as adjuvant therapy for NMIUCB. However, in this previous study, Kubota et al. [13] reported a significantly more favorable recurrence rate in patients with NMIUCB who received adjuvant UFT therapy compared to that in those without any anticancer therapy. This discrepancy may be explained by the different characteristics of patients included in these 2 studies. In fact, Kubota et al. [13] showed a marked prophylactic effect of adjuvant UFT therapy in patients with a single small tumor less than 1 cm in diameter, while approximately 50 and 35% of patients included in the present study had multiple tumors and large tumors greater than 3 cm in diameter, respectively.

UFT has been shown to be resolved into GBL and its acid form, γ-hydroxybutyric acid, which exists in chemical equilibrium under physiological conditions [18]. Recently, GBL was demonstrated to inhibit tumor-induced angiogenesis based on a dorsal air sac assay performed in mice with 5 kinds of cancer cell lines [15]. In the present study, the serum level of GBL in the treatment group was significantly greater than that in the control group. However, there were no significant differences in the serum level of VEGF in addition to those of other major angiogenic factors, including bFGF, PDGF and IL-8, between these 2 groups. Based on our present findings, therefore, the anti-angiogenic activity of GBL might be exerted independently of major angiogenic factors, including VEGF. Nagai et al. [17] however, reported that serum GBL levels, which increased following the administration of UFT, inhibited the angiogenesis induced by VEGF in patients with advanced cervical cancer. That is, VEGF protein expression in approximately 60% of cervical cancer tissues was decreased by UFT therapy, and there was an inverse correlation between changes in serum GBL and VEGF levels after treatment with UFT. This is likely to be affected by several factors, such as the dose of UFT, origin of the cancer and method of assay, and whether VEGF is involved in the inhibition of angiogenesis induced by GBL. Accordingly, further studies are necessary to clarify this issue.

Here, we would like to emphasize the limitations of this study. First, a sample size of 48 patients for such a common disease as NMIUCB is not large enough, and the observation period in this series might have been too short to evaluate the actual recurrence rate. Second, the effects of UFT on angiogenesis were evaluated based on serum levels of angiogenic factors, which may not precisely reflect the production of these factors by tumor cells. Finally, this study failed to show the preventive effect of the adjuvant use of UFT on intravesical recurrence following TUR of NMIUCB despite the significant increase in GBL induced by UFT T. Therefore, it is necessary to develop a more optimal schedule of adjuvant UFT therapy to effectively reduce the proportion of patients developing intravesical recurrence. Considering several studies showing the important role of angiogenesis in the intravesical recurrence of NMIUCB [19, 20], one possible approach is to increase the dose of UFT up to 600 mg/d for all patients in order to achieve a more potent inhibitory effect on the angiogenic activity of NMIUCB. In fact, Shida et al. [14] reported that the proportion of responders in patients with bladder cancer receiving 600 mg/d of UFT was twice as high as that in those receiving 300 mg/d of UFT in Phase II studies.

In conclusion, adjuvant UFT therapy in patients with NMIUCB significantly induced its metabolite, GBL, accompanied by no significant changes in major angiogenic factors, including bFGF, PDGF and IL-8, and failed to reduce postoperative intravesical recurrence. Therefore, we should consider enhancing the anti-angiogenic effect of GBL by using an alternative administration schedule of UFT to more effectively prevent intravescial recurrence of NMIUCB following TUR.

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原　著　Original Article

筋層非浸潤性膀胱癌における経尿道的膀胱腫瘍切除術（TURBT）後塩酸ピラルビシン（THP）3日間連続膀胱内注入療法の膀胱内再発に関する検討

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要旨：

（目的）筋層非浸潤性膀胱癌と診断され，TURBT後塩酸ピラルビシン（THP）の膀胱内注入を術当日から3日間連続施行した症例を対象として，膀胱内非再発率，再発関連因子などを後ろ向きに分析し今後の治療に役立てることを目的とした．

（対象と方法）1995年3月から2009年4月の間に，膀胱CISの併発がなくかつ上部尿路上皮癌の合併や既往歴のない初発未治療筋層非浸潤性膀胱癌と診断され，TURBT後塩酸ピラルビシン（THP）30mg/注射用水40mlの膀胱内注入を術当日，翌日，翌々日の3日間連続施行した184症例を対象とした．腫瘍の数，腫瘍サイズ，異型度，T因子と再発との関係を単変量および多変量解析で検討した．経過観察期間中央値は55.1カ月である．

（結果）症例の大部分がEAUガイドラインによる再発リスク分類の中リスク例に相当した．非再発率は2相性に変化し，治療後1年半から2年間は急峻に低下し，その後はなだらかな低下を示した．1，2，3，5年非再発率は，82.7%，75.3%，72.3%，67.4%であった．日本版再発リスク分類の中リスク群から，Taかつlow gradeかつ腫瘍数2～4個を低中リスク群として3年非再発率を求めると85.1%であった．同様に，EAUガイドラインを参考とした分類で，中リスク群を再発スコア1～3と4～9の2群に分けると，再発スコア1～3の3年非再発率は，85.3%であった．腫瘍の数，腫瘍サイズ，異型度，T因子と再発との関係を調べると，単変量解析では腫瘍の数，異型度，T因子が再発と関連していた（$p<0.05$）．多変量解析では腫瘍の数とT因子が再発と関連していた（$p<0.05$）．

（結論）筋層非浸潤性膀胱癌に対する治療においては，再発スコア値の低い中リスク非G3例やCIS非併発例に対するTURBT後THP30mg3日間連続膀注療法は，アジュバント療法の一つとして考慮してもよい意味のある方法と思われた．しかし，再発スコア値の高い中リスク例に対しては，リスクに応じた抗癌剤の維持膀注療法ないしBCG膀注療法等の追加治療を考慮するのが良いと思われた．

（日泌尿会誌　103(4)：610～616，2012）

キーワード：筋層非浸潤性膀胱癌，塩酸ピラルビシン膀胱内注入，再発

緒　言

筋層非浸潤性膀胱癌の場合，NCCNガイドライン(2006)，EAUガイドライン(2008)[1]ともに，再発・進展リスクに応じた経尿道的膀胱腫瘍切除術（以下TURBTと略す）後の抗癌剤即時膀胱内注入あるいはBCG膀胱内注入を推奨している．

本邦の膀胱癌診療ガイドライン2009年版[2]でも，低リスク筋層非浸潤性膀胱癌ではTURBT後の抗癌剤即時単回注入が推奨されている（推奨グレードA）．中リスク筋層非浸潤性膀胱癌では，TURBT後の補助療法として単回注入のみでは不十分であり，抗癌剤即時注入に続いて維持療法が推奨されている（推奨グレードA）が，維持投与のスケジュールについては結論が得られていない．

現在のようなガイドラインの無かった1990年代当時，TURBT後の膀胱内注入療法は，術後2～3回/週から1回/週まで様々な方法[3]で行われていた．我々は，TURBT後でカテーテルが留置されているため膀胱内注入が簡単で，術後1回注入よりも3回連日注入のほうがより効果が期待できると考え，1995年よりTURBT当日から3日間連続での塩酸ピラルビシン（THP）30mg膀胱内注入療法を実施してきた．今回，その成績について検討したので報告する．

