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FGFR3 and *TP53* mutation analysis in inverted urothelial papilloma: incidence and etiological considerations

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Urothelial papillomas and low-grade urothelial carcinomas have shown a high incidence of fibroblast growth factor receptor 3 (*FGFR3*) mutations and are associated with a favorable prognosis. The association of *FGFR3* mutations with inverted papillomas is less known. We analyzed 20 cases of inverted papilloma in the urinary tract. Mutations of *FGFR3* (exons 7, 10, and 15) and *TP53* genes were evaluated by DNA sequencing in these cases. Point mutations of the *FGFR3* gene were identified in 45% (9 of 20) of inverted papillomas with four cases exhibiting mutations at multiple exons. Seven cases had exon 7 mutations containing R248C, S249T, L259L, P260P, and V266M. Two cases had exon 10 and 15 mutations including A366D, H412H, E627D, D641N, and H643D; five cases had N653H. The most frequent mutation was identified at R248C. None of the inverted papillomas exhibited mutations in *TP53*. During a mean follow-up of 78 months, none had recurrence or developed urothelial carcinoma. These findings support the concept that low-grade and low-stage urothelial neoplasms arise in a background of molecular changes that are distinctly different from the molecular changes of high-grade and high-stage urothelial cancers.
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The fibroblast growth factor receptor 3 (*FGFR3*) is a member of a family of tyrosine kinase receptors and is composed of an extracellular ligand-binding domain, a transmembrane region, and a cytoplasmic domain with tyrosine kinase activity. Ligand binding causes receptor dimerization and subsequent activation of intracellular tyrosines. Activating mutations of *FGFR3* gene lead to constitutive activation of the receptor subsequently inducing the downstream molecular pathogenesis. Activating point mutations of *FGFR3* have been associated with autosomal dominant dwarfism and severe achondroplasia. An oncogenic role for *FGFR3* in human cancer has emerged recently and *FGFR3* mutations were reported to be associated with multiple myeloma, urothelial, and cervical cancers.

Urothelial carcinomas harboring *FGRFR3* mutations, in general, tend to be of low histological grade and of low pathological stage, and consequently are associated with a more favorable clinical outcome.[1–3] Inverted papillomas of the urinary tract are uncommon. If strict histological criteria are adhered to in making the diagnosis, their biological behavior is almost invariably benign.[4] Although a number of studies have addressed *FGFR3* mutation status in urothelial carcinoma, there is a paucity of

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information concerning *FGFR3* abnormalities in inverted papilloma of the urinary tract. Because inverted papillomas lack aggressive biological behavior, and *FGFR3* mutation appears to be associated with relatively indolent behavior in low-grade and low-stage urothelial carcinomas, we tested the frequency and specific types of *FGFR3* mutations, in conjunction with *TP53* mutation status, in 20 cases of inverted papilloma.

Materials and methods

Tumors Samples and Microdissection

Twenty urothelial lesions diagnosed as inverted papilloma were analyzed. All the cases were reviewed retrospectively and fulfilled the diagnostic criteria using the 2004 WHO classification of the genitourinary tumors.[5] None had prior or current urothelial carcinoma *in situ* (CIS) or papillary transitional cell carcinoma (urothelial carcinoma).

Paraffin-embedded tissue was collected from each of the 20 cases of inverted papilloma. Histological sections were prepared from formalin-fixed, paraffin-embedded tissue. The slides were stained with hematoxylin and eosin (HE) for microscopic evaluation after deparaffinization with xylene and ethyl alcohol. Laser-assisted microdissection of the tumor tissues was performed on the lightly HE-stained sections using a PixCell II Laser Capture Microdissection System (Arcturus Engineering, Mountain View, CA, USA).[6,7] Approximately 600–1000 cells of each tumor were microdissected from the 4-μm histological sections as demonstrated in Figure 1. Microdissected normal tissue from the same patient served as a control. The dissected tissue was incubated in 50 μl of digesting buffer containing 10 mM Tris-HCl, 1 mM EDTA, 1% Tween 20, and 5 mg/ml of proteinase K (pH 8.3) at 37°C overnight. The samples were boiled for 10 min to inactivate proteinase K. The genomic DNA from each sample was dissolved in 30 μl of dd H_2O after phenol–chloroform extraction (phenol/chloroform/isoamyl alcohol = 25:24:1).

