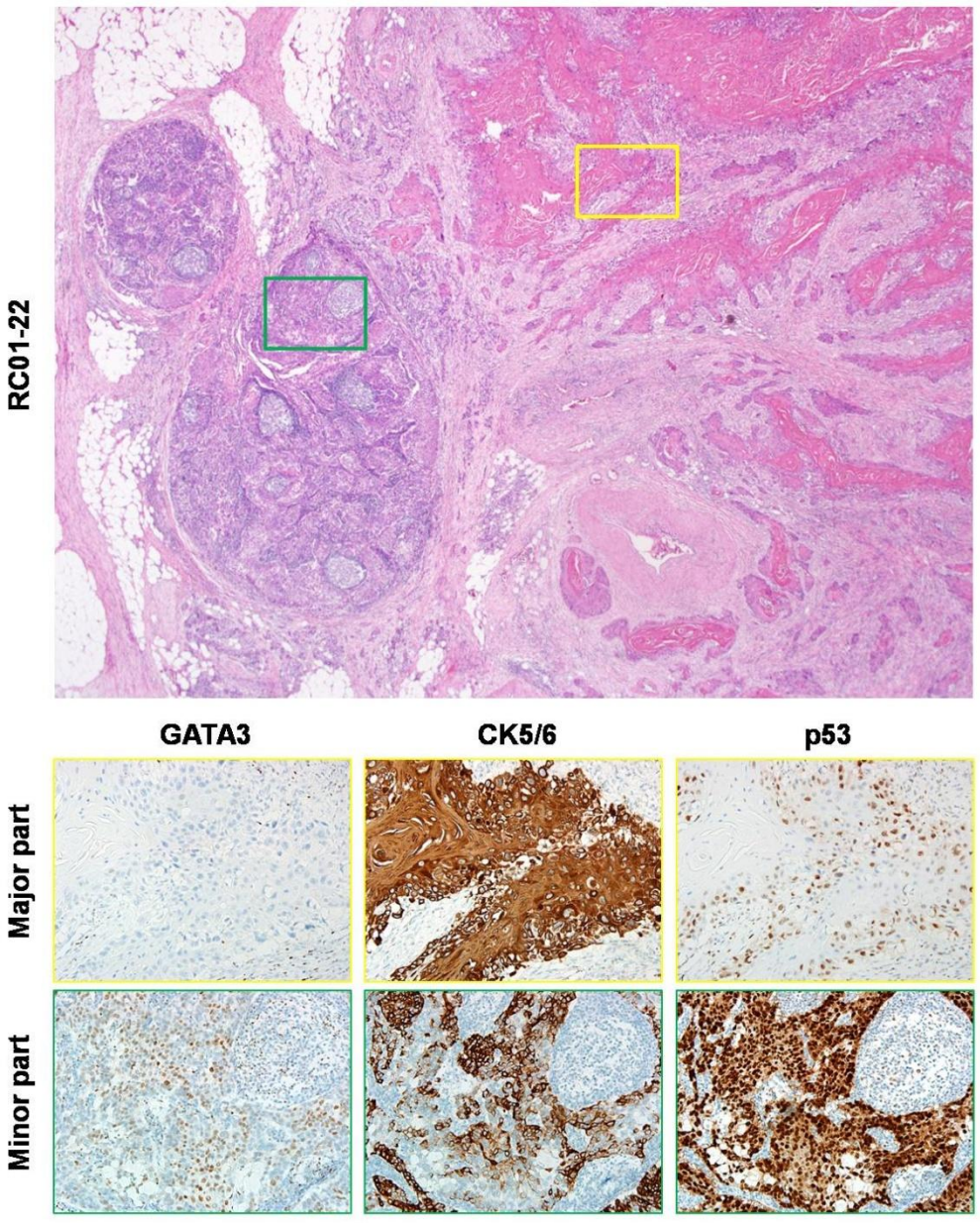

Figure 6. Intratumoral heterogeneity in case RC01-22. The major component demonstrates squamous differentiation with diffuse CK5/6 staining, whereas the minor component is characterized by heavy lymphocytic infiltration and aberrant p53 staining.

