# Mutations of Fibroblast Growth Factor Receptor 3 Gene (FGFR3) in Transitional Cell Carcinoma of Urinary Bladder in Thai Patients [Revision-2a]†

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† *A part of this work has been presented and published in the proceedings of the ASEAN+3 Graduate Research Congress 2012, March, 2012, Chiang Mai, Thailand.*

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***Objective:*** *Determine the incidence of FGFR3 mutations in Thai patients with bladder transitional cell carcinoma (TCC), and evaluate their correlation with pathological characteristics.*
***Material and Method:*** *One hundred twenty two frozen tissue samples from TCC patients were analyzed for mutations in exons 7, 10, and 15 of FGFR3 by polymerase chain reaction and direct DNA sequencing.*
***Results:*** *FGFR3 mutations were detected in 22 of 122 cases (18%) studied, all of which were found within previously identified hotspots, including S249C (13 cases; 59%) and R248C (4 cases; 18%) in exon 7, and Y375C (5 cases; 23%) in exon 10, but no mutations in exon 15. Sixty-five patients (53%) were categorized as non-muscle-invasive TCC (pTa-pT1). The incidence of mutations is significantly higher in non-muscle-invasive tumors (28%) compared to the muscle-invading group (7%) ($p<0.01$). Patients with grade (G) 1 TCC have significantly higher mutation frequency (40%) compared to other grades (4%) ($p<0.01$). When T stage and grade were considered together, mutations were most commonly found in Ta-T1/G1 TCC (18/45 cases, 40%). Mean follow-up period was 45.1 months. Two-year and four-year overall survival (OS) was 70% and 56% respectively. Three-year OS in non-muscle-invasive TCC (80%) is significantly higher than that of muscle invading TCC (41%) ($p<0.01$). However, three-year OS in cases with an FGFR3 mutation (73%) is not significantly different from cases without a mutation (61%). In 16 cases with an FGFR3 mutation and recurrent disease, no mutations were detected in metachronous disease.*
***Conclusion:*** *The overall incidence of FGFR3 mutations in Thai patients with TCC was lower than similar reports from other ethnic groups. In the presented cases, although FGFR3 mutations were frequently detected in low-grade, non-muscle-invasive TCC, identical mutation was not conserved in metachronous disease, thereby precluding the use of this marker in detection of tumor recurrence.*

***Keywords:*** *Urinary bladder cancer, FGFR3, Transitional cell carcinoma*

***J Med Assoc Thai 2013; 96 (8): 976-83***
***Full text. e-Journal:*** *http://jmat.mat.or.th*

Urinary bladder cancer is a common malignancy worldwide. The global age standardized incidence rate (ASR) is 10.1 per 100,000 in males and 2.5 per 100,000 in females[1]. In Thailand, ASR of bladder cancer is 4.6 and 1.0 cases per 100,000 in males and females respectively[2]. The most common form of bladder cancer arises from urothelial cells, known as transitional cell carcinoma (TCC). The tumor can be further subcategorized according to its histopathological features into superficial, non-invasive TCC, which usually shows a well-differentiated (low-grade) histology, and invasive TCC, which tends to have a high histologic grade[3]. Whether muscle-invasive TCC develops from a superficial disease remains unresolved. Molecular genetic studies have suggested that these 2 categories of TCC arise via distinct pathways[4,5]. A cDNA microarray

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study also suggested that each category displays a distinct genetic expression profile[6].

An activating mutation in fibroblast growth factor receptor 3 gene (FGFR3) is associated with TCC tumor development[5]. FGFR3 encodes a 115 kD a plasma membrane receptor protein involved in cellular proliferation and differentiation[7]. Studies have detected mutations of FGFR3 in 50 to 70% of low-grade disease and <10% in muscle-invasive subtype[8,9]. This evidence makes FGFR3 an interesting candidate as a potential disease marker for superficial TCC, and as a molecular target for treatment. However, before this marker can be bought into clinical use, there are two points that need to be clarified: consistency of the mutation frequency throughout various ethnic groups, and the degree of conservation of the mutated gene in recurrent or metastatic disease.

In the present study, the authors examined mutations of FGFR3 in Thai patients with TCC and evaluated their association with pathological category and clinical outcome. In addition, in cases in which the primary tumor harbored mutations and metachronous disease occurred, tissue samples were studied for possible mutations.