受付日：2010年9月16日，受理日：2012年1月21日

表1　対象184例の患者背景

・症例数：184例（男性：151例，女性：33例）		
・年齢：25-92歳（平均年齢：69.9歳）		
・腫瘍の数	単発	49例（26.6%）
	2-4個	66例（35.9%）
	5個以上	69例（37.5%）
・腫瘍サイズ	<1cm	30例（16.3%）
	1-3cm	140例（76.1%）
	3cm<	14例（ 7.6%）
・T因子	Ta	58例（31.5%）
	T1	126例（68.5%）
・異型度	G1	46例（ 25%）
	G2	116例（ 63%）
	G3	22例（ 12%）
・併発CIS	なし	184例
・EAU再発リスク分類*	低リスク群	14例（ 7.6%）
	中リスク群	168例（91.3%）
	高リスク群	2例（ 1%）
・日本版再発リスク分類#	低リスク群	26例（14.1%）
	中リスク群	137例（74.4%）
	高リスク群	21例（11.4%）
・観察期間：	2.7カ月-181.4カ月（中央値：55.1カ月）	

（*：EAU guideline（2008）を参考にした　#：膀胱癌診療ガイドライン2009年版による）

対象・方法

1）対象

1995年3月～2009年4月までの間に，当センターで膀胱CISの併発がなくかつ上部尿路上皮癌の合併や既往歴のない初発未治療筋層非浸潤性膀胱癌と診断され，2）の治療方法で述べる治療を受けかつpT1以下であった184症例を対象とした．対象184例の患者背景を表1に示す．男性151例，女性33例で平均年齢は69.9歳（25歳～92歳），経過観察期間の中央値は55.1カ月である．

2）治療方法

TURBT後数時間以内に，塩酸ピラルビシン（THP）30mg/注射用水40mlを膀胱内に注入し1時間保持．この膀注を翌日，翌々日の3日間連続施行した．術後3～5年間は可能な限り3カ月毎に膀胱鏡検査と尿中細胞診を行った．5年以上再発を認めない例には，検査間隔を延ばすとともに尿中細胞診や膀胱エコー検査などにより再発の有無をチェックした．膀胱鏡により初回再発を認め，生検陽性または尿細胞診陽性が確認された例を再発と定義した．

3）統計学的方法

TURBT施行日を起算日とし，Kaplan-Meyer法により非再発率を算出した．有意差検定にはlog-rank検定と一般化Wilcoxon検定を用いた．多変量解析にはCoxの比例ハザードモデルを用いた．

結　果

対象症例の患者背景

今回対象となった症例の男女比は4.6：1であった．腫瘍数では多発例が多く（73.4%），腫瘍サイズは1～3cm大が多くを占め（76.1%），T1例が68.5%，G2例が63%と多かった．今回の184症例をEAUガイドライン（2008）[1]の再発リスク分類を参考にして分類すると，低リスク14例，中リスク168例，高リスク2例となり大部分が中リスク例に相当した（表1）．ただし，今回のデータには膀胱癌取り扱い規約（第3版）[4]出版以前のデータが多く含まれている．そのため，同第2版[5]の腫瘍の数の分類のデータを生かすためにやむをえず腫瘍数の再発スコアに限り腫瘍数2～4個を再発スコア2，腫瘍数5個以上を再発スコア4と変更して処理している．一方，日本版再発リスク分類[2]で分類すると，低リスク26例，中リスク137例，高リスク21例となりEAUガイドラインを参考とした分類に比べ低リスク例と高リスク例が増加していた（表1）．

非再発率

非再発率は2相性に変化していた．治療後1年半から2年間は急峻に低下し，その後はなだらかな低下を示した．1，2，3，5年非再発率は，各々82.7%，75.3%，72.3%，67.4%であった（図1）．非再発率を腫瘍サイズ別に調べると，腫瘍サイズの大きいほうが非再発率は低値であったが，サイズ別に有意差を認めなかった．腫瘍数と非再発率の関係を調べると，単発例に比べ多発例では腫瘍数が多いほど低値を示した．単発例と2～4個あるいは単発例と5個以上の多発例との間には各々有意差（$p<0.05$）を認めた（図2）．T因子と非再発率の関係においては，T1がTaに較べ低値を示した（$p<0.05$）（図3）．異型度と非再発率の関係においては，異型度が高くなるほど低値を示し，G1とG3との間に有意差（$p<0.05$）を認めた（図4）．

EAUガイドライン[1]を参考にした再発リスク分類の低リスク群14例と中リスク群168例の非再発率を比較すると，低リスク群では再発例がないのに対し，中リスク群の3年非再発率は69.6%と低値であった（$p<0.05$）（図5）．

日本版再発リスク分類では，低リスク群での再発例がなかったが，中リスク群の3年非再発率70.7%に対し，高リスク群では42.5%とリスクが高くなるに従って低値を示し，各群間に有意差を認めた（図6）．日本版再発リスク分類は，低リスク群と高リスク群の検出に優れ，高リスク群における早期の再発と非再発率の低値が明瞭であった．

次に，日本版再発リスク分類の中リスク群から，Taかつlow gradeかつ腫瘍数2～4個を低中リスク群として14例を抽出し，3年非再発率を求めると85.1%であった（図7）．同様に，EAUガイドラインを参考にした分類の中リスク群を再発スコア1～3と4～9の2群に分け，

