RESEARCH ARTICLE

c-KIT Positive Schistosomal Urinary Bladder Carcinomas are Frequent but Lack KIT Gene Mutations

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Abstract

Urinary bladder squamous cell carcinoma (SCC), one of the most common neoplasms in Egypt, is attributed to chronic urinary infection with Schistosoma haematobium (Schistosomiasis). The proto-oncogene c-KIT, encoding a tyrosine kinase receptor and implicated in the development of a number of human malignancies, has not been studied so far in schistosomal urinary bladder SCCs. We therefore determined immunohistochemical (IHC) expression of c-KIT in paraffin sections from 120 radical cystectomies of SCCs originally obtained from the Pathology Department of Suez Canal University (Ismailia, Egypt). Each slide was evaluated for staining intensity where the staining extent of >10% of cells was considered positive. c-KIT overexpression was detected in 78.3% (94/120) of the patients, the staining extents in the tumor cells were 11-50% and >50% in 40 (42.6%) and 54 (57.4%) respectively. The positive cases had 14.9%, 63.8%, 21.3% as weak, moderate and strong intensity respectively. Patients with positive bilharzial ova had significantly higher c-KIT expression than patients without (95.2% vs. 38.9%, P=0.000). Mutation analysis of exons 9-13 was negative in thirty KIT positive cases. The high rate of positivity in SBSCC was one of the striking findings; However, CD117 may be a potential target for site specific immunotherapy to improve the outcome of this tumor.

Keywords: c-KIT - immunostaining - gene mutation - schistosomiasis - squamous cell carcinoma - urinary bladder

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Introduction

Urinary bladder carcinoma (UBC) is the first ranking tumor in Egyptian males and it represents 34.26% of male malignant tumors; it is characterized by high frequency of SCCs due to Schistosomiasis which induces squamous metaplasia of the urothelium (Mokhtar et al., 2007). Muscle invasion is the usual presentation of Schistosomal bladder cancer; this invasive behavior is secondary to the aggressive nature of the disease (Badr et al., 2004).

The proto-oncogene *c-KIT* encodes a 145-160 kDa, type III transmembrane tyrosine kinase receptor known as *c-KIT* or CD117 (Vliagoftis et al., 1997; Tian et al., 1999), which belongs to the same family of receptors as platelet-derived growth factor and colony-stimulating factor (Yarden et al., 1987; Vliagoftis et al., 1997). Expression of *c-KIT* is essential to the development of some cell types including melanocytes, germ cells, mast cells, erythrocytes, and interstitial cells of Cajal (Zsebo et al., 1990; Tsuura et al., 1994). In addition, expression of this receptor may be seen in other histologically normal cell types, such as breast epithelial cells, renal tubule cells, astrocytes, Purkinje cells, parotid acini, and endometrial cells (Natali et al., 1992a; 1992b; Elmore et al., 2001). Steel factor (SLF), also known as KIT ligand or stem-cell factor, is the cognate ligand for KIT. Binding of SLF to KIT results in receptor homodimerization, activation of KIT tyrosine kinase activity, and resultant phosphorylation of a variety of substrates. In many cases, these substrates are themselves kinases and serve as effectors of intracellular signal transduction (Blume-Jensen et al., 1991; Lev et al., 1991).

Three general mechanisms of KIT activation in tumor cells have been widely described: (1) autocrine and/or paracrine stimulation of the receptor by its ligand, SLF; (2) cross-activation by other kinases and/or loss of regulatory phosphatase activity and (3) acquisition of activating mutations (Turner et al., 1882; DiPaola et al., 1997; Ingram et al., 2000). KIT expression has been detected in a variety of different tumor entities. In gastrointestinal stromal tumors (GISTs), the frequency of KIT immunohistochemical positivity was found so high (90-95%) and was considered a prerequisite for the histologic diagnosis of many tumor types (Holst et al., 1999; Hornick and Fletcher 2002; Sela et al., 2003).

In terms of therapeutic strategies, it has become important to evaluate the frequency of *c-KIT* expression and mutation in malignancies since the emergence of the targets of the tyrosine kinase inhibitor imatinib mesylate (STI571; Gleevec;) a potential therapeutic agent which inhibits the action of BCL-ABL in chronic myeloid leukemia and *c-KIT* in gastrointestinal stromal tumor

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(GIST) and mast cell disease (Savage and Antman, 2002). In fact, its excellent effect against these tumors has been reported in clinical trials (Demetri et al., 2002; Pardanani et al., 2003). Imatinib mesylate is also effective against small cell carcinoma of lung in vitro (Heinrich et al., 2002).