FGFR3 Mutation Analysis

Exons 7, 10, and 15 of *FGFR3* were amplified by PCR using the previously reported primers.[8–10] PCR was performed with 3 μl of isolated genomic DNA in a final volume of 50 μl containing 2.3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 μM deoxynucleotide triphosphates, 2 μM each primer, and 2 U Taq DNA polymerase (Bio-Rad, Hercules, CA, USA). Each PCR protocol had an initial denaturing step of 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at 55°C (exons 7 and 15) for 30 s or at 58°C (exon 10) for 30 s, and at 72°C for 30 s, and then followed by a single final extension step at 72°C for 7 min. The PCR products were purified by QIAquick PCR Purification kit (Qiagen Sciences, Germantown, MD, USA). DNA concentration of PCR products was measured and adjusted to 20 ng per microliter. Purified PCR product was sequenced by ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

TP53 Mutation Analysis

TP53 DNA in exons 5, 7, and 8 was amplified by PCR using the established primers.[11–13] PCR was performed with 3 μl of isolated genomic DNA in a final volume of 50 μl containing 2.3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 μM deoxynucleotide triphosphates, 2 μM each primer, and 2 U Taq DNA polymerase (Bio-Rad). Each PCR protocol had an initial denaturing step of 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s, and then followed by a single final extension step at 72°C for 7 min. The PCR products were purified by QIAquick PCR Purification kit (Qiagen Sciences). DNA concentration of PCR products was measured and adjusted to 20 ng per microliter. Purified PCR product was sequenced by ABI Prism 3100 Genetic Analyzer.

Results

The inverted papillomas were diagnosed according to accepted criteria.[5] The median age at diagnosis was 58 years (range 37–75 years). Eighteen of the patients were men whereas two were women. The most common location of the lesion was the bladder neck (5 of 20 cases), whereas lateral wall (3 of 20), trigone (4 of 20), ureteral orifice (2 of 20), ureter (4 of 20), and base and dome (1 of 20 each) were also reportedly involved. The most common presenting complaint was hematuria. The mean clinical follow-up was 78 months (range 11–220 months). None developed recurrence or urothelial carcinoma.

FGFR3 mutation analysis was performed in all tumors. The mutations detected in inverted papillomas included R248C, S249T, L259L, P260P, and V266M at exon 7; A366D and H412H at exon10; E627D, D641N, H643D, and N653H at exon 15 (Table 1; Figures 1 and 2). Previously described missense mutations and activating mutations that were detected include R248C and S249T.[14] To the best of our knowledge, mutations of L259L, P260P, V266M, A366D, H412H, E627D, D641N, H643D, and N653H were not reported in previously published papers. Missense or candidates of activating mutations that were detected include V266M, A366D, E627D, D641N, H643D, and N653H. Finally, the L259L, P260P, and H412H mutations are silent mutations, probably of no significant biological impact. Overall, 9 of 20 inverted papillomas (45%) demonstrated point mutations, with 2 identified in three cases and 3 mutations in one case. The most common mutation was R248C, which was observed