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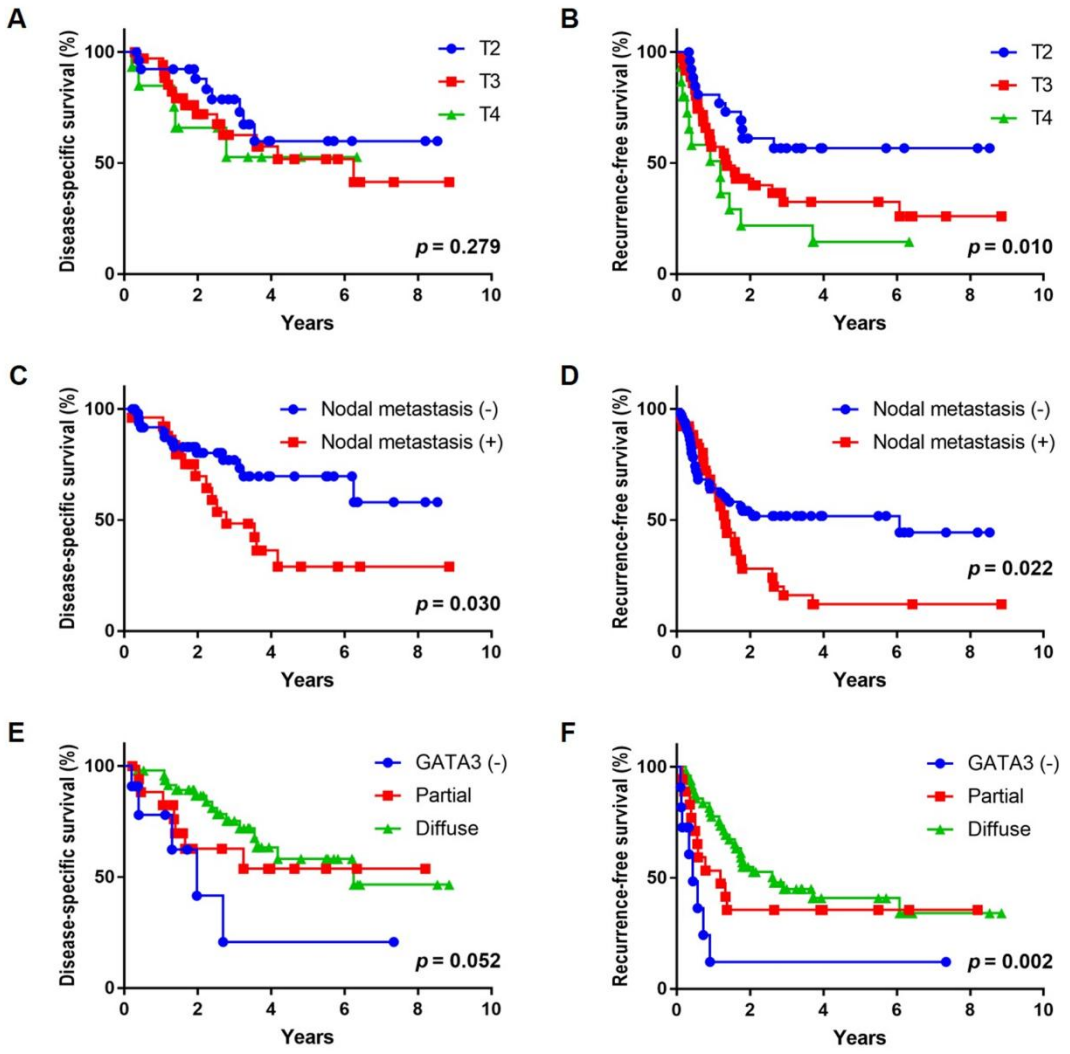

Figure 7. Kaplan–Meier curves regarding the 78 patients with chemotherapy-naïve MIBC. (A, B) DSS and RFS stratified by T stage. (C, D) DSS and RFS stratified by nodal status. (E, F) DSS and RFS stratified by GATA3 expression.