図1　184例の非再発率

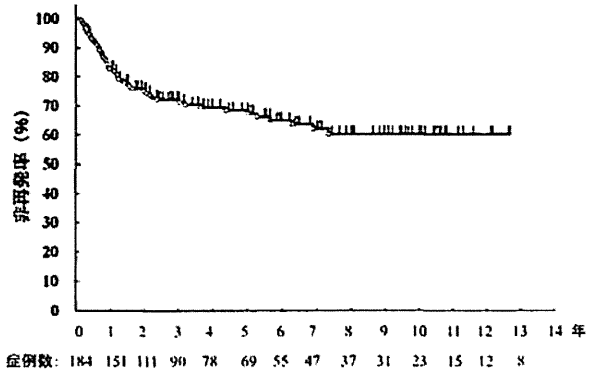


図2　腫瘍の個数別非再発率

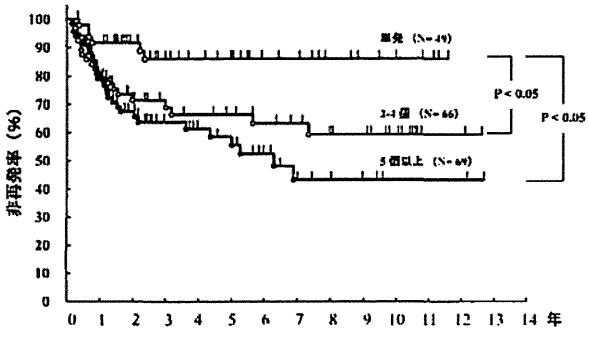


図3　T因子別非再発率

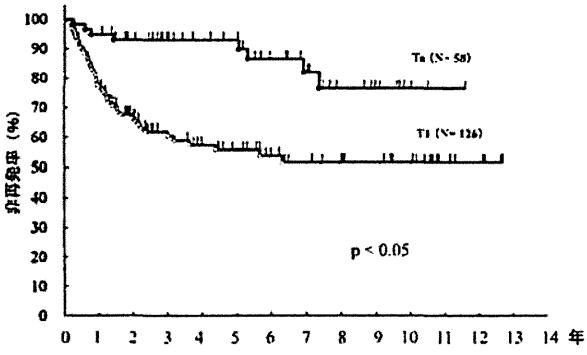


図4　腫瘍のgrade別非再発率

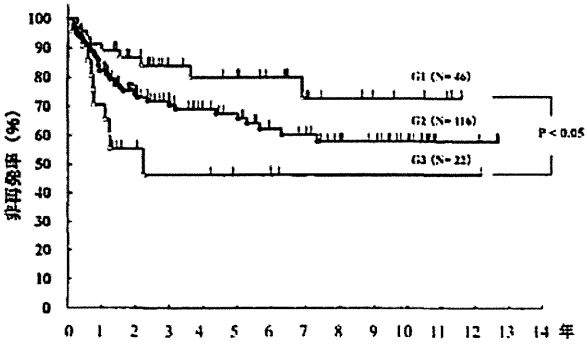


図5　EAUガイドラインを参考に分類した低リスク群，中リスク群と中リスク群を再発スコア1～3と4～9の2群に分離独立させた場合の各リスク群別非再発率

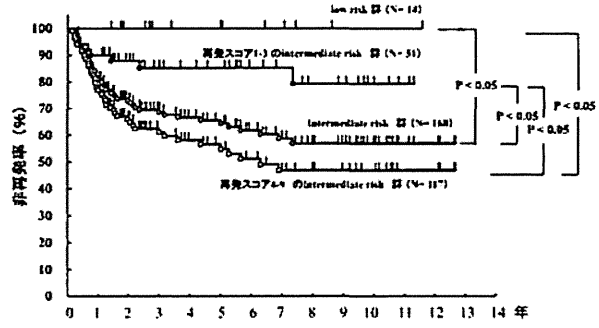


図6　日本版再発リスク群別非再発率

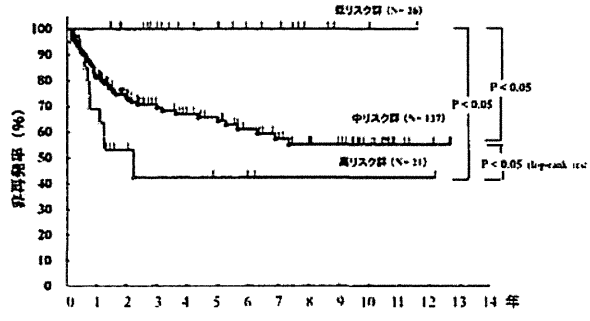
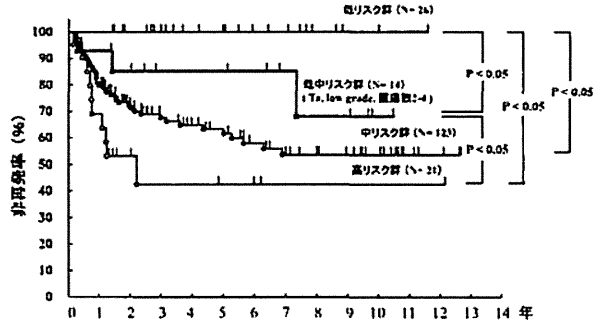


図7　低中リスク群を分離独立させた日本版再発リスク群別非再発率



再発スコア1～3　51例の3年非再発率を求めると85.3%であった（図5）．日本版の低リスク群と低中リスク群の間には有意差を認めた（図7）が，EAUガイドラインを参考に分類したlow risk群と再発スコア1～3のintermediate risk群の間には有意差を認めなかった（図5）．

腫瘍の数，腫瘍サイズ，異型度，T因子と再発との関係をCoxの比例ハザードモデルを用いて解析すると，腫瘍の数とT因子が再発と有意に関連していた（表2）．

考　察

低～中リスク筋層非浸潤性膀胱癌の場合，TURBT後に抗癌剤の膀胱内注入療法が行われている．低リスク例における即時単回注入療法の再発予防効果はメタ解析で

表2 Coxの比例ハザードモデルによる腫瘍数，腫瘍サイズ，異型度，T因子と再発との関係

	subgroup	n	ハザード比	95%CI	p value
腫瘍数	単発	49	1.0		
	2-4個	66	2.24	0.873-5.732	0.093
	5個以上	69	3.05	1.236-7.524	0.016
腫瘍サイズ	<1cm	30	1.0		
	1-3cm	140	1.10	0.500-2.413	0.816
	3cm<	14	1.19	0.395-3.567	0.760
異型度	G1	46	1.0		
	G2	116	1.30	0.602-2.785	0.508
	G3	22	1.86	0.721-4.808	0.199
T因子	Ta	58	1.0		
	T1	126	3.00	1.341-6.721	0.008

表3 TURBT後抗癌剤膀注の報告例

報告	症例数	腫瘍個数	初発/再発	TURBT後の膀注回数	薬剤	非再発率（%） 1年	2年	3年	備考
Okamura et al.	84例	殆どが単発	殆どが初発	直後1回膀注	THP 30mg	92.4	82.7	78.8	多くは低リスク？
Cancer.2002	86例	殆どが単発	殆どが初発	TURのみ		67.0	55.7	52.6	
川口ら	26例	単/多	初/再	翌日から7日間連日	THP 30mg	83.7	67		TURのみと有意差なし
臨泌.2005	37例	単/多	殆どが初発	TURのみ		77.6	54.6		
Koga et al.	77例	単/多	初発	直後（2回）+維持膀注17回	epi-ADM 30mg			85.2	長期維持膀注のほうが再発予防効果あり
J.Urol.2004	73例	単/多	初発	直後（2回）+維持膀注7回	epi-ADM 30mg			63.9	
自験例2010	184例	単/多	初発	直後から3日間連日	THP 30mg	82.7	75.3	72.3	殆どが中リスク例

確認されかつ推奨されている[6]．中リスク例では即時単回注入のみでは不十分と考えられ，引き続いての膀注療法が必要とされているが，薬剤の種類，薬液の濃度，投与のスケジュールなどに関しては一定の見解が得られていない[2,7]のが実情である．

筋層非浸潤性膀胱癌のTURBT後の膀胱腔内再発様式には，腫瘍細胞の他部位への播種，微小残存病変からの発生，新たな腫瘍の発生の3種類が考えられる．我々の非再発率もHinotsuら[8]と同じように2相性の変化，すなわちTURBT後1年半から2年間は急峻に低下し，その後はなだらかな低下を示したのはそれらに合致する結果と思われる．今回の184症例を対象とした再発に関連する因子の解析結果においては，単変量解析では腫瘍の数，異型度(G1とG3の間でのみ)，T因子が，多変量解析では腫瘍の数とT因子が再発と関連していた．しかし，G2とG3間での異型度別や腫瘍サイズ別での有意差は認められなかった．原因としては，今回の治療方法の対象として選ばれた症例群が高リスク例の少ないbiasのかかった症例群で，G3例や腫瘍サイズの大きな例が少ないことや，症例数自体が少ないためなどが考えられる．

TURBT後の膀注療法に期待されることは，一つにはTURBTによる完全切除後の予防的あるいはアジュバント療法としての効果がある．もう一つとしては，完全に切除しきれなかった遺残腫瘍の根絶または新たな腫瘍発生の抑制の二つが考えられる．前者のためには単回～複数回のTURBT後即時膀注療法が考えられ，後者のためには術後維持膀注療法が考えられる．我々の行ってきたTURBT後即時3日間連続膀注法は前者を目的とするものである．

今回の報告は，我々の行ってきた方法の成績を後ろ向きに検討したものである．残念ながらTURBT単独群とTURBT+3日間連続膀注群をランダム化比較試験(RCT)で比較したものではないため，3日間連続膀注の効果がどのくらいあるのか不明である．そこで，我々の成績を本邦の他施設の成績と比較してみた(表3)．Okamuraら[9]の術後単回膀注の成績は，我々の成績に較べ非常に良好であるが，低リスク例が多く含まれているためかと思われる．1年非再発率に関する川口ら[10]の7日間連続膀注と我々の成績は似たような成績であり，7日間連続膀注は必要ないと思われる．

