WORLD JOURNAL OF SURGICAL ONCOLOGY

CASE REPORT Open Access

Multiple primary malignant neoplasms of the glottis, renal pelvis, urinary bladder, oral floor, prostate, and esophagus in a Japanese male patient: a case report

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Abstract

Owing to recent advances in diagnostic and surgical techniques for cancer, a patient diagnosed with two or more neoplasms is not rare. We report on the case of a 58-year-old male with multiple primary malignant neoplasms, who suffered from three histological types of malignant neoplasm in six organs, namely the glottis, renal pelvis, urinary bladder, oral floor, prostate, and esophagus in chronological order. The first neoplasm was a squamous cell carcinoma of the glottis diagnosed in 2006. The second and third neoplasms were urothelial carcinomas of the right renal pelvis and urinary bladder, respectively, diagnosed in 2008. The remaining three neoplasms were diagnosed in 2010, namely a squamous cell carcinoma of the oral floor, an adenocarcinoma of the prostate, and a squamous cell carcinoma of the esophagus. The glottic cancer and esophageal cancer were treated by external radiation therapy. The malignant neoplasms of the oral floor and those which originated in the urinary tract were surgically resected. All neoplasms except the malignant neoplasm of the oral floor were well controlled. The patient died of cervical lymph node metastasis from the squamous cell carcinoma of the oral floor in January 2011. As far as we know, the present report is the first one on this combination of primary malignant neoplasms.

Keywords: Metachronous, Multiple primary malignant neoplasms, p53 staining, Synchronous

Background

Early detection and development of novel treatment modalities are improving longevity of patients with cancer. Consequently, the increasing possibility of suffering from multiple primary malignant neoplasms (MPMN) is emerging as a common problem for cancer survivors; MPMN were first described by Billroth et al. [1]. In 1932, Warren and Gates proposed three criteria for the diagnosis of a second primary cancer: i) each tumor must present a definite clinical and histological picture of malignancy; ii) each tumor must be histologically distinct; and iii) the probability that one was a metastatic lesion from the other must be excluded [2]. Moertel proposed new definition of MPMN, where synchronous malignancies are those that occur within 6 months of the diagnosis of a previous malignant neoplasm and metachronous ones are those that occur more than 6 months apart [3]. According to the Surveillance, Epidemiology, and End Results (SEER) Program Coding and Staging Manual 2004, synchronous tumors are multiple tumors diagnosed within 2 months of the original/initial diagnosis, and metachronous ones are multiple tumors or lesions that occur more than 2 months after the original/initial diagnosis [4].

In this report, we describe the rare case of a patient who suffered from three histological types of malignant neoplasm in six organs, namely a squamous cell carcinoma (SCC) of the glottis, oral floor, and esophagus, a

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urothelial carcinoma (UC) of the right renal pelvis and urinary bladder, and an adenocarcinoma (AC) of the prostate.

Case presentation

A 58-year-old male, a manager of a tavern, habitual drinker (one bottle of beer a day for 36 years), and heavy smoker (20 cigarettes a day for 33 years), was referred to our department for asymptomatic gross hematuria on February 2008. Two years before, he had been diagnosed with a SCC of the glottis, pT1N0M0 (stage I) which was treated by 70 Gy of external radiation therapy at the University of Tokyo Hospital. Since November 2007, he had been diagnosed with alcoholic liver cirrhosis and had received conservative medical treatment at a nearby hospital. He did not have any family history of malignant neoplasms. An abdominal computed tomography (CT) showed right hydronephrosis and tumor of the right renal pelvis. A retrograde pyelography also supported the findings of the abdominal CT. We conducted cystoscopy prior to the surgery; however, no distinct bladder tumors could be seen. We diagnosed him with right renal pelvic tumor and performed right nephroureterectomy on April 2008. The pathological diagnosis was UC of the right renal pelvis, high-grade, pT3N0M0 (stage III) including carcinoma *in situ* in the renal pelvis. Adjuvant chemotherapy was not administered due to chronic liver dysfunction and thrombocytopenia. Except for alcoholic liver cirrhosis, no other medical conditions or treatment-induced immunosuppression were recorded. After the surgery, follow-up was conducted by a cystoscopy every 3 months and an abdominal CT scan every 6 months.

In September 2008, cystoscopy revealed multiple bladder tumors. He underwent transurethral resection of bladder tumor (TURBT) in October 2008. The pathological finding was UC of the urinary bladder, high-grade, pTisN0M0 (stage 0is). He received weekly intravesical instillation of bovine Bacille-Calmette Guérin (Connaught strain, 81 mg/once a week) for 8 weeks to prevent recurrence of the bladder tumor.