## Material and Method

### *Patients and specimen collection*

The research has been approved by the Research Ethical Committee of the Faculty of Medicine, Prince of Songkla University (55-098-10-1-3). Frozen specimens from 122 primary TCC cases, who were operated on at Songklanagarind Hospital, Thailand between February 2007 and October 2011, were retrieved for pathological review and DNA extraction. Cases with bladder tumor of other histologic types were excluded. Stage and grade of tumor were assigned according to TNM staging system and World Health Organization grading system[10]. Staging work-ups included chest x-ray and bone scan. Patients in pTa and pT1 categories were considered as non-muscle-invasive TCC and when categorized pT2 and above were in muscle-invasive group[11]. Treatment of TCC included transurethral resection or radical cystectomy, depending on the extent of tumor and patient's physical status. Radiation therapy was reserved for patients with residual tumor after the operation and for inoperable cases. Post-operative surveillance for recurrence was performed by cystoscopy approximately every 4 months in the first 2 years following the original procedure and every 6 months thereafter. Recurrence was defined as histologically proven TCC occurring at either the urinary bladder or a distant organ.

In cases with a mutation in the primary tumor and the patient later developed recurrence or a second primary tumor, a second specimen was retrieved in order to study the index mutation in the metachronous tumor.

### *FGFR3 mutation detection*

DNA was extracted from tumor tissue with a QIAamp DNA mini kit (Qiagen, Germany), according to the manufacturer's protocol. DNA quality and purity were determined by spectrophotometry in a NanoDrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). DNA from formalin-fixed paraffin-embedded (FFPE) tissue was extracted using the method described by Wu et al[12], together with the QIAamp DNA mini kit.

The reference sequence of FGFR3 used in the present study was from GenBank database (accession No. NG_012632.1). FGFR3 exons 7, 10 and 15, reported as mutation hotspots, were PCR amplified. PCR was performed in a 50 µl reaction. Volume containing 1X PCR buffer (Roche, Germany), 2.5 U of FaststartTaq DNA polymerase (Roche, Germany), 200 µM of dNTP mix (Qiagen, Germany), 200 mM of each primer, $MgCl_2$ 0.75 mM for exon 7, 1.25 mM for exon 10 and 1.50 mM for exon 15 and 250 ng of tumor DNA. Except for primers for exon 15 amplification, which were from Wuechner et al[13], the FGFR3 primers used in the present study were newly designed as shown in Table 1. Thermal cycling conditions performed in an MJ thermocycler; Biorad, California, USA, were as follows: 95°C for 5 min;

**Table 1.** Primers used for FGFR3 sequencing (in part)

| Exon | Primer | Sequence (5' to 3') | PCR product size |
|---|---|---|---|
| 7 | FGFR3 E7F | CGG CAG TGG CGG TGG TGG TG | 296 base pairs |
| | FRFR3 E7R | GCC CAG GAG CCC CAG CGG | |
| 10 | FGFR3 E10F | AAC GCC CAT GTC TTT GCA GCC GA | 256 base pairs |
| | FGFR3 E10R | GCC TGG CGG GCA GGC AGC | |

39 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec; and a final heating at 72°C for 10 min. Amplicons were analyzed by agarose gel-electrophoresis, purified using a QIAquick PCR purification kit (Qiagen, Germany), following the manufacturer's protocol and sequenced in both directions BigDye Terminator Ready reaction kit (Applied Biosystems, Foster City, USA) in an ABI PRISM 3130 Genetic analyzer (Applied Biosystems, Foster City, USA).

***P53 mutation detection***

Additional screening for P53 mutations covering Exons 5-8 also used PCR-direct sequencing method. The primers and PCR conditions followed the authors' previous work[14].

***Statistical analysis***

Descriptive statistics were used for the demographic characteristics of the patients. Association between mutation status and other clinicopathological parameters were examined by Chi-square test or Fishers' exact test where appropriate. Follow-up interval was calculated from the first date of diagnosis to March 2012. Recurrence and second primary tumors were considered as events in the analysis of event-free survival. Two-year and four-year OS and event-free survival (EFS) rates were compared between cases with and without FGFR3 mutation by Log-rank test. Kaplan-Meier curves were constructed to illustrate survival probability according to mutation status. Statistical significance is deemed at p-value <0.05. All analyses were performed using Intercool Stata program version 6.0 (Stata Corporation, Texas, USA) statistical package.