Kogaら[11]のTURBT直後膀注（2回）＋長期維持膀注（1年間）の成績は非常に良好であるが，TURBT直後膀

注（2回）＋短期維持膀注（3カ月間）の成績は我々の成績より不良である．KogaらのTURBT直後膀注(2回)の方法は，TURBT後24時間以内に1回，2〜3日以内に2回目の膀注をし，以後維持膀注に移行する方法である．我々の方法と異なるのは，薬剤，TURBT直後膀注のタイミングと回数，維持膀注の有無である．Kogaらと我々の患者背景に大差はないと思われる．これらの結果から示唆されることは，もし維持膀注が必要と判断される場合には，短期の維持膀注ではなく長期の維持膀注をすべきということであろう．この点に関しては，再発リスクがintermediate riskの場合の抗癌剤の推奨維持膀注期間がEAUガイドラインでは6〜12カ月(grade of recommendation：B)であることからも妥当と考えられる．しかし，昨今の医療事情を考慮し費用対効果の面からの検討も必要であろう．

そこで，我々の行ってきた3日間連続膀注法が妥当と考えられるような対象症例があるのか，あるのならどのような症例が該当するのかについて検討をした．

我々の方法による成績で特徴的なのは，低リスク例での再発がないことである．EAUガイドラインによれば，低リスク例の1，5年予測再発率は15%，31%である．日本版低リスク群でも再発例を認めていない．低リスク例におけるこのような良好な成績は，TURBT後即時単回注入よりも3日間連続膀注のほうがアジュバント療法としての効果が高いことを示唆していると考えられる．そうであるならば，低リスク例の場合より強いアジュバント療法が必要であるが長期の維持膀注ないしBCG膀注までは必要ないような中リスク例，すなわち低リスクに近い中リスク例が我々の方法のよい対象となりうるのではないかと考えた．日本版再発リスク分類の中リスク群から，低中リスク群（Taかつlow gradeかつ腫瘍数2〜4個）14例の3年非再発率を求めると85.1%であり（図7)，EAUガイドラインを参考に分類した中リスク群のなかの再発スコア1〜3　51例の3年非再発率は85.3%であった（図5)．これらの成績は，Kogaら[11]のTURBT直後膀注(2回)＋長期維持膀注の成績と比べ遜色ないと思われる．このような結果から，Taかつlow gradeかつ腫瘍数2〜4個の例や，EAUガイドラインを参考とした分類の再発スコアが1〜3と低い中リスク非G3例やCIS非併発例に対しては，TURBT後THP 30mg 3日間連続膀注療法は，アジュバント療法の一つとして考慮してもよい意味のある方法と思われた．しかし，再発スコアのより高い中リスク群に対してはさらに維持膀注療法ないしBCG膀注療法[12]等を考慮するのが良いと思われた．

最後に，今回の分析に際しての再発リスク分類に関しては，日本版では低リスク群と高リスク群の抽出に優れていると思われる．しかし，中リスク群に関してはT因子や腫瘍数などでより細かく分類することが可能でないかと思われた．EAUガイドライン[1]では，中リスク群を再発スコア1〜4と5〜9の2群に分けて1年と5年の予測再発率を記載している．日本版での中リスク群に関してもより細かく分類するほうが実際的と思われる．

EAUガイドラインを参考にした再発リスク分類に際しては，膀胱癌取り扱い規約（第2版)[5]の腫瘍の数の分類を生かすために，やむをえず便宜的に腫瘍数2〜4個を再発スコア2，腫瘍数5個以上を再発スコア4と変更して分類した．そのため，高リスク群が日本版より少なくなった可能性を否定できない．EAUガイドラインの低リスク群はG2例を含まぬため，日本版よりさらに限定された群である．そのため，日本版の低リスク群がEAUガイドラインの再発スコア1〜9の中リスク群に分類される例が生じるとともに，EAUガイドラインでは，G3T1癌も中リスク群に分類される場合も考えられる．中リスク群には，高リスクに近い中リスク例から低リスクに近い中リスク例までの幅広い症例が含まれることに留意すべきである．従って，実際の臨床では，日本版あるいはEAUガイドラインの中リスク群をリスク別により細分して治療に当たるのが良いと思われた．

結　語

筋層非浸潤性膀胱癌に対する治療においては，再発スコア値の低い中リスク非G3例やCIS非併発例に対するTURBT後THP 30mg 3日間連続膀注療法は，アジュバント療法の一つとして考慮してもよい意味のある方法と思われた．しかし，再発スコア値の高い中リスク例に対しては，リスクに応じた抗癌剤の維持膀注療法ないしBCG膀注療法等の追加治療を考慮するのが良いと思われた．

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THE INTRAVESICAL RECURRENCE AFTER 3-DAY CONSECUTIVE INTRAVESICAL INSTILLATION OF PIRARUBICINE HYDROCHLORIDE (THP) FOLLOWING TRANSURETHRAL RESECTION OF BLADDER TUMOR (TURBT) FOR NON-MUSCLE-INVASIVE BLADDER CANCER

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Abstract:

(Purpose) We estimated the results of 3-day consecutive intravesical instillation of pirarubicine hydrochloride (THP) following transurethral resection of bladder tumor (TURBT) for non-muscle-invasive bladder cancer retrospectively.

(Patients and methods) Through March 1995 to April 2009, a total of 184 patients were instilled 3-day consecutive intravesical instillation of pirarubicine hydrochloride (THP) (30 mg/40 ml in disinfected distilled water) started within a few hours after TURBT. 184 patients were diagnosed as untreated fresh non-muscle-invasive urothelial bladder cancer with no concomitant carcinoma in situ (CIS), no concurrent upper urinary tract urothelial cancer and no past history of upper urinary tract urothelial cancer. Number of tumors, tumor size, tumor grade and clinical tumor stage were analyzed in relation to tumor recurrence by univariate and multivariate analyses. Median follow-up were 55.1 months.

(Results) Using EAU guideline on non-muscle invasive urothelial carcinoma of the bladder, 168 patients were classified at intermediate risk of tumor recurrence, 14 patients were at low risk of tumor recurrence and 2 patients were at high risk of tumor recurrence. The shape of non-recurrence rate curve showed two phase decrease pattern, namely, early hasty decrease within 1.5 or two years and late gentle decrease thereafter. The 1, 2, 3, 5-year non-recurrence rate were 82.7%, 75.3%, 72.3% and 67.4% respectively. The 3-year non-recurrence rate of low score group (recurrence score 1–3) at intermediate risk of tumor recurrence was 85.3%. Univariate analysis revealed that number of tumors, tumor grade and clinical tumor stage were related to tumor recurrence ($p<0.05$). By multivariate analysis, number of tumors and clinical tumor stage were related to tumor recurrence ($p<0.05$).

(Conclusions) In patients of low score group at intermediate risk of tumor recurrence without grade 3 urothelial carcinoma and concomitant bladder CIS, 3-day consecutive intravesical instillation of pirarubicine hyorochloride (THP) following TURBT for non-muscle-invasive bladder cancer would be a significant adjuvant therapy. But in patients of high score group at intermediate risk of tumor recurrence, it seemed better to do additional maintenance intravesical chemotherapy or intravesical BCG therapy.

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Keywords: non-muscle-invasive bladder cancer, intravesical instillation of pirarubicine hydrochloride, recurrence

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ORIGINAL ARTICLE – UROLOGIC ONCOLOGY

Prognostic Value of Renin–Angiotensin System Blockade in Non-muscle-invasive Bladder Cancer

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ABSTRACT

Background. To determine whether the administration of renin–angiotensin system (RAS) inhibitors, such as angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs), affect disease outcome in non-muscle-invasive bladder cancer (NMIBC).