In October 2009, he underwent TURBT again for recurrent bladder cancer. The pathological finding was UC of the urinary bladder, high-grade, pTis. UC was also found at the prostatic urethra, high-grade, pTis. We recommended a radical cystectomy and urethrectomy with urinary diversion. On December 2009, he became aware of a mass beneath his tongue. We found a papillary superficial tumor on the left side of the oral floor. Upon consultation with the department of oral surgery, the tumor was resected in January 2010. The pathological finding was a well differentiated SCC of the oral floor, pT1N0M0 (stage I). Just 2 weeks after the oral surgery, we performed radical cystectomy and urethrectomy with an ileal conduit urinary diversion. The pathological findings were UC *in situ* involving the urethra and prostatic duct, high-grade, pTisN0M0 (stage 0is) and incidental AC of the prostate, Gleason score 3 + 3, pT1aN0M0 (stage IIa).

In March 2010, 6 weeks after resection of the tumor of the oral floor, a head and neck CT scan suggested a submental lymph node swelling. He underwent bilateral cervical lymphadenectomy and 17 lymph nodes were resected. As a result, only one submental lymph node was diagnosed as a metastasis of well differentiated SCC of the oral floor. On June 2010, he experienced dysphagia. During an upper gastrointestinal endoscopy, biopsies for erosive lesions at the upper and middle portions of the esophageal mucosa were performed. The pathological finding was a well differentiated SCC of the esophagus, pT1N0M0 (stage I) and external radiation therapy with total 68.4 Gy was performed. From July 2010 onwards, cervical lymph node metastasis grew rapidly and gradually caused a tracheal obstruction. He underwent tracheotomy on November 2010. After receiving palliative care, he died of cancer cachexia on January 2011. An autopsy was not performed.

The malignant neoplasms in six organs are listed sequentially in Table 1. Figure 1 shows hematoxylin-eosin staining and immunohistochemical staining using anti-p53 antibody of SCC and UC. It is reported that nuclear immunoreactivity of p53 is a good surrogate of *TP53* mutations [5]. Immunoreactivity of p53 in tumor cells was different among SCCs; the glottic cancer and esophageal cancer in the upper portion were diffusely positive, while the oral cancer, esophageal cancer in the middle portion, and cervical lymph node metastasis of the oral cancer were completely negative. As for the renal pelvic cancer and bladder cancer, immunoreactivity of p53 was weakly positive in scattered cancer cells.

Discussion

The actual risk of developing MPMN in Japan is unknown since cancer registration systems have yet to be legislated in Japan. Demandante et al. reviewed the literature published from 1966 to 2000 and reported that the prevalence of MPMN varied from 0.734% to 11.7% [8]. Based on epidemiological data from the National Cancer Institute's SEER Program in 2003, it is estimated that approximately 16% of new cancers reported to their registry represent a second- or high-order malignancy [9]. The Osaka Cancer Registry, one of the regional cancer registration systems in Japan, has been operating since December 1962 and has accumulated about one million cancer incidence data in Osaka prefecture [10]. Using the Osaka Cancer Registry data, Tabuchi et al. reported that 10-year cumulative risk of metachronous second primary cancer in Japanese male patients was 10.2% at 50 to 59 years of age, 16.2% at 60 to 69 years of age, and 21.8% at 70 to 79 years of age [11].

Table 1 List of three histological types of malignant neoplasm in six organs

Month/Year	Organ	Histology	Immunoreactivity of p53	ICD-O-3		pT-stage
				Site code	Histology code	
April/2006	Glottis	SCC	Diffusely positive	C320	8071/31	pT1
April/2008	Renal pelvis	UC	Weakly positive	C659	8120/33	pT3
October/2008	Urinary bladder	UC	Weakly positive	C675	8120/32	pTa
				C676	8120/23	pTis
January/2010	Oral floor	SCC	Completely negative	C041	8071/31	pT1
January/2010	Prostate	AC	Not examined	C619	8140/32	pT1a
June/2010	Esophagus			C154	8070/31	pT1
	Upper: 22 cm from incisor	SCC	Diffusely positive			
	Middle: 30 cm from incisor	SCC	Completely negative			

We classified malignant neoplasms by the International Classification of Diseases for Oncology version 3 (ICD-O-3) [6]. The T-stage was based on the Union for International Cancer Control TNM classification of malignant tumors (7th Edition) [7].
SCC, Squamous cell carcinoma; UC, Urothelial carcinoma; AC, Adenocarcinoma.

This is an extremely rare case of MPMN in terms of the number of malignant neoplasms and their combination. We could not find out any case reports of the same combination of neoplasms on either the PubMed or the Japan Medical Abstracts Society databases. It is difficult to estimate the risk of quintuple cancers; however, Rabbani et al. estimated the risk of MPMN in patients with renal cell carcinoma [12]. Of 551 patients, they reported that the incidence of double cancer was 26.9%, triple cancer was 6.2%, quadruple cancer was 1.1%, and quintuple cancer was 0.2% [12]. Liu et al. studied the types of second primary malignancies among the patients with head and neck cancer [13]. The types of second primary malignancy with adjusted hazard ratio (95% confidence interval) were esophageal cancer 3.47 (2.40 to 5.03), prostate cancer 0.94 (0.45 to 1.95), bladder cancer 0.90 (0.39 to 2.10), among others [13]. Powell et al. considered the prognosis of patients with MPMN [14] by dividing patients with MPMN into synchronous and metachronous cases according to the classification proposed by Moertel [3]. Overall survival ratio was significantly lower in the patients with synchronous MPMN than those with metachronous MPMN (adjusted hazard ratio 0.50, P <0.001).