In the present study, we investigated the expression and mutation frequency of *c-KIT* in urinary bladder SCCs from Egyptian patients and its relation with Schistosomiasis and various clinicopathologic variables, since this could provide us with useful information concerning whether the drud Imatinib mesylate might be effective against this aggressive malignancy. Imatinib mesylate acts by specifically inhibiting tyrosine kinase receptor that is characteristic of a particular cancer cell, rather than non-specifically inhibiting and killing all rapidly dividing cells (Gambacorti-Passerini, 2008).

Materials and Methods

A total of 120 archieval formalin-fixed, paraffin embedded tumor tissues from patients diagnosed at the Suez Canal University Hospital, Ismailia, Egypt with urinary bladder SCC and treated by radical cystectomy from 2002-2007, in addition to five cystoscopic controls of normal urinary bladder mucosa taken from patients not harboring tumors were included in this study. Demographic data of all cases were summarized from the patients' files from the Urology Department, Faculty of Medicine, Suez Canal University.

Histopathological methods

Representative H&E stained sections were examined to evaluate the histo-pathological type, grade, stage and nodal metastasis. Schistosomiasis infection was confirmed by the presence of ova on histological sections and according to this; the cases were divided into 84 tumors (70.0%) positive for Bilharziasis and the rest of the tumors were negative. According to WHO grading system (Mostofi et al., 1973), six tumors (5.0%) were low grade (grade1), sixty two tumors (51.7%) were intermediate grade (grade2) and fifty two tumors (43.3%) were high grade (grade3). Pathologic staging was performed according to the 2002 TNM (tumor, lymph nodes, and metastasis) classification system (Greene et al., 2002) into 4 (3.3%) T1, 74 (61.7%) T2, 26 (21.7%) T3 and 16 (13.3%) T4. Forty four patients (36.7% of cases) had lymph node infiltration on pathological examination. Also the presence of hyperplasia, metaplasia, and dysplasia in adjacent mucosa were assessed.

Immunohistochemistry (IHC)

IHC staining was performed with the avidin-biotin complex method of Hsu et al (Hsu et al., 1981). In brief, serial 5 m-thick sections of formalin-fixed, paraffin-embedded tissue samples were used. Slides were deparaffinized twice in xylene for 5 min and rehydrated through graded ethanol solutions to distilled water. Endogenous peroxidase activity was inactivated by incubation in 0.3% hydrogen peroxide diluted in methanol for 30 min. Nonspecific binding sites were blocked using Protein Block (DAKO) for 20 min. Antigen retrieval was carried out by microwave heating sections in citrate buffer (DAKO Target Retrieval Solution S1699, DAKO Corporation, Carpintera, CA, USA) for 15 min. IHC was performed with anti-CD117 antibody from DAKO (A4502). In a comparison of multiple antibodies, A4502 had previously yielded the highest specificity and the least background (Went et al., 2004). A4502 was applied at a dilution of 1:300 at room temperature for 2.5 h followed by biotinylated secondary antibody (DAKO) at room temperature for 30 min and then with streptavidin-biotin peroxidase complex kit (Dako) for 30 min. Sections were exposed to 3,3-Diaminobenzidine for 3-5 min as the chromogen, the slides were counterstained with Mayer's hematoxylin and mounted. Sections from gastrointestinal stromal tumors were used as positive controls. A preabsorption experiment using CD117 peptide stock solution (Neomarkers PP1518; NeoMarkers, Freemont, CA) was used as a negative control.

Quantitative analysis of IHC

All slides were evaluated under light microscope without knowledge of the patient's clinical status. The entire section was scanned at low magnification, and hot spots were preferentially scored. At least 1,000 cells were analyzed in each slide. The cutoff value of 10% were accepted as positive staining is based on the criteria used in the literature for *c-KIT* staining. Staining was considered true positive if the reaction product was localized to the cell membrane alone or to the cell membrane and cytoplasm simultaneously, cytoplasmic staining alone proved to be false-positive in all preabsorption control samples (Went et al., 2004). Staining extent was scored as (1) when 10-50% of the cells were stained, and (2) when >50% of the cells were stained. Staining intensity was evaluated only in positive cases and scored as (1) for weak staining (faint, light yellow), (2) for moderate staining (brown), and (3) for strong staining (dark brown) (Aydin et al., 2008).