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Table 1. Primer sequences for mutation analysis

Gene	Region	Primer sequence	PCR product	Reference
TERT	Promotor	Forward: 5′ GCCGGGCTCCCAGTGGATTCG 3′ Reverse: 5′ GCTTCCCACGTGCGCAGCAGGA 3′	220 bps	[61]
FGFR3	Exon 7	Forward: 5' AGTGGCGGTGGTGGTGAGGGAG 3' Reverse: 5' TGTGCGTCACTGTACACCTTGCAG 3'	120 bps	[10]
	Exon 10	Forward: 5' CAGGCCAGGCCTCAACGCCC 3' Reverse: 5' AGGCCTGGCGGGCAGGCAGC 3'	270 bps	[13]
	Exon 15	Forward: 5' AGTGCATCCACAGGGACCTG 3' Reverse: 5' GTGTGGGAAGGCGGTGTTG 3'	200 bps	[62]*
HRAS	Exon 3	Forward: 5' TGGGGAGACGTGCCTGTTGG 3' Reverse: 5' GGTTCACCTGTACTGGTGGA 3'	157 bps	[63]*

*With modifications

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Table 2. Immunoreactive score (IRS) by Remmele and Stegner's criteria

A: Percentage of positive cells	**B**: Intensity of staining
0: no positive cells	**0**: no color reaction
1: <10% positive cells	**1**: mild reaction
2: 10–50% positive cells	**2**: moderate reaction
3: 51–80% positive cells	**3**: intense reaction
4: >80% positive cells	
Final IRS (A × B): 0–12	

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Table 3. Clinical characteristics and mutation status of patients with inverted papilloma, PUNLMP, and low-grade NIPUC

Gene	**Inverted papilloma (A)** (n=21)	**PUNLMP (B)** (n=30)	**Low-grade NIPUC (C)** (n=34)	***P*-value**	
				A vs. (B+C)	**B vs. C**
Age (year)				0.465	0.237
Median (range)	56 (39–81)	57 (28–82)	65 (34–86)		
Sex				0.044	0.772
Male	20	22	26		
Female	1	8	8		
***TERT* promoter**				<0.001	0.178
Wild type	21 (100%)	20 (67%)	17 (50%)		
Mutant	0 (0.00%)	10 (33%)	17 (50%)		
FGFR3				0.009	0.104
Wild type	19 (90%)	21 (70%)	17 (50%)		
Mutant	2 (10%)	9 (30%)	17 (50%)		
HRAS				<0 .001	0.570
Wild type	5 (24%)	27 (90%)	29 (85%)		
Mutant	16 (76%)	3 (10%)	5 (15%)		

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Table 4. Detailed mutation status of inverted papilloma, PUNLMP, and low-grade NIPUC

Gene	Inverted papilloma **(A)** (n=21)	PUNLMP (B) (n=30)	Low-grade NIPUC (C) (n=34)
***TERT* promoter**			
Wild type	21 (100%)	20 (67%)	17 (50%)
Mutant	0 (0%)	10 (33%)	17 (50%)
C228A		1 (3%)	1 (3%)
C228T		7 (23%)	12 (35%)
C250T		2 (7%)	4 (12%)
FGFR3			
Wild type	19 (90%)	21 (70%)	17 (50%)
Mutant	2 (10%)	9 (30%)	17 (50%)
S249C (C746G)		6 (20%)	10 (29%)
G372C (G1108T)		1 (3%)	
S373C (A1111T)			1 (3%)
Y375C (A1118G)	2 (10%)	1 (3%)	4 (12%)
K652E (A1954G)			1 (3%)
K652T (A1955T)		1 (3%)	1 (3%)
HRAS			
Wild type	5 (24%)	27 (90%)	29 (85%)
Mutant	16 (76%)	3 (10%)	5 (15%)
Q61K (C181A)	6 (29%)	1 (3%)	
Q61R (A182G)	10 (48%)	2 (7%)	2 (6%)
Q61L (A182T)			3 (9%)