Regarding the etiology of multiple primary malignancies, several factors have been incriminated such as genetic, hormonal (e.g., sex steroid), iatrogenic (e.g., chemotherapy, radiation therapy, hormonal and immunosuppressive medications), and immunologic factors [15,16]. In particular, tobacco smoking causes cancers of the lung, oral cavity, naso-, oro-, and hypopharynx, nasal cavity and accessory sinuses, larynx, esophagus, stomach, pancreas, colorectum, liver, kidney (body and pelvis), ureter, urinary bladder, uterine cervix and ovary (mucinous), and myeloid leukemia [17]. In the present case, alcoholic liver cirrhosis, tobacco smoking, and external radiation therapy for glottic cancer may have played a role in MPMN development. Several investigators have observed that patients with cancers of these sites developed a second primary cancer within the same anatomic region [18-20]. Wynder et al. publicized that continuation of smoking habit after diagnosis of the index cancer increased the risk for development of a second primary lesion [21]. Heavy alcohol consumption also predisposes certain sites to tumorigenesis, but primarily in conjunction with tobacco usage. In spite of cessation of smoking and drinking habits after the diagnosis of the glottic cancer, our patient developed MPMN.

Hereditary forms of adult cancers are also frequently associated with multiple primary cancers [22,23]. If the nature of the hereditary predisposition is similar to that of childhood cancers, i.e., an inherited mutation that reduces the subsequent number of mutations necessary in each cell of the target organ, then such individuals may have a high rate of spontaneous tumors and a unique sensitivity to environmental agents [24]. Kotnis et al. conducted a case control study to assess genetic predisposition in a biologically-enriched clinical model system of tobacco-related cancers occurring as MPMN. They found that tobacco habit and three genetic polymorphisms, including *Tp53* (Arg72Arg), *XRCC1* (Arg399His), and *meH* (Tyr113His), formed the best model for developing tobacco-associated MPMN [25].

In 1953, Slaghter et al. proposed field cancerization, a process whereby the epithelial lining has been continuously exposed to tobacco and/or alcohol, leading to extensive premalignant and malignant cytologic changes and an increased risk for multiple independent tumor development [26]. Field cancerization in the epithelium has become accepted as playing a role in the development of MPMN in the same system; however, molecular studies have supported an alternative theory of a common