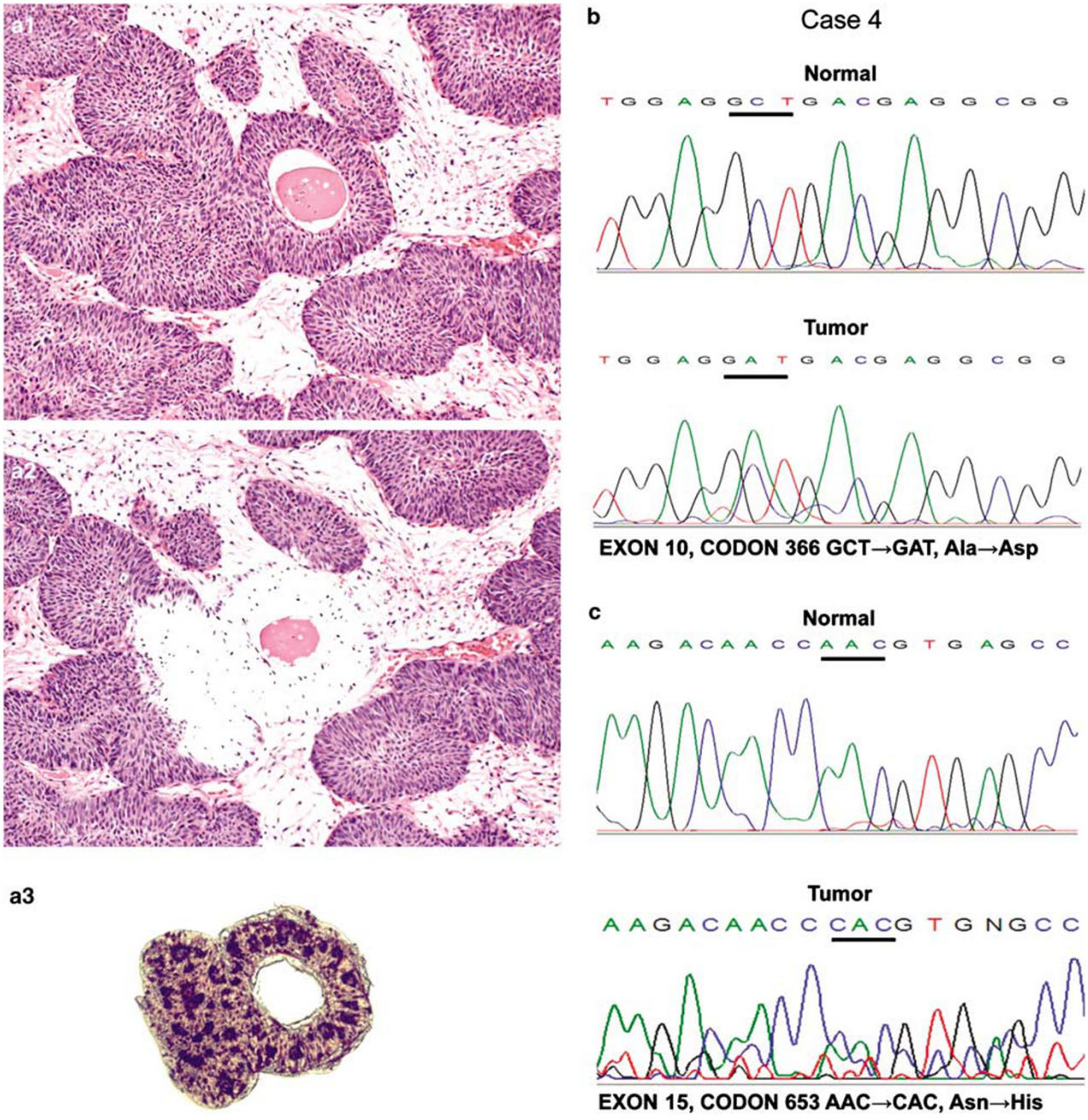

Figure 1 Urothelial inverted papilloma (case 4). (**a**) Laser microdissection of inverted papilloma. (**a1**) Lesion (inverted papilloma) before microdissection. (**a2**) Lesion after microdissection. (**a3**) Laser capture lesional cells. (**b**) *FGFR3* gene mutation detected by direct sequencing. Upper panel: normal tissue (control); lower panel: point mutation at exon 10, A366D, GCT→GAT. (**c**) *FGFR3* gene mutation detected by direct sequencing. Upper panel: normal tissue (control); lower panel: point mutation at exon 15, N653H, AAC→CAC.

in three cases. D641N mutations were identified in two cases. The other mutations were found only once in separate cases.

No mutations were identified in normal tissue from the same specimen in patients with inverted papilloma. No mutations in *TP53* genes were identified in any inverted papillomas. Although more studies are needed to determine whether the mutations identified in this series have a causative effect on the development of inverted papillomas, the presence of the mutations within the papillomas and the absence in normal tissue support this hypothesis.