Methods. A total of 330 patients with initially diagnosed NMIBC were identified. We retrospectively investigated the clinical outcomes after transurethral resection of bladder tumor (TUR-BT) in patients who did or did not receive RAS inhibitors. The median follow-up period was 4.1 years.

Results. A total of 128 patients (38.8 %) experienced subsequent tumor recurrence, and stage progression was observed in 17 patients (5.2 %) during follow-up. Fifty-one patients (15.5 %) had received ACEI or ARB administration at transurethral resection. Multivariate analysis demonstrated that tumor multiplicity, absence of bacillus Calmette-Guérin instillation, and no administration of ACEI or ARB ($P = 0.010$, hazard ratio 2.26) were independent risk factors for subsequent tumor recurrence. The 5-year recurrence-free survival rate was 78.4 % in patients administered ACEIs or ARBs, and 53.3 % in their counterparts ($P = 0.011$).

Conclusions. The absence of RAS inhibitor administration was an independent risk factor for subsequent tumor recurrence in patients with initially diagnosed NMIBC. Our data support further investigation of the role of RAS inhibitors as a potential therapy to decrease tumor recurrence in NMIBC.

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Bladder cancer accounts for more than 90 % of all newly diagnosed urothelial carcinoma.[1] At presentation, approximately 75 % to 85 % of patients are found to have non-muscle-invasive disease (i.e., stage Ta, Tis, or T1).[2] When transurethral resection of bladder tumor (TUR-BT) is used alone, the recurrence rates are as high as 70 %, and the risk of progression to muscle invasive disease is 30 %.[3] Adjuvant intravesical therapy has been used in the treatment of non-muscle-invasive bladder cancer (NMIBC) to decrease its risk of recurrence and progression.

Intravesical bacillus Calmette-Guérin (BCG) immunotherapy, the most commonly used intravesical treatment for high-risk NMIBC, is endorsed in the European Association of Urology and American Urological Association practice guidelines.[2,4] Intravesical BCG yields a complete response in 55 % to 65 % of papillary tumors and in 70 % to 75 % of carcinoma-in situ, and up to 83 % if 3-week maintenance therapy is provided.[4–7] Despite the overall effectiveness of BCG, the disease of a large population of patients with NMIBC does not respond, patients experience recurrence shortly after therapy, or patients cannot tolerate its adverse effects. Because of these limitations, it is important that further research be conducted to identify potent therapeutic agents in an attempt to improve disease control in patients with NMIBC.

Angiotensin II (Ang II) is a key biological peptide in the renin–angiotensin system (RAS) and plays a role in electrolyte homeostasis and blood pressure control. Increasing evidence suggests that the angiotensin type 1 receptor (AT1R) is expressed in various malignancies, and AT1R expression was found to be significantly involved in tumor growth, metastasis, and angiogenesis.[8–11] In previous studies, we have shown that Ang II–AT1R signaling led to potent induction of vascular endothelial growth factor, and that the inhibition of RAS may have an antiangiogenic effect on bladder cancer.[12–15] Using human bladder cancer specimens, we also showed that AT1R expression was an independent

predictor for subsequent tumor recurrence in NMIBC, and patients with high AT1R expression tended to have tumor recurrence even if they had undergone BCG therapy.[16]

In the present study, we retrospectively reviewed the data of 330 patients with initially diagnosed NMIBC. We examined the association between clinicopathological parameters and the use of angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs), then analyzed the clinical outcomes to identify the prognostic impact of administration of ACEIs and ARBs.

MATERIALS AND METHODS

After institutional review board approval, the medical records between 1999 and 2009 archived at Keio University Hospital were reviewed. During this period, more than 950 cases were treated by TUR-BT, and consequently 419 patients were identified as having NMIBC at our institution. After excluding patients who were initially diagnosed at another institution or who were followed for less than 6 months, we identified a total of 357 patients initially diagnosed with NMIBC in our study population. We excluded 6 patients who underwent cystectomy as uncontrollable NMIBC after TUR-BT. After excluding 21 patients who received RAS inhibitors after surgery, data from the remaining 330 patients were analyzed. The median follow-up was 4.1 years (range 0.8–12.2 years).

These patients were assessed by urine cytology and cystoscopy every 3 months for 2 years after TUR-BT, every 6 months for the next 3 years, and then every 6 to 12 months thereafter. Intravenous urography, ultrasonography, and/or computed tomography scanning were performed to evaluate the extravesical lesions and upper urinary tract once a year. The use of intravesical therapy depended on the discretion of the attending physician. BCG treatment was begun 4 to 5 weeks after TUR-BT and continued weekly for 6 to 8 weeks. Intravesical chemotherapy also followed the same schedule.

Tumor recurrence was defined as a new tumor appearing in the bladder, and stage progression was defined as confirmed histological muscle invasion (pathological stage greater than pT2) or detectable distant metastasis. The independent variables included in the present study were age, gender, tumor grade, pathological T stage, tumor multiplicity, the appearance of carcinoma-in situ for transurethral resection specimens, the status of postoperative intravesical treatments (BCG instillation and/or intravesical chemotherapy), and the use of antihypertensive drugs at TUR-BT.

All patients were interviewed by attending physicians at least two times, such as at the time of diagnosis and at TUR-BT, and data concerning their medications administered at surgery were routinely collected. Data concerning their medication after surgery were obtained from medical records and interviews by physicians. The use of antihypertensive drugs at TUR-BT was reviewed, focusing on the use of RAS inhibitors (ACEIs or ARBs) and other agents (calcium-channel blockers, β-blockers, and diuretics).

The associations between patient clinicopathological parameters and the status of RAS inhibitors were analyzed and validated by the Chi-square test. Recurrence-free and progression-free survival curves were constructed by the Kaplan–Meier method and were compared by the log rank test. Univariate and multivariate Cox proportional hazard regression models with stepwise forward selection were used to identify factors associated with subsequent tumor recurrence and stage progression. Differences among groups were regarded as significant at $P < 0.05$. These analyses were performed with SPSS software, version 17.0 (SPSS, Chicago, IL).

RESULTS

The mean age of all cohorts was 68 years (range 37–94 years). Men accounted for 84.5 % (279 patients) and women 15.5 % (51 patients) of the cohort. Table 1 lists the clinicopathological parameters in the 330 patients.

TABLE 1 Clinicopathological parameters in the 330 study patients

Characteristic	*n* (%)
Age	
<65 y	148 (44.8)
≥65 y	182 (55.2)
Gender	
Male	279 (84.5)
Female	51 (15.5)
Tumor grade	
G1/2	187 (56.7)
G3	143 (43.3)
Pathological T stage	
pTa	234 (70.9)
pT1	96 (29.1)
Tumor multiplicity	
Yes	181 (54.8)
No	149 (45.2)
Carcinoma-in situ	
Yes	33 (10.0)
No	297 (90.0)
BCG instillation	
Performed	153 (46.4)
Not performed	177 (53.6)
Intravesical chemotherapy	
Performed	35 (10.6)
Not performed	295 (89.4)

BCG bacillus Calmette-Guérin

TABLE 2 Number of patients receiving antihypertensive therapy

Drug	*n*
ACEIs	5
Enalapril	2
Imidapril	1
Temocapril	1
Trandolapril	1
ARBs	46
Candesartan	23
Losartan	10
Valsartan	8
Telmisartan	3
Olmesartan	2
Calcium-channel blockers	90
β-Blockers	20
Diuretics	5

ACEI angiotensin-converting enzyme inhibitor, *ARB* angiotensin II receptor blocker

BCG treatment was provided to 153 patients (46.4 %) after initial TUR-BT, and 35 patients (10.6 %) underwent intravesical chemotherapy. A total of 116 patients (35.2 %) received medications for hypertension at TUR-BT; 51 (15.5 %) were provided ACEIs ($n = 5$) or ARBs ($n = 46$). Other antihypertensive drugs included calcium-channel blockers ($n = 90$), β-blockers ($n = 20$), and diuretics ($n = 5$). The type of ACEI or ARB at surgery is listed in Table 2. Of the 51 patients, 49 continued to receive RAS inhibitors for a mean duration of 3.8 years after initial TUR-BT, while patient data concerning the duration of RAS inhibitor administration after surgery were not available for 2 patients.