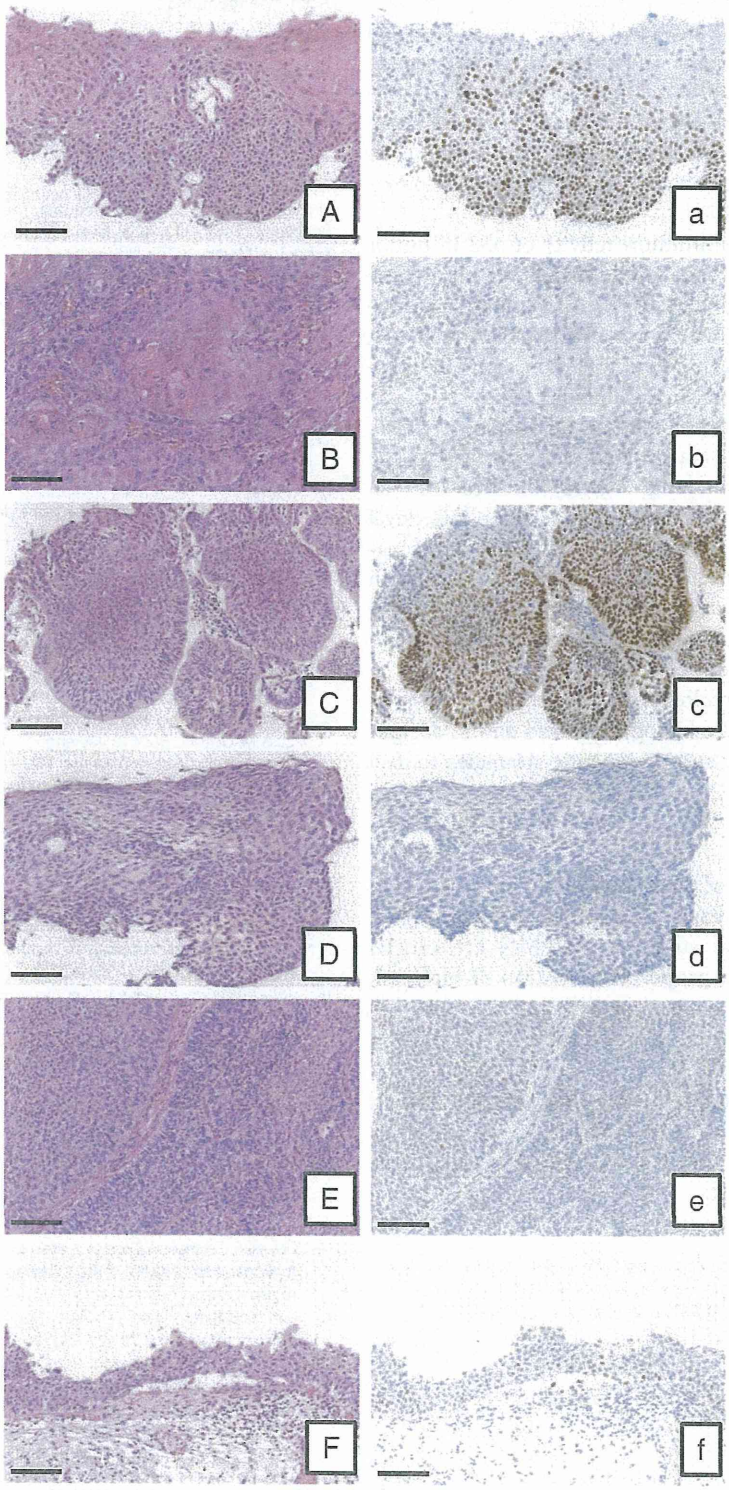

Figure 1 The expression of p53 in SCC and UC. Tissue samples were stained by hematoxylin-eosin (left column) and anti-p53 antibody diluted 1:50 (Clone DO-7, Leica Biosystems, Wetzlar, Germany; right column). Immunoreactivity of p53 in tumor cells was identified by nuclear brown color. **(A and a)** Glottic cancer; **(B and b)** Oral cancer; **(C and c)** Esophageal cancer (upper portion); **(D and d)** Esophageal cancer (middle portion); **(E and e)** Renal pelvic cancer; **(F and f)** Bladder cancer. Scale bars, 100 μm.

clonal origin [27]. These studies suggest that a proportion of second primary head and neck SCC is clonally related to an index tumor, despite the presence of intervening normal mucosa and significant separation by normal mucosa. The same types of carcinogens and oncogenes seem to be responsible for the development of neoplasms in cases of other associations such as breast and endometrial/ovarian tumors [28-31].

In the present case, immunoreactivity of p53 in tumor cells was different among SCCs, suggesting that these cancers were polyclonal, although distinct genetic abnormalities including loss of heterogeneity, gene mutations, and influence of oncogenic viral infection may be necessary to definitely determine polyclonality.

Another point of interest may be the origin of UC of the renal pelvis and urinary bladder. According to the report from the International Agency for Research on Cancer [32], high-grade UC is likely to have high level amplifications and *TP53* mutations. In the present patient, however, immunoreactivity of p53 in the both high-grade UCs was very weak. The atypical finding ironically suggests UC of the renal pelvis and urinary bladder might be of monoclonal origin.

Modern molecular biologic techniques might help in the understanding of the biology involved and in designing effective diagnostic and therapeutic strategies to deal with these tumors.

Conclusions

A rare case of MPMN of three histological types of malignant neoplasm in six organs was reported. Epidemiological and clinicopathological studies of MPMN are crucial for early detection and proper intervention of high risk patients.

Consent

The study was conducted with the approval of the Ethics Committee of the University of Tokyo. Written informed consent was obtained from the patient for publication of the case report.

Abbreviations

AC: Adenocarcinoma; CT: Computed tomography; MPMN: Multiple primary malignant neoplasms; SCC: Squamous cell carcinoma; SEER: Surveillance, Epidemiology, and End Results; TURBT: Transurethral resection of bladder tumor; UC: Urothelial carcinoma.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YM1 and MS were involved in acquisition of clinical documents and drafting the manuscript. TM reviewed the pathological slides and helped to draft the manuscript. YM1, MS, YM2, TF, HF, HN, HK, and YH involved in the treatment of this patient. MS conceived of the study. YM2, TF, HF, TN, HN, HK, and YH were involved in the review of literature and completing the manuscript. YM1, MS, TM, YT, and YH wrote a revised manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

No funding source supported this case report. We thank to all medical staff of the University of Tokyo Hospital who contributed to treat and care for the present patient.

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Received: 6 February 2014 Accepted: 15 September 2014
Published: 22 September 2014

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doi:10.1186/1477-7819-12-294
Cite this article as: Mukaiyama *et al.*: Multiple primary malignant neoplasms of the glottis, renal pelvis, urinary bladder, oral floor, prostate, and esophagus in a Japanese male patient: a case report. *World Journal of Surgical Oncology* 2014 12:294.

Selective Inhibitory Effect of Imidafenacin and 5-Hydroxymethyl Tolterodine on Capsaicin Sensitive C Fibers of the Primary Bladder Mechanosensitive Afferent Nerves in the Rat

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Purpose: Imidafenacin and fesoterodine are used to treat overactive bladder. Imidafenacin, fesoterodine and its active metabolite 5-hydroxymethyl tolterodine are muscarinic receptor antagonists. It is believed that these agents act on afferent nerves in addition to smooth muscle. We investigated the effects of imidafenacin and 5-hydroxymethyl tolterodine on single unit afferent activity of mechanosensitive capsaicin sensitive and insensitive primary bladder afferent nerve fibers in rats.