PCR-SSCP and direct sequencing

Thirty urinary bladder carcinomas showing intense CD117 staining were selected for *c-KIT* mutation analysis. The formalin-fixed tissues were deparaffinized and the DNA was extracted according to the protocols provided by Qiagen (Basel, Switzerland). We performed PCR-single-strand conformation polymorphism (SSCP) in an effort to analyze mutations in the *c-KIT* gene. PCR was carried out at a final concentration of 1x PCR buffer (10x PCR buffer; 150 mM Tris-HCl, pH 8.0, 500 mM KCl), 50 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.5 mM of each forward and reverse primers, and 1.25 U of 'AmpliTaq Gold' (PE Applied Biosystems, Branchburg, NJ) in a total volume of 20 μl. All PCRs were initiated at 95°C for 9 min, followed by 35 three-step cycles for exon 9-13 of the *c-KIT* gene at 94°C for 30 s, 65°C for 30 s and 72°C for 30s. Two microliters of PCR products were mixed with 20 μl of SSCP gel loading dye, heated for 5 min at 95°C and then chilled on ice. Approximately 6 μl of this mixture was loaded in each well of a 10% acrylamide gel, electrophoresed at 10, 15 and 20°C for 3 h at 300 V, and

the gel subsequently stained with Syber Gold to visualize the PCR product. The primers designed for the PCR and the sequence reactions are listed in Table 1. Small areas of gels from the position of mobility shift bands were cut out by razor blades, immersed in 70 µl distilled water and heated at 90°C for 5 min to extract the DNA from the gels. 4 µl of each extracted DNA solution were amplified by PCR with the same primers used in PCR-SSCP and sequenced to detect mutations using eluted DNA as a template for a Big Dye Terminator Cycle Sequencing Ready Reaction kit, and analyzed on an ABI PRISM 310 Genetic analyzer (Applied Biosystems, Forest City, CA). All mutations were verified by repeat sequence analysis in the sense and antisense directions.

Statistical analyses

Pathological stage was grouped into two subgroups: ≤T2 and >T2. Tumor grade was categorized into two subgroups: ≤GII and >GII. Lymph node metastases were recorded as negative or positive. Bilharzial ova were recorded as present or absent. Data analyses were conducted to assess a) the association between *c-KIT* expression and clinicopathological parameters including patients age, gender, tumor grade, stage, size, lymph node metastasis, Distant metastasis, LVSI, TIL and presence of bilharzial ova. Data were analyzed using SPSS 11.0 software. The X^2 test or Fisher's exact probability test were used to compare categorical variables between two groups. Significance was defined as $P<0.05$ (Dawson and Trapp, 2000).

Results

Clinicopathological features of the studied cases

The age of the studied cases ranged between 39-72 years with median age of 49.6 years. Seventy four patients (61.7%) were ≤50 years old, and 38.3% of patients were >50 years old. Out of 120, ninety six (80%) were males and twenty four (20%) were females with male to female ratio of 4:1. Tumor size ranged from 2.0-7.5 cm (average 4.2 cm), eighty patients out of 120 (66.7%) showed tumor size >4 cm. All patients except four had advanced disease (T2 or above) at presentation. Pathologic stage was identified into the lamina propria T1 (4 patients, 3.3%), muscularis propria T2 (74 patients, 61.7%), perivesical fat T3 (26 patients, 21.7%), and adjacent structures T4 (16 patients, 13.3%) respectively. Among patients, 36.7% (44/120) had lymph node metastases and (LVSI) in 56 of 120 patients (46.7). Eight patients had distant metastases at presentation.

Table 1. Primer Sequence for Kit SSCP Analysis

Exon	Direction	5' → 3'
9	Forward	5'-ATTTATTTTCCTAGAGTAAGCCAGGG-3'
	Reverse	5'-ATCATGACTGATATGGTAGACAGAGC-3'
10	Forward	5'-TGGTAGAGATCCCATCCTGC-3'
	Reverse	5'-TGGGGAGAAAGGGAAAAATAG-3'
11	Forward	5'-TTGTTCTCTCTCCAGAGTGCTC-3'
	Reverse	5'-AAGGTGACATGGAAAGCCC-3'
12	Forward	5'-CAGCACCATCACCACTTACC-3'
	Reverse	5'-AGCAAAAAGCACAACTGGC-3'
13	Forward	5'-AGATGCTCAAGCGTAAGTTC C-3'
	Reverse	5'-CCTGACAGACAATAAAAGGCAG-3'.