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Table 5. Clinicopathological features of the 91 patients who received radical cystectomy

Variable	Value	Variable	Value
Age (year)		**GATA3**	
Range	39–89	Negative (<10%)	11 (12.1%)
Median	67	Partial (10%–80%)	22 (24.2%)
Sex		Diffuse (>80%)	58 (63.7%)
Male	64 (70.3%)	**CK20**	
Female	27 (29.7%)	Negative (<10%)	52 (57.1%)
Neoadjuvant chemotherapy		Partial (10%–80%)	21 (23.1%)
No	78 (85.7%)	Diffuse (>80%)	18 (19.8%)
Yes	13 (14.3%)	**CK5/6**	
T stage		Negative (<10%)	39 (42.9%)
T2	30 (33.0%)	Partial (10%–80%)	19 (20.9%)
T3	40 (44.0%)	Diffuse (>80%)	33 (36.3%)
T4	21 (23.1%)	**p53 score**	
Lymph node metastasis		0	13 (14.3%)
Absent	60 (65.9%)	1	18 (19.8%)
Present	31 (34.1%)	2	26 (28.6%)
Ki-67 index (%)		3	33 (36.3%)
Range	5.79–96.30	4	1 (1.1%)
Median	56.51		

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Table 6. Correlation among GATA3, CK20, CK5/6, and Ki-67 index

Correlation in percentage (95% confidence interval)

	GATA3	CK20	CK5/6	Ki-67
GATA3	1.000	0.543 (0.375 – 0.677)	-0.459 (-0.611 – -0.274)	-0.321 (-0.499 – -0.117)
CK20		1.000	-0.521 (-0.660 – -0.348)	-0.377 (-0.545 – -0.179)
CK5/6			1.000	0.263 (0.054 – 0.450)
Ki-67				1.000

Correlation in immunoreactive score* (95% confidence interval)

	GATA3	CK20	CK5/6	Ki-67
GATA3	1.000	0.549 (0.381 – 0.682)	-0.413 (-0.574 – -0.220)	-0.333 (-0.509 – -0.130)
CK20		1.000	-0.462 (-0.614 – -0.278)	-0.371 (-0.541 – -0.173)
CK5/6			1.000	0.244 (0.034 – 0.433)
Ki-67				1.000

*Applies to GATA3, CK20 and CK5/6.

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Table 7. Association between Ki-67 and other IHC markers

IHC Marker	Ki-67 index (%)		
	Range	Mean ± SD	*p* value
GATA3			
Negative or partial	15.0–96.3	63.8 ± 23.7	0.006
Diffuse	5.8–92.0	49.5 ± 23.1	
CK20			
Negative	5.8–96.3	61.2 ± 24.2	0.002
Partial or diffuse	8.5–84.1	45.9 ± 21.5	
CK5/6			
Negative or partial	5.8–95.4	48.6 ± 23.6	0.001
Diffuse	8.5–96.3	65.3 ± 21.8	
p53			
Each score*			
0	22.3–87.9	58.9 ± 22.0	
1	5.8–66.9	35.9 ± 19.4	0.004 (0 versus 1)
2	8.5–82.6	47.2 ± 21.2	0.079 (1 versus 2)
3	14.3–96.3	69.1 ± 21.1	<0.001 (2 versus 3)
			0.164 (0 versus 3)
Non-aberrant versus aberrant			
Non-aberrant (1 or 2)	5.8–82.6	42.5 ± 21.0	<0.001
Aberrant (0, 3, or 4)	14.3–96.3	66.0 ± 21.4	

Abbreviation: SD, standard deviation

*Score 4 (cytoplasmic) pattern was present in only one case and not listed here.