Materials and Methods: Female Sprague Dawley® rats were anesthetized. Single unit afferent activity was recorded from the L6 dorsal roots and classified by conduction velocity as that of Aδ or C fibers. After measuring control single afferent activity during constant filling cystometry the procedure was repeated with intravenous administration of imidafenacin (0.3 to 30 μg/kg) or 5-hydroxymethyl tolterodine (0.01 to 1 mg/kg) at cumulative doses with or without intravesical capsaicin or oxotremorine-M instillation.

Results: A total of 116 single unit afferent fibers were isolated from 91 rats, including 19 Aδ and 97 C fibers. Neither imidafenacin nor 5-hydroxymethyl tolterodine significantly affected the overall single unit afferent activity of Aδ or C fibers. Based on capsaicin sensitivity C fibers were divided into capsaicin sensitive and insensitive groups. Each antimuscarinic inhibited the single unit afferent activity of capsaicin sensitive C fibers but not of capsaicin insensitive C fibers at the highest dose. Moreover, oxotremorine-M facilitated single unit afferent activity in a proportion of C fibers. The facilitated single unit afferent activity was significantly attenuated by the highest dose of imidafenacin.

Conclusions: These findings demonstrate that imidafenacin and 5-hydroxymethyl tolterodine can selectively inhibit capsaicin sensitive C fibers among mechanosensitive bladder afferents by antagonizing bladder muscarinic receptors.

Key Words: urinary bladder; neurons, afferent; capsaicin; imidafenacin; 5-hydroxymethyl tolterodine

Abbreviations and Acronyms

5-HMT = 5-hydroxymethyl tolterodine

ATP = adenosine triphosphate

CAP = capsaicin

mAChR = muscarinic acetylcholine receptor

OAB = overactive bladder

Oxo-M = oxotremorine-M

RTX = resiniferatoxin

SAA = single unit afferent activity

TRPV = transient receptor potential vanilloid

Accepted for publication September 4, 2014.

Supported by Ministry of Education, Culture, Sport, Science and Technology of the Japanese Government Grants-in-Aid for Scientific Research 40159588 (YI) and 80595257 (NA), and Kyorin Pharmaceutical and Pfizer research grants.

Study received University of Tokyo institutional animal care and use committee approval.

* Financial interest and/or other relationship with Pfizer, Astellas, Kyorin, Kissei, Ono and Taiho.

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‡ Financial interest and/or other relationship with Astellas, Asahi Kasei, Kissei, Kyorin, Ono, Taiho, Pfizer, Nippon-Shinyaku, RaQualia, Eli Lilly Japan and Daiichi-Sankyo.

ANTIMUSCARINIC agents such as imidafenacin and fesoterodine have been used as first line pharmacological treatment of OAB.[1] Imidafenacin has higher affinity for the M1 and M3 subtypes than for the M2 receptor subtype[2] with organ selectivity for the bladder.[3] Fesoterodine and its

0022-5347/15/1934-0001/0
THE JOURNAL OF UROLOGY®

http://dx.doi.org/10.1016/j.juro.2014.09.005
Vol. 193, 1-10, April 2015
Printed in U.S.A.

Dochead: Investigative Urology FLA 5.2.0 DTD ■ JURO11781_proof ■ 19 December 2014 ■ 8:21 pm ■ EO: JU-14-757

active metabolite 5-HMT (previously named SPM 7605) block all mAChR subtypes in the human bladder.[4]

The pharmacological action of antimuscarinics is presumed to be the suppression of detrusor contractions.[1] However, recent observations indicated that oxybutynin and darifenacin, which are nonselective and M3 receptor subtype selective antimuscarinic agents, respectively, inhibited the activity of mechanosensitive primary bladder afferent nerves in rats.[5,6] RTX and CAP can activate TRPV1, which is expressed on urothelial cells and bladder primary afferent fibers that run suburothelially.[7] Our previous studies demonstrated that approximately a third and two-thirds of mechanosensitive bladder C-fiber afferents can be classified as CAP sensitive and insensitive, respectively, and TRPV4 agonists and exogenous ATP activated only CAP insensitive C fibers.[8,9] Recent animal studies revealed that imidafenacin and tolterodine, of which the effects are mediated by the active metabolite 5-HMT, improved cerebral infarction induced detrusor overactivity in rats by suppressing RTX sensitive C fibers.[10,11] The antidiuretic effect of imidafenacin but not of atropine occurs through the activation of RTX sensitive C fibers in the rat bladder.[12] These results suggest that imidafenacin and 5-HMT act on bladder afferent function through RTX sensitive C fibers and possibly TRPV1 mediated C fibers.

We investigated the direct effect of imidafenacin and 5-HMT on the SAA of primary mechanosensitive bladder afferent nerves and determined the relationship with CAP sensitivity in urethane anesthetized rats.