Discussion

The *FGFR3* gene on chromosome 4p16 is involved in cell signaling pathways and angiogenesis as well as cell proliferation, development, and differentia-

Table 1 Detection of *FGFR*3 and *TP53* mutations in inverted urothelial papillomas

Case		FGFR3			*TP53*		
		Exon 7	*Exon 10*	*Exon 15*	*Exon 5*	*Exon 7*	*Exon 8*
1	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	S249T, TCC→ACC[a]	No mutation	No mutation	No mutation	No mutation	No mutation
2	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
3	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	P260P, CCG→CCT[b]	No mutation	No mutation	No mutation	No mutation	No mutation
4	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	R248C, CGC→TGC[a]	A366D, GCT→GAT[c]	N653H, AAC→CAC[c]	No mutation	No mutation	No mutation
5	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
6	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
7	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
8	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	R248C, CGC→TGC[a]	No mutation	H643D, CAC→GAC[c]	No mutation	No mutation	No mutation
9	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
10	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	L259L, CTG→TTG[b]	No mutation	No mutation	No mutation	No mutation	No mutation
11	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
12	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
13	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
14	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	V266M, GTG→ATG[c]	H412H, CAC→CAT[b]	No mutation	No mutation	No mutation	No mutation
15	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
16	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	R248C, CGC→TGC[a]	No mutation	E627D, GAG→GAT[c]	No mutation	No mutation	No mutation
17	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	D641N, GAC→AAC[c]	No mutation	No mutation	No mutation
18	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
19	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	D641N, GAC→AAC[c]	No mutation	No mutation	No mutation
20	N	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation
	T	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation

N, normal control; T, inverted papilloma.
[a]Missense mutation and activating mutation.
[b]Silent mutation.
[c]Missense mutation and candidate of activating mutation.

tion. *FGFR3* mutations are associated with autosomal dominant skeletal disorders such as achondroplasia and thanatophoric dysplasia.[15] More recently, *FGFR3* mutations have been identified in some human neoplasms, including multiple myeloma and carcinoma of the uterine cervix, but not in neoplasms of the stomach, rectum, colon, prostate, ovary, breast, brain, lung, skin, esophagus,

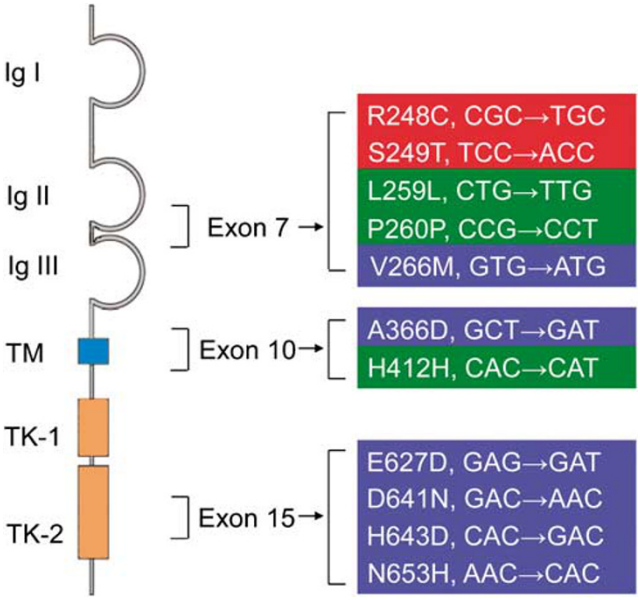

Figure 2 Schematic illustration of *FGFR3* mutation positions in the inverted urothelial papillomas. Mutations were presented as the type of mutation and amino-acid changes designated to the *FGFR3* protein structure. Ig I, Ig II, and Ig III: immunoglobulin-like domains; TM: transmembrane domain; TK-1 and TK-2: tyrosine kinase domains. Red-coded mutations are activating mutations published previously; blue-coded are missense mutations that are candidate of activating mutations; green-coded are silent mutations.

Table 2 Distinct carcinogenesis pathways for low- and high-grade urothelial tumors

	FGFR3 *mutation*	p53 *mutation*
Urothelial tumors	Papillary, noninvasive	High grade, invasive
Molecular pathway	Ras and PIK3 kinase pathway	Cell-cycle regulation and apoptosis pathway
Key genes involved	*Ras*, *STAT1*, *cyclin D1*	*p21*, *Bax*, *Bcl2*, *Rb*
Smoking association	Negative	Positive
Prognosis	Better prognosis	Poor prognosis in general
Grade	Low grade	High grade
Stage	Low stage	High stage

or kidney.[16] In this report, we found a high frequency of *FGFR3* mutations in inverted urothelial papilloma.