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Table 8. Cox regression analysis of clinical outcomes in the 78 chemotherapy-naïve patients with MIBC using tumor staging and percentages of IHC staining

Variable	Univariate		Multivariate	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
RFS				
T stage (T2 reference)	1.837 (1.230–2.745)	0.003	1.697 (1.083–2.658)	0.021
Nodal metastasis (N1–3 vs. N0)	1.944 (1.091–3.463)	0.024	1.821 (0.968–3.424)	0.063
GATA3 (by percentage)	0.342 (0.156–0.751)	0.008	0.270 (0.120–0.608)	0.002
CK20 (by percentage)	0.593 (0.276–1.274)	0.181		
CK5/6 (by percentage)	1.580 (0.818–3.051)	0.173		
p53 (aberrant vs. non-aberrant)	1.053 (0.594–1.866)	0.860		
Ki-67 index	0.713 (0.199–2.555)	0.603		
DSS				
T stage (T2 reference)	1.343 (0.786–2.294)	0.281		
Nodal metastasis (N1–3 vs. N0)	2.257 (1.059–4.811)	0.035		
GATA3 (by percentage)	0.414 (0.149–1.147)	0.090		
CK20 (by percentage)	0.597 (0.208–1.718)	0.339		
CK5/6 (by percentage)	1.443 (0.608–3.428)	0.406		
p53 (aberrant vs. non-aberrant)	1.249 (0.583–2.675)	0.567		
Ki-67 index	0.442 (0.084–2.321)	0.335		
OS				
T stage (T2 reference)	1.460 (0.887–2.403)	0.136		
Nodal metastasis (N1–3 vs. N0)	1.702 (0.838–3.457)	0.142		
GATA3 (by percentage)	0.484 (0.183–1.281)	0.144		
CK20 (by percentage)	0.539 (0.197–1.471)	0.227		
CK5/6 (by percentage)	1.663 (0.746–3.709)	0.214		
p53 (aberrant vs. non-aberrant)	1.214 (0.597–2.467)	0.592		
Ki-67 index	0.390 (0.083–1.824)	0.232		

Abbreviations: HR, hazard ratio; CI, confidence interval

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Table 9. Cox regression analysis of clinical outcomes in the 78 chemotherapy-naïve patients with MIBC using tumor staging and immunoreactive scores (IRS) of GATA3, CK20 and CK5/6 staining

Variable	Univariate		Multivariate	
	HR (95% CI)	*p* value	HR (95% CI)	*p* value
RFS				
T stage (T2 reference)	1.837 (1.230–2.745)	0.003	1.639 (1.053–2.551)	0.029
Nodal metastasis (N1–3 vs. N0)	1.944 (1.091–3.463)	0.024	1.806 (0.962–3.391)	0.065
GATA3(by IRS)	0.911 (0.849–0.977)	0.01	0.897 (0.834–0.965)	0.004
CK20 (by IRS)	0.963 (0.905–1.025)	0.238		
CK5/6 (by IRS)	1.034 (0.973–1.098)	0.283		
DSS				
T stage (T2 reference)	1.343 (0.786–2.294)	0.281		
Nodal metastasis (N1–3 vs. N0)	2.257 (1.059–4.811)	0.035		
GATA3 (by IRS)	0.924 (0.844–1.013)	0.091		
CK20 (by IRS)	0.967 (0.889–1.052)	0.438		
CK5/6 (by IRS)	1.042 (0.963–1.127)	0.309		
OS				
T stage (T2 reference)	1.460 (0.887–2.403)	0.136		
Nodal metastasis (N1–3 vs. N0)	1.702 (0.838–3.457)	0.142		
GATA3 (by IRS)	0.934 (0.858–1.017)	0.117		
CK20 (by IRS)	0.956 (0.882–1.036)	0.270		
CK5/6 (by IRS)	1.047 (0.973–1.126)	0.222		

Abbreviations: HR, hazard ratio; CI, confidence interval

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