During the median follow-up of 4.1 years, 128 patients (38.8 %) experienced tumor recurrence, and disease stage progression was observed in 17 patients (5.2 %). Table 3 summarizes the use of ACEIs and ARBs. Univariate and multivariate analyses were performed to determine risk factors for subsequent tumor recurrence and stage progression (Table 4). Multivariate analysis revealed that

TABLE 3 Clinicopathological parameters in 330 patients according to the status of ACEI or ARB administration

Characteristic	Patients not administered ACEI/ARB, *n* (%) ($n = 279$)	Patients administered ACEI/ARB, *n* (%) ($n = 51$)	*P* value
Age			0.647
<65 y	127 (45.5)	21 (41.2)	
≥65 y	152 (54.5)	30 (58.8)	
Gender			0.055
Male	231 (82.8)	48 (94.1)	
Female	48 (17.2)	3 (5.9)	
Tumor grade			0.166
G1/2	163 (58.4)	24 (47.1)	
G3	116 (41.6)	27 (52.9)	
Pathological T stage			0.045
pTa	204 (73.1)	30 (58.8)	
pT1	75 (26.9)	21 (41.2)	
Tumor multiplicity			0.993
Yes	153 (54.8)	28 (54.9)	
No	126 (45.2)	23 (45.1)	
Carcinoma-in situ			0.960
Yes	28 (10.0)	5 (9.8)	
No	251 (90.0)	46 (90.2)	
BCG instillation			0.880
Performed	130 (46.6)	23 (45.1)	
Not performed	149 (53.4)	28 (54.9)	
Intravesical chemotherapy			0.085
Yes	26 (9.3)	9 (17.6)	
No	253 (90.7)	42 (82.4)	
Use of non-ACEI/ARB drugs			<0.001
Yes	65 (23.3)	32 (62.7)	
No	214 (76.7)	19 (37.3)	

BCG bacillus Calmette-Guérin, *ACEI* angiotensin-converting enzyme inhibitor, *ARB* angiotensin II receptor blocker

TABLE 4 Risk factors for predicting on tumor recurrence in 330 patients with initially diagnosed NMIBC using univariate and multivariate analyses

Characteristic	Cox regression analysis of tumor recurrence			Cox regression analysis of stage progression		
	Univariate	Multivariate		Univariate	Multivariate	
	P value	HR (95 % CI)	*P* value	*P* value	HR (95 % CI)	*P* value
Age	0.227			0.151		
<65 y						
≥65 y						
Gender	0.074			0.623		
Male						
Female						
Tumor grade	0.264			0.001		0.003
G1/2						
G3					6.62 (1.88–23.2)	
Pathological T stage	0.374			0.012		
pTa						
pT1						
Tumor multiplicity	0.014		<0.001	0.027		
Yes		2.13 (1.43–3.16)				
No						
Carcinoma-in situ	0.852			0.155		
Yes						
No						
BCG instillation	0.066		0.001	0.013		
Performed						
Not performed		1.93 (1.31–2.83)				
Intravesical chemotherapy	0.748			0.076		
Yes						
No						
Use of non-ACEI/ARB drugs	0.794			0.057		
Yes						
No						
Use of ACEI/ARB drugs	0.006		0.010	0.778		
Yes						
No		2.26 (1.22–4.19)				

HR hazard ratio, *CI* confidence interval, *BCG* bacillus Calmette-Guérin, *ACEI* angiotensin-converting enzyme inhibitor, *ARB* angiotensin II receptor blocker

tumor multiplicity ($P < 0.001$, hazard ratio [HR] 2.13), absence of BCG instillation ($P = 0.001$, HR 1.93), and no ACEI or ARB administration ($P = 0.010$, HR 2.26) were independent risk factors for subsequent tumor recurrence. Kaplan–Meier analysis showed that the 5-year recurrence-free survival rate was 78.4 % in patients who received ACEIs and ARBs and 53.3 % in their counterparts ($P = 0.017$) (Fig. 1a). On the other hand, multivariate analysis concerning stage progression showed that only tumor grade 3 ($P = 0.003$, HR 6.62) in transurethral resection specimens was an independent predictor of subsequent stage progression in our population (Table 4). No significant difference was observed between patients who did or did not receive ACEIs or ARBs in both univariate and multivariate analyses concerning stage progression.

Next, we clarified whether the use of ACEIs or ARBs could provide additional effects on subsequent tumor recurrence or stage progression in a subgroup of patients treated with BCG ($n = 153$). Multivariate analyses demonstrated that tumor multiplicity ($P = 0.005$, HR 2.03) and no receipt of ACEI or ARB ($P = 0.041$, HR 2.60) were also independent risk factors for subsequent tumor recurrence (Table 5). Kaplan–Meier analysis revealed that the 5-year recurrence-free survival rate was 78.3 % in patients who received ACEIs and ARBs and 44.5 % in their counterparts ($P = 0.049$) (Fig. 1b). However, in the present study, multivariate analyses including the use of RAS inhibitors revealed no significant indicators for subsequent stage progression in the subgroup of patients who received BCG therapy after initial TUR-BT.

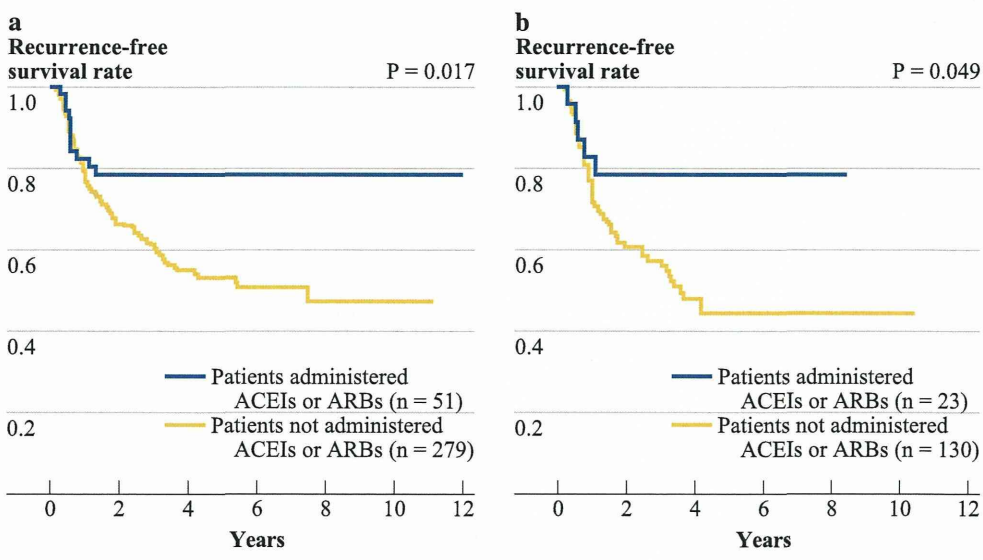

FIG. 1 **a** Recurrence-free survival rate after TUR-BT in 330 patients according to receipt of ACEI or ARB. **b** Recurrence-free survival rate after TUR-BT in 153 patients who received BCG therapy, according to the receipt of ACEI or ARB

DISCUSSION

In the present study, we retrospectively investigated the use of RAS inhibitors (ACEIs and ARBs) and other standard prognostic factors in 330 patients with initially diagnosed NMIBC. Kaplan–Meier analysis revealed that subsequent tumor recurrence was significantly decreased in patients who received ACEIs or ARBs. Multivariate analysis also showed that in addition to other standard prognostic factors, no receipt of ACEI or ARB was an independent predictor of subsequent tumor recurrence, while no significant difference was observed concerning stage progression between patients who did or did not receive ACEIs or ARBs. Similar results were observed when we focused on patients treated with BCG therapy after TUR-BT. These results suggest that RAS inhibitor administration may improve disease control by decreasing tumor recurrence after TUR-BT in patients with initially diagnosed NMIBC.