## Results

***Demographic data***

Sixty-five patients (53%) were categorized as non-invasive TCC (pTa-pT1), of which 45 cases (69%) had a low-grade (grade 1) tumor (Table 2). Synchronous tumor in ureter or kidney was recorded in 12 cases (10%) and distant metastasis was presented in eight cases (7%). A history of tobacco smoking was recorded in 74 cases (62%) and alcoholic drinking in 48 cases (40%). There is a significant association between male and smoking habit ($p<0.01$) and alcohol drinking ($p<0.01$). The definitive treatment in 80 cases (66%) was a transurethral resection, while 42 cases (34%) underwent a total cystectomy with or without adjuvant radiation therapy.

***Incidence of FGFR3 mutation***

FGFR3 mutations in the exons were detected in 22 cases (18%). Comprising of R248C in four cases (18%), S249C in 13 cases (59%), and Y375C in five cases (23%). The incidence of mutation is significantly higher in the non-muscle-invasive group (28%) compared to the muscle invasion group (7%) ($p<0.01$). Patients with TCC grade 1 have a significantly higher mutation incidence (40%) compared to those in other grades (4%, $p<0.01$) (Fig. 1). When pT stage and grade were considered together, mutations were most commonly found in pTa-pT1/G1 TCC, 18/45 cases (40%) (Table 3). The occurrences of mutations are not significantly related to sex, age, metastatic status, smoking, or alcoholic drinking. Mutations were detected in two TCC cases, who later developed pulmonary metastasis, but the index mutation was detected in the metastatic site only in one of the two cases.

***Incidence of P53 mutations***

On screening of P53 mutations in 122 TCC cases, mutations were detected in seven cases (6%). The mutations included four cases of E171V and each of T211I and R249S. Of the seven cases, five were high-grade when two were low-grade. There were two cases with concomitant FGFR3 and P53 mutation, one case of high-grade tumor, and one case of pT1

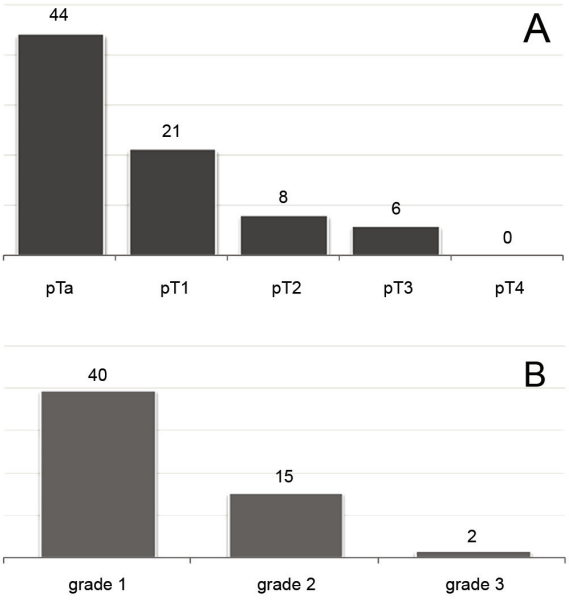


**Fig. 1** FGFR3 mutation frequency (percent) according to pT stage (A) and histologic tumor grade (B).

**Table 2.** Demographic characteristics of 122 TCC patients and comparison between cases with and without FGFR3 mutations

| | No. of cases (%) | FGFR3 | | |
|---|---|---|---|---|
| | | Wild type (%) | Mutant (%) | p-value |
| Total | 122 (100) | 100 (82) | 22 (18) | |
| Mean age (years) | 70.5 | 71.1 | 67.7 | 0.15 |
| Sex | | | | |
| Male | 103 (84) | 85 (82) | 18 (17) | 0.71 |
| Female | 19 (16) | 15 (79) | 4 (21) | |
| Smoking | | | | |
| Yes | 74 (62) | 63 (85) | 11 (15) | 0.31 |
| No | 45 (38) | 35 (78) | 10 (22) | |
| Alcohol drinking | | | | |
| Yes | 48 (40) | 39 (81) | 9 (19) | 0.70 |
| No | 71 (60) | 59 (83) | 12 (17) | |
| T-stage | | | | |
| pTa | 18 (14) | 10 (56) | 8 (44) | <0.01 |
| pT1-2 | 84 (69) | 71 (85) | 13 (15) | |
| pT3-4 | 20 (16) | 19 (95) | 1 (5) | |
| Tumor grade | | | | |
| G1 | 48 (39) | 29 (60) | 19 (40) | <0.01 |
| G2 | 13 (11) | 11 (85) | 2 (15) | |
| G3 | 60 (50) | 59 (98) | 1 (2) | |
| Associated ureteric TCC | | | | |
| Yes | 12 (10) | 12 (100) | 0 (0) | 0.23 |
| No | 110 (90) | 88 (80) | 22 (20) | |
| Distant metastasis | | | | |
| Yes | 8 (7) | 6 (75) | 2 (25) | 0.61 |
| No | 113 (93) | 94 (83) | 20 (7) | |