METHODS

Animals

We used 111 adult female Sprague Dawley rats at ages 9 to 11 weeks weighing 180 to 250 gm. Rats were maintained under standard laboratory conditions with a 12:12-hour light-dark cycle and free access to food and water. The protocol was approved by the University of Tokyo institutional animal care and use committee and conformed to NIH (National Institutes of Health) guidelines for the care and use of experimental animals.

Afferent Measurement Experimental Procedure

Rats were anesthetized with urethane (1.2 gm/kg intraperitoneally). Body temperature was maintained at 38C by a heated blanket. Single afferent fiber measurements were made as previously described.[8,9] Briefly, the left pelvic nerve was dissected from the surrounding tissue proximal to the major pelvic ganglion. A pair of silver electrodes was placed around the pelvic nerve. A PE-50 catheter (Clay-Adams®) was inserted in the bladder. The 2 L6 dorsal roots were cut near the entrance to the spinal cord after laminectomy. Fine filaments were dissected from the left L6 dorsal root and placed across shielded bipolar silver electrodes. Clearly different unitary action potentials of afferent fibers originating from the bladder were identified by electrical stimulation of the left pelvic nerve and bladder distension with saline using the Spike2 (http://ced.co.uk/) impulse shape recognition program. Action potentials of a maximum of 3 fibers were investigated at the same time during a single bladder filling. Conduction velocity of the identified action potential was calculated from the latency of the response to electrical stimulation and the conduction distance between stimulation and recording sites, which was based on our anatomical data. Fibers were grouped based on conduction velocity. Those with a conduction velocity of less than 2.5 m per second and 2.5 or greater were considered to correspond to unmyelinated C and myelinated Aδ fibers, respectively.[13]

To facilitate CAP permeability after intravesical instillation protamine sulfate solution (10 mg/ml, 0.3 ml) was intravesically instilled and kept in the bladder for 60 minutes just before measurement. Our previous studies demonstrated that this does not lead to any significant difference in bladder compliance or SAA before or after protamine sulfate exposure.[9] In contrast, a study showed that the intravesical Oxo-M sites of action are likely the muscarinic receptors near the lumen based on the fast onset of action of intravesical Oxo-M on cystometry and the known properties of Oxo-M, a quaternary structure and hydrophilic properties.[14] Considering this information, we did not use protamine sulfate to facilitate Oxo-M permeability. SAA was recorded during constant filling cystometry using saline at a rate of 0.08 ml per minute. Filling continued until 30 cm H_2O intravesical pressure was attained. Bladder compliance was calculated between the start and end of bladder filling. The afferent activity caused by pelvic nerve stimulation was also recorded before and after bladder filling, and confirmed to correspond to that caused by bladder filling.

At the beginning of the experiments recording was repeated 3 consecutive times at 5-minute intervals to evaluate reproducibility. The third recording served as the control (before drug administration) value. Five experimental protocols were subsequently performed in separate rats. 1) Imidafenacin (0.3, 3 and 30 μg/kg cumulatively) or 5-HMT (0.01, 0.1 and 1 mg/kg cumulatively) was administered intravenously. Three minutes after each administration the 3 cycle recordings were performed to evaluate the dose dependence of the immediate drug effect during each 5-minute interval (fig. 1, *A*). [F1] 2) Imidafenacin was administered only at the highest dose (30 μg/kg intravenously). At 20 minutes after administration recording was performed to evaluate time dependence (fig. 1, *B*). 3) Higher doses of imidafenacin (cumulatively 3 and 30 μg/kg intravenously) or 5-HMT (cumulatively 0.1 and 1 mg/kg intravenously) were administered. Three minutes after each dose 2 cycle recordings were performed. CAP (10^{-5} M) was then instilled in the bladder to evaluate the relationship to CAP sensitivity (fig. 1, *C*). 4) Oxo-M (25 μM) was instilled in the bladder at 0.04 ml per minute for 8 minutes to evaluate whether imidafenacin would antagonize mAChR