Several meta-analyses have shown that adjuvant intravesical treatment (chemotherapy or BCG) reduces subsequent tumor recurrence. Though the choice between further chemotherapy or BCG instillation depends on the patient risk profile, the efficacy of BCG therapy is promising, and randomized trials have also shown better outcomes for reducing tumor recurrence compared with transurethral resection alone or topical chemotherapy.[3–5,17,18] However, some tumors often refract or relapse after BCG therapy, while some patients cannot tolerate its adverse effects.[19] Therefore, identifying a new strategy that is both effective and tolerable would be a striking breakthrough in the management of NMIBC.

RAS inhibitors, such as ACEIs and ARBs, are widely used to treat hypertension. Concern regarding the potential role of Ang II in angiogenesis and promotion of tumor growth has been growing in the past few decades.[8–11] In addition, Lever et al. reported the first clinical evidence that long-term use of ACEIs induces potent protective effects against carcinogenesis.[20] To date, in addition to cardiovascular functions regulated by the systemic RAS, the potential role of local RAS in malignancy has been investigated, and an increasing body of evidence suggests the efficacy of RAS inhibitors in various cancer treatments.[21–23]

We previously reported that AT1R expression was significantly associated with intratumoral neovascularization in human bladder cancer specimens, and higher AT1R expression could be an important factor for identifying patients at higher risk of tumor recurrence.[16] Because greater vascular endothelial growth factor expression and stronger microvessel density have been shown to be associated with an earlier and significantly greater recurrence rate, we hypothesized that administration of RAS inhibitors (ACEIs and ARBs) may affect the subsequent prognosis in patients with NMIBC.[24–26] Multivariate analyses in the present study showed that ACEI or ARB use was an independent predictor of tumor recurrence but not stage progression. Similar results were also observed in the analyses of patients treated with BCG therapy.

Although tumor behavior is regulated by many factors, it may be controversial as to whether RAS inhibition is a key mechanism involved in suppressing tumor recurrence in NMIBC. However, our recent work has elucidated that Ang II–AT1R signaling has a significant impact on the tumor microenvironment by promoting macrophage mobilization and infiltration into the tumor bed via signaling pathways involving monocyte chemoattractant protein-1 (MCP-1).[27] MCP-1 has been identified as a prominent regulator of the growth, survival, invasiveness, and migration of tumor cells.[9,28] Therefore, in addition to

TABLE 5 Risk factors for predicting on tumor recurrence in 153 patients after receiving BCG therapy using univariate and multivariate analyses

Characteristic	Cox regression analysis of tumor recurrence		
	Univariate	Multivariate	
	P value	HR (95 % CI)	*P* value
Age	0.207		
<65 y			
≥65 y			
Gender	0.548		
Male			
Female			
Tumor grade	0.038		
G1/2			
G3			
Pathological T stage	0.086		
pTa			
pT1			
Tumor multiplicity	0.010		0.005
Yes		2.03 (1.24–3.32)	
No			
Carcinoma-in situ	0.293		
Yes			
No			
Use of non-ACEI/ARB drugs	0.982		
Yes			
No			
Use of ACEI/ARB drugs	0.032		0.041
Yes			
No		2.60 (1.04–6.47)	

BCG bacillus Calmette-Guérin, *HR* hazard ratio, *CI* confidence interval, *ACEI* angiotensin-converting enzyme inhibitor, *ARB* angiotensin II receptor blocker

tumor angiogenesis, we believe that the regulation of these potential factors may contribute to improving disease outcome.

This study has several limitations. First, it was performed in a retrospective manner, and unknown sources of bias may exist in the findings. Because of the limited sample size of patients with ACEIs or ARBs, we could not fully evaluate the differences in doses or types of ACEIs and ARBs. Not all patients received one immediate postoperative instillation of chemotherapy, which may have had an effect on tumor recurrence. BCG instillation with the maintenance schedule and second TUR-BT were not commonly practiced at our institution during this period, and if they had been, it may have improved the results. We believe that further evaluation with a larger clinical data set and a prospective study design is warranted in order to clarify the accurate prognostic role of RAS inhibitors in the treatment of NMIBC.

In conclusion, the results of our retrospective analysis suggest that RAS inhibitors may improve prognostic outcomes in patients with NMIBC. Because RAS inhibitors are already used as antihypertensive drugs without severe adverse effects, our data support further investigation of the role of RAS inhibitors as a potential therapy to decrease tumor recurrence in NMIBC.

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p300 mediates cellular resistance to doxorubicin in bladder cancer

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Abstract. Bladder cancer is one of the most common urogenital malignancies. At the non-invasive stage, bladder cancer can be completely resected transurethrally. However, 70% of patients experience intravesical tumor recurrence within 5 years. Patients with advanced bladder cancer frequently receive a chemotherapy regimen containing doxorubicin. However, doxorubicin resistance is a major obstacle to cancer chemotherapy. Previously, we reported that the histone acetyltransferase p300/CBP-associated factor is involved in doxorubicin resistance in bladder cancer. However, the role of another histone acetyltransferase, p300, in bladder cancer resistance to doxorubicin remains unclear. In this study, we investigated the molecular mechanism of doxorubicin resistance in bladder cancer with regard to p300. The result showed that p300 expression was reduced in doxorubicin-resistant bladder cancer cells and in response to doxorubicin exposure. Furthermore, p300 suppression rendered bladder cancer cells resistant to doxorubicin. Taken together, the results from this study indicate that p300 may be a promising molecular therapeutic target through the modulation of cellular sensitivity to doxorubicin in bladder cancer.

Introduction

Bladder cancer is one of the most common urogenital malignancies. At the non-invasive stage, bladder cancer can be completely resected transurethrally. However, 70% of patients experience intravesical tumor recurrence within 5 years. To prevent recurrence of non-invasive bladder cancer, the instillation of bacillus Calmette-Guérin, doxorubicin and mitomycin C has been empirically adapted, but these can suppress intravesical recurrence only modestly (1). However, anticancer agents, including doxorubicin, gemcitabine and cisplatin are administered for advanced bladder cancer (2). However, most bladder cancers recur with a terminal prognosis, even though these anticancer agents are empirically effective. Therefore, more effective intravesical and systemic chemotherapy for bladder cancer is required.