Most tumors were moderately (51.7%) or poorly (43.3%) differentiated, whereas only 6 tumors were well differentiated (5.0%). Regarding the tumor site, it represented (40.0%) in the lateral walls of the bladder, (26.7%) in the bladder trigone and 18.3% of cases were multicentric, followed by the dome and bladder neck (15.0%). The mucosa surrounding the tumor showed squamous metaplasia (86.7%), squamous cell carcinoma in situ (16.7%), squamous metaplasia with dysplasia (13.3%), urothelial hyperplasia (18.3%).

IHC analyses of c-KIT expression

C-KIT immunostaining rsults in the urinary bladder SCCs are shown in Table 2 and Figures 1-3

Table 2. *C-KIT* IHC Staining in Urinary Bladder SCC Regarding Expression, Intensity, and Extent of Staining

		SCC
Staining expression No. (%)	Positive	94 (78.3)
	Negative	26 (21.7)
Staining intensity No. (%)	Weak	14 (14.9)
	Moderate	60 (63.8)
	Strong	20 (21.3)
Staining extent No. (%)	>10-50%	40 (42.6)
	>50	54 (57.4)

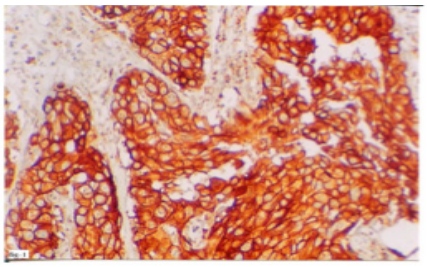

Figure 1. Strong Membranous Immunoreactivity of *c-KIT* in Grade II Squamous Carcinoma of the Urinary Bladder (Hematoxylin and DAB, X 400)

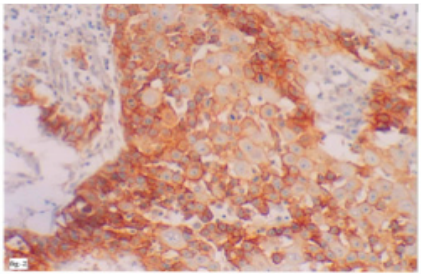

Figure 2. Heterogeneous *c-KIT* Expression of Grade III Squamous Cell Carcinoma (Hematoxylin and DAB, X 200)

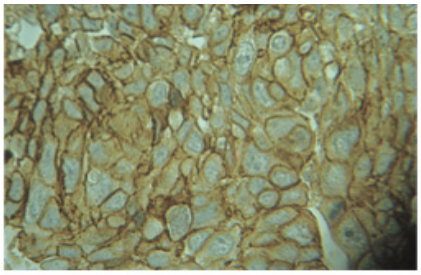

Figure 3. Simultaneous Membranous and Cytoplasmic Immunostaining of *c-KIT* of Intermediate Intensity (Hematoxylin and DAB, X 400)

Staining expression (Table 2): C-KIT expression was localized to the cell membrane alone or to the cell membrane and cytoplasm simultaneously. Its expression was not demonstrated in all specimens of normal urothelium. According to our scoring criteria, positive *C-KIT* expression was demonstrated in 94 of 120 (78.3%) of patients with SCCs of the urinary bladder. 26 samples (21.7%) were negative (20 samples with no staining, six with less than 10% staining).

Staining pattern (Figure 1-3): C-KIT was not expressed in specimens of histologically normal or hyperplastic urothelium and stromal cells surrounding the tumor. Cases of squamous metaplasia showed surface epithelial staining in 26/52 (50.0%). The dysplastic urothelium showed basal and suprabasal expression of c-KIT, but the full thickness of carcinoma in situ was positive. *c-KIT* was expressed homogeneously and intensely in the cell membrane of most cases of SCC (Figure 1); the heterogeneous expression pattern was seen in few cases (Figure 2). In some cases, the staining was detected in the cell membrane and cytoplasm simultaneously (Figure 3).