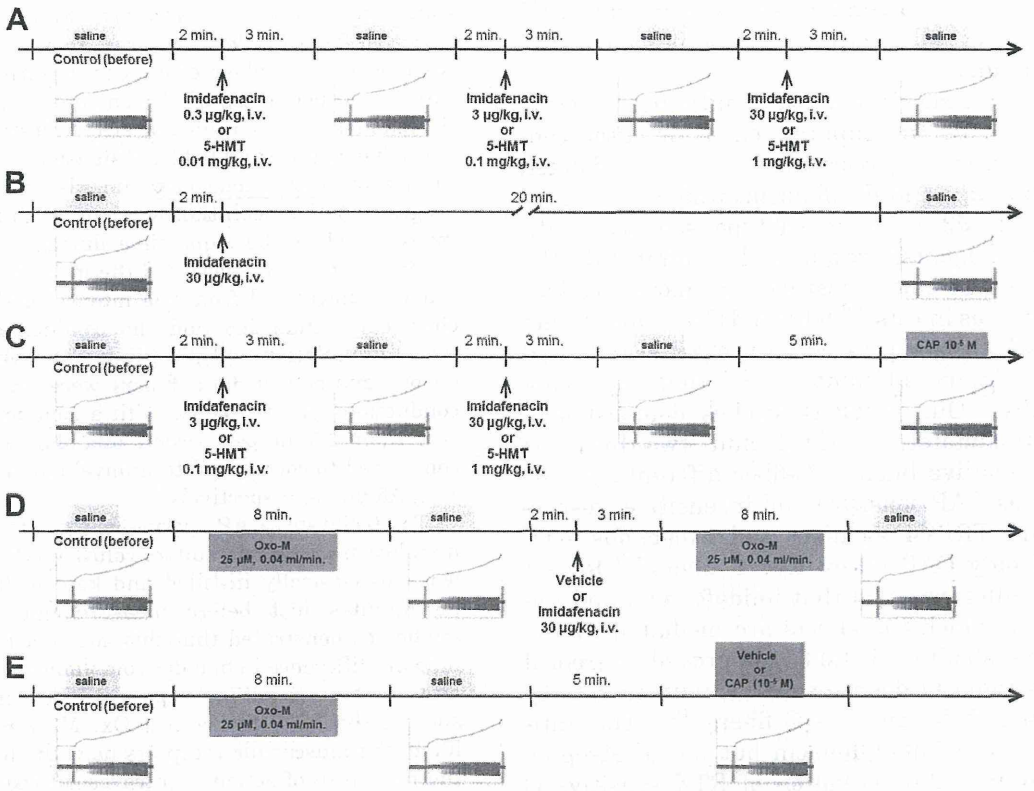

Figure 1. Experimental model and procedures, including cumulative drug with 5 minutes between each recording (*A*), single imidafenacin administration with measurement repeated 20 minutes later (*B*), drug and CAP with 5 minutes between each recording (*C*), Oxo-M and vehicle or imidafenacin (*D*), and Oxo-M and subsequent vehicle or CAP (*E*). *i.v.*, intravenously.

activation by Oxo-M. After Oxo-M instillation if C-fiber SAA was facilitated compared to that before instillation this fiber was defined as Oxo-M sensitive. Vehicle or imidafenacin (30 µg/kg) was administered intravenously 3 minutes before the second Oxo-M instillation (fig. 1, *D*). 5) To address whether Oxo-M facilitates distension evoked firing of CAP sensitive and CAP insensitive afferents Oxo-M (25 µM) was instilled in the bladder at 0.04 ml per minute for 8 minutes. After Oxo-M instillation if C-fiber SAA was facilitated compared to the control (before instillation), this fiber was defined as Oxo-M sensitive. Five minutes later vehicle or CAP (10^{-5} M) was instilled intravesically to evaluate the effect of CAP on Oxo-M sensitive or insensitive C-fiber activity (fig. 1, *E*).

The relationship of nerve activity to pressure was established by comparing nerve activity and intravesical pressure at 1-second intervals. These values were subsequently averaged at a 5 cm H_2O pressure interval during the filling phase. Average total unitary activity was calculated as a function of intravesical pressure. Afferent nerve activity is shown in Hz and a percent of control activity based on pressure integrated for the whole filling phase.

C fibers were classified as CAP sensitive or insensitive based on SAA increases over those of controls at CAP instillation with 150% considered the minimum threshold for sensitivity.[8,9] Classification as Oxo-M sensitive or insensitive was defined the same way.

Drugs

We used protamine sulfate, CAP (Sigma-Aldrich®), Oxo-M (Tocris Bioscience, Bristol, United Kingdom), imidafenacin (4-(2-methyl-1*H*-imidazol-1-yl)-2,2-diphenylbutanamide) and 5-HMT (R-enantiomer: 2-((R)-3-diisopropylammonium-1-phenylpropyl)-4-(hydroxymethyl)phenol). Protamine sulfate was dissolved in distilled water. CAP was dissolved in absolute ethanol as a stock solution (10^{-3} M) and stored at −80C. Drugs were subsequently diluted on the day of the experiment using saline. Oxo-M was dissolved in saline. Imidafenacin was dissolved in saline with 1 M hydrochloric acid, subsequently neutralized with 1 M sodium hydroxide and serially diluted to desired concentrations. We dissolved 5-HMT (10 mg/ml) in 10% *N,N*-dimethylacetamide, 10% Cremophor® and 80% saline. Subsequent dilutions were made in saline. Doses were chosen according to previous studies in rats.[4,8,9,15–17]

Statistical Analysis

All data are shown as the mean ± SEM. Results of comparisons between 2 groups were analyzed using the paired or unpaired Student t-test. Results of multiple comparisons with the control (before drug administration) were analyzed by 1-way ANOVA followed by the Dunnett (repeated measures) or Friedman test followed by the Tukey test. Results of multiple comparisons between groups were analyzed by 2-way ANOVA followed by

the Tukey test with $p < 0.05$ considered statistically significant.

RESULTS

Bladder Compliance

Bladder compliance significantly increased after moderate and high doses of imidafenacin and 5-HMT as well as CAP (table 1). However, bladder compliance did not significantly change 20 minutes after single administration of the highest dose of imidafenacin (table 1). In addition, after Oxo-M instillation bladder compliance did not significantly change regardless of pretreatment with vehicle or imidafenacin, or subsequent instillation of vehicle or CAP (table 2).