TCC = transitional cell carcinoma

**Table 3.** FGFR3 mutation frequency according to pT stage and tumor grade

| | pTa (%) | pT1 (%) | pT2 (%) | pT3 (%) | pT4 (%) |
|---|---|---|---|---|---|
| Grade 1 | 8/15 (53) | 10/30 (33) | 1/3 (33) | - | - |
| Grade 2 | 0/3 (0) | 0/5 (0) | 2/5 (40) | - | - |
| Grade 3 | 0/12 (0) | 0/28 (0) | 1/16 (6) | 0/3 (0) | - |

grade 1 that later progressed to pT4 and high-grade tumor.

***Association with clinical outcomes***

The mean follow-up period was 45.1 months. Two-year and four-year OS rates were 70% and 56% respectively, while the two-year and four-year EFS rates were 41% and 21% respectively. The three-year OS rate in the non-invasive TCC group (80%) is significantly higher than in the muscle invading TCC group (41%) ($p<0.01$) (Fig. 2A), while that in grade 1 or 2 TCC cases (77%) is significantly better than in grade 3 cases (47%) ($p = 0.02$). Three-year OS rate in cases with FGFR3 mutations was 73%, not significantly different than in cases without mutation (61%) (Fig. 2B). All cases of non-low grade tumor with FGFR3 mutations were alive as of the preparation of this report, with a mean follow-up period of 28.4 months.

***FGFR3 mutation in metachronous disease***

Among the 19 superficial TCC cases that had an FGFR3 mutation, 10 cases had a total of 16 episodes of recurrence within the period of the study, with

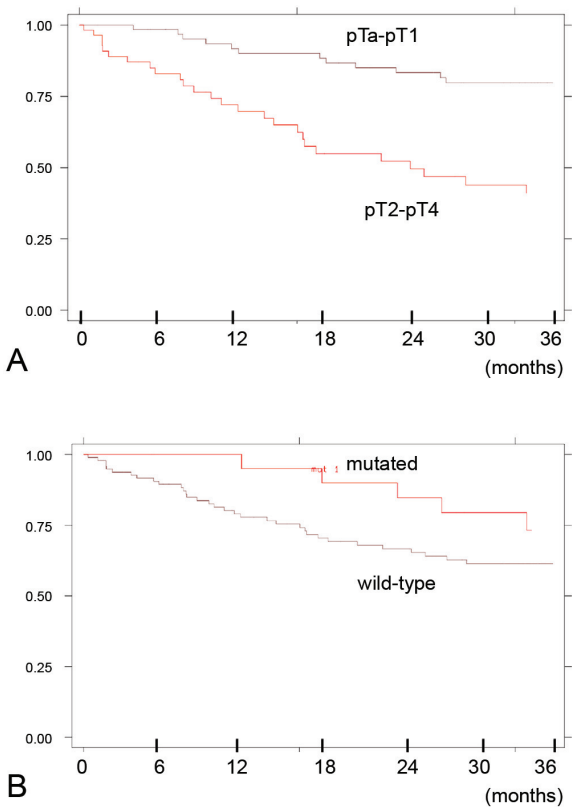


**Fig. 2** Survival probability of 122 TCC cases analyzed according to their pT-stage (A) and FGFR3 mutation status (B). pT-stage has a significant association with survival (log-rank $p<0.01$), while mutation status had no significant survival association ($p = 0.14$).

four cases at a higher tumor grade. In addition, there were another two cases with synchronous pulmonary metastasis and two cases of second primary tumors, including a case with squamous cell carcinoma at the base of the tongue and a case of lymphoma. Of the 16 local recurrence and four secondary tumors studied, mutation was detected in one pulmonary metastasis. None of the local recurrent tumors showed conservation of the identical FGFR3 mutation detected in the primary TCC.