Staining intensity and extent (Table 2): weak staining intensity (1) was observed in 14/94 (14.9%) of cases and moderate intensity (2) in 60/94 (63.8%), while the remaining 20 (21.3%) cases demonstrated strong (score 3) staining intensity. Regarding the extent of staining, 42.6% (n=40) cases demonstrated positive *c-KIT* staining in > 10-50% of the tumor cells, while 54/94 (57.4%) cases showed overexpression in >50% of the cells.

Table 3. *c-KIT* Expression According to Clinicopathological Characteristics of the Squamous Cell Carcinoma Patients.

Characteristics	Patients No. (%)	*c-KIT* positive No. (%)	P-value
All cases	120	94 (78.3)	
Age			1.0+
≤50 years	74 (61.7)	58 (78.4)	
>50 years	46 (38.3)	36 (78.3)	
Gender			0.71+
Male	96 (80.0)	76 (79.2)	
Female	24 (20.0)	18 (75.0)	
Tumor size			0.004+*
≥4 cm	40 (33.3)	20 (50.0)	
>4 cm	80 (66.7)	74 (92.5)	
Stage			0.35+
≤T2	78 (65.0)	64 (82.1)	
>T2	42 (35.0)	30 (71.4)	
Bilharzial ova			0.000+*
Present	84 (70.0)	80 (95.2)	
Absent	36 (30.0)	14 (38.9)	
Grade			0.30++
≤GII	68 (56.7)	50 (73.5)	
>GII	52 (43.3)	44 (84.6)	
LVSI**			0.79++
Positive	56 (46.7)	44 (78.6)	
Negative	64 (53.3)	50 (78.1)	
TIL***			0.07++
Present	56 (46.7)	38 (67.9)	
Absent	64 (53.3)	56 (87.5)	
Node metastasis			0.12+
Positive	44 (36.7)	40 (42.6)	
Negative	76 (63.3)	54 (57.4)	
Distant metastasis			0.0005+*
Positive	8 (6.7)	6 (63.8)	
Negative	112 (93.3)	2 (21.2)	

*P-value <0.05 is significant, **LVSI,lymphovascular space invasion, ***TIL, tumor infiltrating lymphocytes, +Fisher exact test, ++chi-square test

Comparisons with clinicopathological parameters

The relationship between *c-KIT* expression and various clinicopathologic variables of SCCs are listed in Table 3. Patients with positive Bilharzial ova demonstrated significantly higher rates of *c-KIT* expression as compared to patients without ova (95.2% vs. 38.9%, p=0.000). Most of the *c-KIT* positive immunostaning were seen in tumors >4 cm in diameter. In general, a significant correlation was found between *c-KIT* positive immunostaining and the increased tumor size (p=0.004). Moreover, patients with distant metastasis demonstrated significantly *c-KIT* overexpression as compared to patients without metastasis (63.8% vs. 21.2%, P= 0.0005), On the other hand, there was no significant correlation of *c-KIT* expression within the studied clinicopathological characteristics including age (P=1.0), gender (P=0.71), tumor grade (P=0.30), pathologic T stage (P=0.35), LVSI (P=0.79), TIL (P=0.07) or lymph node metastasis (P=0.12).

Mutational analysis of c-KIT gene

Mutation analysis of exons 9-13 was negative in thirty KIT- positive tumors. No mutational shifted bands were detected in any of the DNA single strands of the studied tumors.

Discussion

Schistosomal urinary bladder SCCs are known to be an aggressive carcinoma de novo; this is suggested by high frequency of mutant p53 expression (73%) and high proliferation index (87%) (Badr et al., 2004). This has led to numerous studies in an effort to understand the cytogenetic and molecular biology of Schistosomal SCCs. One of these studies demonstrated mutations in exons 5-8 of the p53 gene (Warren et al., 1995) while another study demonstrated alterations in the P15 and P16 genes (Swellam et al., 2004). To the best of our knowledge, the current study is the first to evaluate the expression and mutation of *c-KIT* in Schistosomal SCCs.