Primary Mechanosensitive Bladder Afferent SAA

We isolated a total of 139 single unit afferent fibers from 111 rats. Of the units 19 and 120 corresponded to the criteria for myelinated Aδ and unmyelinated C fibers (mean conduction velocity 4.23 ± 0.40 and 1.71 ± 0.04 m per second, respectively).

Neither cumulatively administered imidafenacin nor 5-HMT significantly affected the SAA of Aδ or C fibers even at the highest dose when evaluated 3 minutes after each dose (supplementary table, http://jurology.com/, and fig. 2, *A* and *B*). In addition, 20 minutes after the highest dose of imidafenacin was administered neither Aδ nor C-fibers SAA was significantly changed (supplementary table, http://jurology.com/, and fig. 2, *C*).

C-fiber afferent activity was divided into 2 groups based on CAP sensitivity. Of 45 discriminated C-fiber single units 19 and 26 were classified as CAP sensitive and insensitive, respectively. Upon imidafenacin or 5-HMT administration CAP sensitive fiber sensitivity decreased significantly at the highest dose (30 μg/kg or 1 mg/kg, respectively, figs. 3, *A* and *C*, and 4, *A*, *C*, *D* and *F*). However, CAP insensitive C-fiber activity showed no significant changes after administration of either drug (supplementary table, http://jurology.com/, and figs. 3, *B* and *D*, and 4, *B*, *C*, *E* and *F*).

In the Oxo-M instillation study C fibers were also divided into 2 groups, that is 15 Oxo-M insensitive and 32 Oxo-M sensitive fibers (100% vs 94% and 100% vs 209% of control activity, respectively, before vs after Oxo-M instillation) (fig. 1, *D* and *E*). In Oxo-M sensitive fibers the facilitatory responses of C-fiber SAA to Oxo-M rather slightly increased after intravenous vehicle administration (figs. 5, *A* and 6, *A* and *C*). In contrast, the SAA response significantly decreased after the highest dose of imidafenacin (30 μg/kg intravenously) (supplementary table, http://jurology.com/, and figs. 5, *B*, and 6, *B* and *D*).

In Oxo-M sensitive C fibers the facilitated afferent activities induced by Oxo-M were significantly decreased upon the second measurement after vehicle instillation at a 5-minute interval (fig. 7, *A* and *C*). Seven of 9 Oxo-M sensitive C fibers were further facilitated by CAP instillation. However, another 2 fibers were not facilitated and the overall response was significantly increased (supplementary table, http://jurology.com/, and fig. 7, *B* and *D*). In contrast, intravesical instillation of CAP did not significantly increase Oxo-M insensitive C-fiber activity (supplementary table, http://jurology.com/, and fig. 8).

Table 1. *Bladder compliance before and after drug and CAP administration*

	Imidafenacin	5-HMT
Cumulative administration + measurement at 5-min intervals		
No. rats	14	9
Dose:		
Low	0.3 μg/kg	0.01 mg/kg
Middle	3 μg/kg	0.1 mg/kg
High	30 μg/kg	1 mg/kg
Mean ± SEM bladder compliance (ml/cm H_2O):		
Control	0.0214 ± 0.0010	0.0146 ± 0.0012
Low dose	0.0227 ± 0.0011	0.0160 ± 0.0014
Middle dose	0.0243 ± 0.0014*	0.0173 ± 0.0016†
High dose†	0.0257 ± 0.0015	0.0180 ± 0.0015
Bolus + measurement 20 mins after administration		
No. rats	14	–
Dose	30 μg/kg	–
Mean ± SEM bladder compliance (ml/cm H_2O):		–
Control	0.0218 ± 0.0009	
Dose	0.0233 ± 0.0010	
Higher doses, CAP (10^{-5} M) + measurement at 5-min intervals		
No. rats	18	15
Dose:		
Middle	3 μg/kg	0.1 mg/kg
High	30 μg/kg	1 mg/kg
Mean ± SEM bladder compliance (ml/cm H_2O):		
Control	0.0157 ± 0.0006	0.0142 ± 0.0006
Middle dose	0.0167 ± 0.0006*	0.0152 ± 0.0007
High dose†	0.0174 ± 0.0006	0.0156 ± 0.0007
CAP†	0.0181 ± 0.0010	0.0174 ± 0.0008

* Significantly different vs control (Friedman and Tukey tests $p < 0.05$).
† Significantly different vs control (Friedman and Tukey tests $p < 0.01$).

DISCUSSION

It was suggested that during the storage phase there is ongoing acetylcholine release from nerves and/or urothelium, acting on mAChRs located on afferent nerves, which may initiate the micturition reflex and contribute to OAB symptoms.[18,19] Previous studies in an experimental model similar to that in the current study showed that intravenous administration of the antimuscarinic agents oxybutynin and darifenacin could inhibit Aδ and C-afferent fiber SAA.[5,6]