## Discussion

The present study focused on the incidence of FGFR3 mutation in Thai patients with superficial TCC of the urinary bladder and on possible correlation with pathological characteristics. The major finding was that the incidence of FGFR3 mutations (18%) in the presented patients was lower than in previous reports on other ethnic groups. Mutations are significantly associated with low tumor grade and T-stage. In addition, it is worth noting that the mutations rate was less than 5% in grade 3 disease and not found at all in pT4 stage cases or in recurrent tumors.

FGFR3 encodes for a 115 KD plasma membrane receptor of fibroblast growth factor involved in cellular proliferation, differentiation and angiogenesis[7]. Receptor autosignaling caused by FGFR3 mutations results in pathological growth and hence tumor development[15]. The majority of mutations of FGFR3 are clustered around exon 7, encoding an extracellular domain, and exon 10 encoding a transmembrane tyrosine kinase, although a lower incidence of mutations within exon 15 has been detected[15,18]. The general incidence of the FGFR3 mutation in urinary bladder TCC has been reported to range from 31% to 53%[3,8,9,19]. The lower overall incidence of FGFR3 mutation detected in Thai TCC in the present study could be partly explained by the relatively lower incidence of low-grade disease. However, when non-muscle-invasive tumors are taken into account, the incidence (53%) in pTaG1 is comparable with other reports[18,20-22]. The significant correlation between FGFR3 mutations and superficial TCC in the present study is consistent with previous findings[3,20,21,23]. In addition, the mutation spectrum in our TCC, predominated by S249C and Y375C, is also in line with other studies[8,16,23,24].

A possible prognostic role of FGFR3 mutations in TCC has been investigated in various ethnic groups, which reported an association with good prognosis[21,25]. However, in the presented cases the authors could not demonstrate a significant association between mutation and clinical outcome, although the authors did find that T-stage and histologic grade are significantly correlated with better survival and that FGFR3 mutations are associated with low-grade disease.

Apart from the incidence of FGFR3 mutations, the present study also investigated whether the identical mutation could be found in recurrent TCC that had a mutation in the primary tumor. Notably, 40% of local recurrences in the presented patients resulted in their tumor grade changing from low to high grade. The question was then raised whether an identical mutation remains in recurrent tumor or metastatic disease. The present study found negative results in all 16 cases that presented with an initial FGFR3 mutation. Recently, Lindgren et al[26] reported that a mutation present in the initial tumor is not always

detectable in the recurring ones[26]. Another study, which examined of 21 metachronous TCC cases with initial FGFR3 mutations, found five cases in which the initial mutation was not present at the higher tumor stage[27]. These earlier reports taken together with the present results, suggest that TCC in an individual is not likely to originate from a single cell clone, thereby reducing the likelihood that FGFR3 mutation will prove to be a useful single marker for detection of tumor recurrence. Evidence of concomitant P53 mutations in our FGFR3-mutated cases that had metachronous high-grade component might suggest that other genetic pathways than FGFR3 are responsible for progression from initial tumor to more aggressive phenotype. Moreover, heterogeneity in mutation status within the same tumor may compromise future development of chemotherapy targeting FGFR3.

In summary, the authors investigated FGFR3 mutations involved in both primary and recurrent TCC in Thai patients. Although FGFR3 mutations were frequently detected in low-grade TCC, the mutation was not consistently found in metachronous disease, a finding that would tend to negate the use of this marker in detection of tumor recurrence. However further studies are needed before a conclusion can be searched.

**Acknowledgement**

The authors want to thank Susisda Korvichiankul, chief urology nurse of Songklanagarind Hospital, for assistance in tumor tissue collection, and Paradee Weerawittayakul Cancer Registry Unit of Songklanagarind Hospital for assistance in providing survival data, and Dave Patterson, consultant of the Office of International Affairs, Faculty of Medicine, Prince of Songkla University, for editing the English language of the manuscript. The study was partially supported by a grant from the Graduate School, Prince of Songkla University.

**Potential conflicts of interest**

None.