A strong association between *FGFR3* mutation and low-grade urothelial carcinoma has been recognized.[1,17] Indeed, *FGFR3* mutations are associated with low-stage, low-grade tumors. *FGFR3* mutations have also been identified in 23% of flat hyperplastic urothelial lesions (7 of 30).[18] A recent study of inverted papillomas of the urinary tract revealed infrequent *FGFR3* mutations (9.8% of cases).[19] Specific codons involved in point mutations in urothelial carcinomas include 248, 249, 372, 375, and 652.[15,20] Tumors harboring more than one *FGFR3* mutation have been identified.[10,21] *FGFR3b*-S249C, the most common mutation in bladder tumors, was found to be tumorigenic *in vitro* and also gave rise to tumors in mice that were xenografted with *FGFR3b*-S249C transfected cells.[22]

Two divergent molecular pathways appear to exist in urothelial tumorigenesis and cancer progression (Table 2). Higher-grade tumors are much less likely to harbor *FGFR3* mutations; in contrast, they often exhibit *TP53* mutations.[20] Low-grade papillary tumors typically harbor activating mutations of *FGFR3*. These tumors tend to be genetically stable even if they do frequently recur. They are often multifocal and may arise from simple urothelial hyperplasia, and usually do not progress to invasion. An alternate molecular pathway is believed to be operative in the carcinogenesis of a group of urothelial carcinomas with entirely different clinical and pathological features, characterized by aggressive behavior and a tendency to be invasive. These tumors are frequently associated with *TP53* mutations, which appear early in tumorigenesis. They typically arise *de novo* and are frequently found in high-grade, flat, and CIS tumors, usually with no previous history of low-grade, noninvasive papillary lesions. These tumors are genetically unstable and tend to accumulate mutations. The *TP53* mutations lead to dysfunction in cell cycle and apoptosis. Mutations in *TP53*, but not *FGFR3*, are known to be influenced by smoking. *FGFR3* and *TP53* mutations have not been identified in normal bladder epithelium from patients with confirmed urothelial carcinoma, suggesting the mutations were somatic.[15,23] Additional molecular studies by Jebar *et al*,[9] have found that *FGFR3* mutations are mutually exclusive of *Ras* gene mutations in urothelial cell carcinoma (Table 2).

Although the concept of two divergent pathways to tumorigenesis is appealing, consideration should be given to the possibility of genetic progression leading to increased aggressiveness in urothelial carcinomas. Specifically, *FGFR3* mutations may signal an early event in tumorigenesis followed by *TP53* mutation. However, analyses of both high- and low-grade tumors have only yielded a relatively small number of tumors harboring both *TP53* and *FGFR3* mutations.[1,13] van Rhijn *et al*[1] hypothesized that an intermediate phenotype in urothelial carcinomas with both *FGFR3* and *TP53* alterations may exist.

In our study, 45% of inverted papillomas exhibited mutations in the *FGFR3* gene, whereas none harbored *TP53* mutations. These findings support the concept that low-grade and low-stage urothelial neoplasms arise in a background of molecular changes that are distinctly different from the molecular changes preceding the onset of high-grade, invasive, biologically aggressive bladder cancers.

The exact mechanism by which activating mutations lead to tumorigenesis is still being deciphered. The *FGFR3* regulates cell growth and differentiation through the banding of fibroblast growth factors. The mechanism of *FGFR3*-mutation-related oncogenesis is believed to cause constitutive activation of the receptor. van Rhijn *et al*[1] identified activating point mutations of *FGFR3* in low-grade urothelial carcinomas by sequencing. Exons 7, 10, and 15 seem especially susceptible to tumor-initiating mutations.[13,14] *FGFR3* mutations were found only in papilloma but not in adjacent normal tissue, suggesting a causative affect.

In addition to identifying two previously described mutations (S249T and R248C), we found several new missense and candidate activating mutations (Figure 2). These mutations include V266M, E627D, A366D, D641N, H643D, and N653H. We also identified three silent mutations (L259L, P260P, and H412H) in inverted papillomas. In conclusion, *FGFR3* mutations are associated with inverted urothelial papillomas. Benign and low-grade papillary urothelial lesions including 'hyperplasia', papillomas, inverted papillomas, PUNLMP, and low-grade papillary urothelial carcinomas may well result from a similar etiology. *TP53* gene mutations were not associated with inverted papillomas.

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