The *c-KIT* gene encodes a tyrosine kinase receptor related to platelet-derived growth factor (PDGF)/colony-stimulating factor 1 (CSF-1) (Yarden et al., 1987). The *c-KIT* pathway has been shown to be involved in a number of physiological and pathological processes, including hematopoiesis, spermatogenesis, melanogenesis, and oncogenesis (Ullrich and Schlessinger, 1990; Natali et al., 1992a). Two mechanisms of *c-KIT* activation exist: autocrine and/or paracrine activation by its ligands and ligand-independent activation by mutation. Ligand-dependent activation of *c-KIT* depends on the engagement of its ligand (the stem cell factor). The binding of *c-KIT* by stem cell factor induces phosphorylation and activation of the *c-KIT* signal transduction pathway. This type of activation occurs during some normal physiological processes, and during oncogenesis of

some cancers (Krystal et al., 1996; DiPaola et al., 1997). It has been shown that some tumor cells express both *c-KIT* protein and stem cell factor which appear to be capable of protecting the cells against apoptosis (Krystal et al., 1996). Ligand-independent constitutive activation of *c-KIT* involves mutations, mainly in exon 11, the region between the transmembrane and tyrosine kinase domains. *c-KIT* abnormal protein derived from mutation is constitutively activated without engagement by its ligand. Stable transfection of mutant *c-KIT* complementary DNA has been shown to induce malignant transformation, suggesting that the mutations contribute to tumor development (Hirota et al., 1998).

In this study we used *c-KIT* antibody of A4502 because previous data suggested its highest sensitivity without background problems. Moreover, A4502 has the advantage that a specific peptide was available for negative control experiments which blocked specific reactions by adding a surplus of KIT antigen (Went et al., 2004). In previous studies, several cutoff levels of *c-KIT* expression were used, varying from 10 (Sela et al., 2003; Sabah et al., 2003; Pan et al., 2005; Aydin et al., 2008), 20% (Lonardo et al., 2003), 25% (Makhlouf et al., 2002) and even to 50% (Holst et al., 1999). We followed the majority of publications which stated a cutoff level of 10% of tumor cells.

In the present study, 94/120 (78.3%) showed *c-KIT* expression by IHC, patients with positive Bilharzial ova expressed *c-KIT* higher than negative patients (95.2% versus 38.9% respectively, P=0.000). Also, the normal urothelium was negative for *c-KIT* immunostaining which is consistent with previous results (Went et al., 2004), suggesting that *c-KIT* was upregulated in urinary bladder SCCs. A previous study on small cell carcinoma of the urinary bladder demonstrated a *c-KIT* overexpression in 27% of the studied cases (Pan et al., 2005). In another study including 3,000 human tumor tissues, *c-KIT* expression was positive in various tumor entities including GIST (100%), seminoma (84%), malignant melanoma (36%), thyroid follicular carcinoma (23%), large-cell (17%), and small-cell carcinomas of the lung (7%). Urothelial cell carcinomas of the invasive type (pT2-4), noninvasive type (pTa), in addition to sarcomatoid carcinomas and SCCs showed *c-KIT* overexpression of 4%, 15%, 12% and 16% of cases respectively (Went et al., 2004). In contrast to our results, the last study which showed lower overexpression of *c-KIT* in urothelial SCCs in 16 % of cases only (Went et al., 2004), is probably due to the few number of cases studied in their experiment (6 cases).

From another point of view, many studies have shown that many KIT-expressing tumors are not responsive to Imatinib therapy (Johnson et al., 2003). This indicates that the response rate may depend on the presence of KIT mutations in the tumor and potentially also on the location and type of mutation (Longley et al., 2001; Kemmer et al., 2004). Interestingly, our data showed no KIT gene mutations by SSCP analysis in Schistosomal bladder SCCs. This indicates that KIT activation may occur via other genetic pathways which may cooperate with KIT gene. Hence, the various tyrosine kinase inhibitors targeting these proteins might still be of clinical value, even in the absence of KIT genetic mutations. Similar results.has been demonstrated in acute myeloid leukemia (Kindler et al., 2004). In the absence of mutation, KIT kinase expression may be associated with the presence of multiple copies of the wild-type KIT gene in cancer cells, so further studies are required to investigate the possible beneficial effects of Imatinib mesylate in *c-KIT* positive urinary bladder cancers.

In conclusion, the present study demonstrated a high expression of *c-KIT* in urinary bladder SCCs without KIT gene mutations. Further studies are necessary to clarify the mechanisms of *c-KIT* expression in order to be effective in developing therapeutic strategies.

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