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## *การกลายพันธุ์ของจีน fibroblast growth factor receptor 3 (FGFR3) ในมะเร็งกระเพาะปัสสาวะชนิด transitional cell ในชาวไทย*

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***วัตถุประสงค์:*** *เพื่อศึกษาอุบัติการณ์การกลายพันธุ์ของจีน FGFR3 ในเนื้อเยื่อมะเร็งกระเพาะปัสสาวะชาวไทย และศึกษาความสัมพันธ์ระหว่างการกลายพันธุ์กับลักษณะทางพยาธิวิทยา อันได้แก่ระยะของโรคและเกรดทางจุลพยาธิวิทยา*

***วัสดุและวิธีการ:*** *เนื้อเยื่อมะเร็งกระเพาะปัสสาวะปฐมภูมิจากผู้ป่วยจำนวน 122 ราย ได้รับการนำมาสกัดสารพันธุกรรมและศึกษาการกลายพันธุ์ของจีน FGFR3 บริเวณ exon ที่ 7, 10 และ 15 โดยเทคนิค polymerase chain reaction และ direct DNA sequencing*

***ผลการศึกษา:*** *พบการกลายพันธุ์ของจีน FGFR3 ในเนื้อเยื่อจากผู้ป่วย 22 ราย (ร้อยละ 18) การกลายพันธุ์เกิดขึ้นกับตำแหน่งของการกลายพันธุ์ที่ได้รับการรายงานพบบ่อยได้แก่ การกลายพันธุ์บน exon ที่ 7 ประกอบด้วย S249C (13 ราย ร้อยละ 59) และ R248C (4 ราย ร้อยละ 18) การกลายพันธุ์บน exon ที่ 10 ได้แก่ Y375C (5 ราย ร้อยละ 23) ไม่พบการกลายพันธุ์บน exon ที่ 15 มะเร็งจากผู้ป่วย 65 ราย (ร้อยละ 53) จัดอยู่ในกลุ่มไม่ลุกล้ำกล้ามเนื้อ (ระยะ pTa และ pT1) อุบัติการณ์ของการกลายพันธุ์ในมะเร็งกลุ่มซึ่งไม่ลุกล้ำ (ร้อยละ 28) สูงกว่าในกลุ่มซึ่งลุกล้ำ (ร้อยละ 7) (p-value <0.01) มะเร็งซึ่งอยู่ในเกรดที่ 1 มีอุบัติการณ์การกลายพันธุ์ร้อยละ 40 ซึ่งสูงกว่าอุบัติการณ์ในมะเร็งเกรดอื่น (ร้อยละ 4) เมื่อพิจารณาระยะของโรคร่วมกับเกรด การกลายพันธุ์เกิดกับมะเร็งระยะไม่ลุกล้ำซึ่งเป็นเกรดที่ 1 มากที่สุด (18/45 ราย หรือ ร้อยละ 40) ระยะเวลาในการติดตามผู้ป่วยเฉลี่ย 45.1 เดือน อัตรารอดชีพระยะ 2 และ 4 ปี อยู่ที่ร้อยละ 70.0 และ 56 ตามลำดับ อัตรารอดชีพระยะ 3 ปี ในมะเร็งกลุ่มไม่ลุกล้ำ (ร้อยละ 80) สูงกว่ากลุ่มลุกล้ำ (ร้อยละ 41) อย่างมีนัยสำคัญทางสถิติ (p-value <0.01) อัตรารอดชีพระยะ 3 ปี ในกลุ่มซึ่งมีการกลายพันธุ์อยู่ที่ร้อยละ 73 เทียบกับร้อยละ 61 ในกลุ่มซึ่งไม่มีการกลายพันธุ์ ความแตกต่างดังกล่าวไม่มีนัยสำคัญทางสถิติ การกลับเป็นซ้ำในผู้ป่วย 16 ราย ซึ่งมีการกลายพันธุ์ของจีน FGFR3 อย่างไรก็ตามเมื่อศึกษาการกลายพันธุ์ในเนื้อเยื่อมะเร็งซึ่งกลับเป็นซ้ำกลับไม่พบการกลายพันธุ์*

***สรุป:*** *ได้ศึกษาการกลายพันธุ์ของจีน FGFR3 ในผู้ป่วยมะเร็งกระเพาะปัสสาวะชาวไทยพบอุบัติการณ์ของการกลายพันธุ์โดยรวมค่อนข้างน้อยกว่าซึ่งได้รับการรายงานในชาติพันธุ์อื่น อย่างไรก็ตามการกลายพันธุ์มีความสัมพันธ์กับมะเร็งซึ่งไม่ลุกล้ำและเกรดต้น การไม่พบการกลายพันธุ์ในโรคซึ่งกลับเป็นซ้ำ อาจชี้ข้อจำกัดในการนำการตรวจการกลายพันธุ์มาใช้เพื่อเฝ้าระวังการกลับเป็นซ้ำ*