Doxorubicin has been administered for many types of solid tumors, including breast, hepatocellular and urothelial cancers. In bladder cancer, doxorubicin is employed as an agent of intravesical instillation into the bladder or by intravenous injection. Several molecules that are associated with the acquisition of doxorubicin resistance have been identified, including detoxifying enzymes, drug-efflux pumps and apoptosis-related genes (3). We have previously established doxorubicin-resistant bladder cancer cell lines (4,5). However, the precise mechanism of doxorubicin resistance and the ability to overcome this resistance remains unresolved.

p300 was originally identified using protein-interaction assays with the adenoviral E1A oncoprotein (6). p300 has been implicated in a number of diverse biological functions, including proliferation, cell cycle regulation, apoptosis, differentiation and DNA damage response (7-10). p300 proteins function primarily as histone acetyltransferases (HATs) and as transcription co-factors for a number of nuclear proteins (11,12). Unlike other HATs, which have substrate specificity for histones, p300 is capable of acetylating all four histones. However, the p300/CBP-associated factor (PCAF) is also the first HAT to be discovered in mammalian systems on the basis of its homology to yeast Gcn5p (13). PCAF is known to acetylate various nuclear proteins in addition to histones (9). We previously reported that PCAF promotes cell growth, cellular invasion and cellular resistance to anticancer agents in bladder cancer cells (14). Furthermore, we also reported that p300 is involved in cell growth as well as cisplatin resistance (15). However, it remains unclear how p300 affects cellular resistance to doxorubicin.

In this study, we investigated the functions of p300 with regard to cellular resistance to doxorubicin in bladder cancer. The results revealed that p300 expression was downregulated in doxorubicin-resistant cells, and that doxorubicin treatment reduced p300 expression. Furthermore, p300 silencing affected resistance to doxorubicin in bladder cancer.

Materials and methods

Cell culture. The human urothelial cancer lines, KK47 and T24, were cultured in Eagle's minimal essential medium (EMEM),

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which was purchased from Invitrogen (Carlsbad, CA, USA) and supplemented with 10% fetal bovine serum (FBS). KK47/ADR and T24/ADR cells were established from KK47 and T24 cells, respectively, as described previously (4,5). All cell lines were maintained in a 5% CO_2 atmosphere at 37˚C.

Antibodies. Anti-p300 (sc-585) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Lamin B1 antibody was purchased from Abcam (Cambridge, MA, USA).

Knockdown analysis using siRNAs. Knockdown analyses using siRNAs were performed as described previously (14-16). Briefly, the following double-stranded RNA 25-bp oligonucleotides were commercially generated (Invitrogen): 5'-AUUAUAGGA GAGUUCACCGGGCAGG-3' (sense) and 5'-CCUGCCCGG UGAACUCUCCUAUAAU-3' (antisense) for p300 siRNA #1; 5'-UAACAGUGACCUCUCCUGACUCAGG-3' (sense) and 5'-CCUGAGUCAGGAGAGGUCACUGUUA-3' (antisense) for p300 siRNA #2.

Western blot analysis. Western blot analyses were performed as described previously (14-16). The protein concentration of the extracts was quantified using a protein assay kit based on the Bradford method (Bio-Rad, Hercules, CA, USA). Nuclear extracts (30 μg) were separated by 4-20% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ, USA) using a semi-dry blotter. The blotted membranes were incubated for 1 h at room temperature with the primary antibodies described above. Membranes were then incubated for 40 min at room temperature with a peroxidase-conjugated secondary antibody. The bound antibody was visualized using an ECL kit (GE Healthcare Bio-Science) and the membranes were exposed to X-ray film (GE Healthcare Bio-Science).

Cytotoxicity analysis. Cytotoxicity analyses were performed as described previously by Shiota *et al* (14-16). Briefly, T24 or KK47 cells ($2.5x10^3$) transfected with 40 nM of the indicated siRNA were seeded into 96-well plates. The following day, the indicated concentrations of doxorubicin were applied. After 48 h, surviving cells were stained with Alamar Blue Assay (TREK Diagnostic systems, Cleveland, OH, USA) for 180 min at 37˚C. The absorbance was then measured at 570 nm using a plate reader (ARVOTM MX, Perkin-Elmer Inc., Waltham, MA, USA).

Results

p300 is downregulated in doxorubicin-resistant bladder cancer cells. First, we investigated the expression level of p300 in these doxorubicin-resistant cells. As shown in Fig. 1, p300 expression was decreased in both doxorubicin-resistant cell lines (KK47/ADR and T24/ADR cells lines) compared with their parental cell lines (KK47 and T24 cells lines) at the protein level.

Exposure to doxorubicin downregulates p300 expression in bladder cancer cells. Subsequently, we examined p300 expression after exposure to doxorubicin. Human bladder cancer KK47 cells were exposed to 10 nM of doxorubicin for various durations. Western blot analyses showed that p300 expression level was downregulated by doxorubicin exposure in a peak at 6 h after doxorubicin exposure (Fig. 2A). Similarly, p300 expression in T24 cells was also reduced by exposure to doxorubicin in a dose-dependent manner (Fig. 2B).

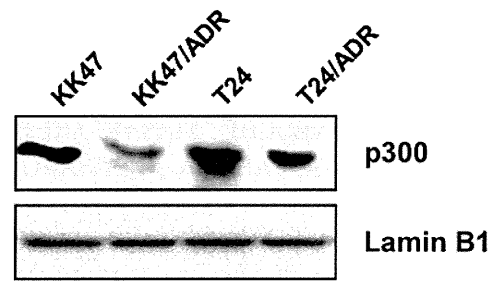

Figure 1. p300 is downregulated in doxorubicin-resistant bladder cancer cells. Nuclear extracts from bladder cancer cells (KK47 and T24 cells) and counterpart doxorubicin-resistant cells (KK47/ADR and T24/ADR cells) were subjected to SDS-PAGE, and Western blot analyses were performed with the indicated antibodies.

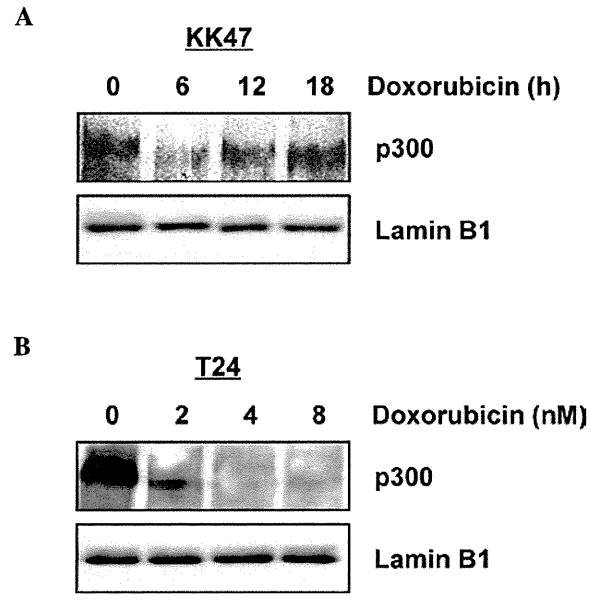

Figure 2. Exposure to doxorubicin downregulates p300 expression in bladder cancer cells. (A) KK47 cells were treated with 10 nM of doxorubicin for the indicated durations. Nuclear extracts were subjected to SDS-PAGE, and Western blot analyses were performed with the indicated antibodies. (B) T24 cells were treated with the indicated concentrations of doxorubicin for 6 h. Nuclear extracts were subjected to SDS-PAGE, and Western blot analyses were performed with the indicated antibodies.

Suppression of p300 renders bladder cancer cells resistant to doxorubicin. Finally, we examined whether p300 affects the cell survival rates after treatment with doxorubicin in KK47 cells. As shown in Fig. 3A, p300 silencing using previously validated p300-specific siRNAs caused KK47 cells to become resistant to doxorubicin. Similarly, cellular resistance to doxorubicin in T24 cells was augmented by p300 shutdown (Fig. 3B) (15,16).

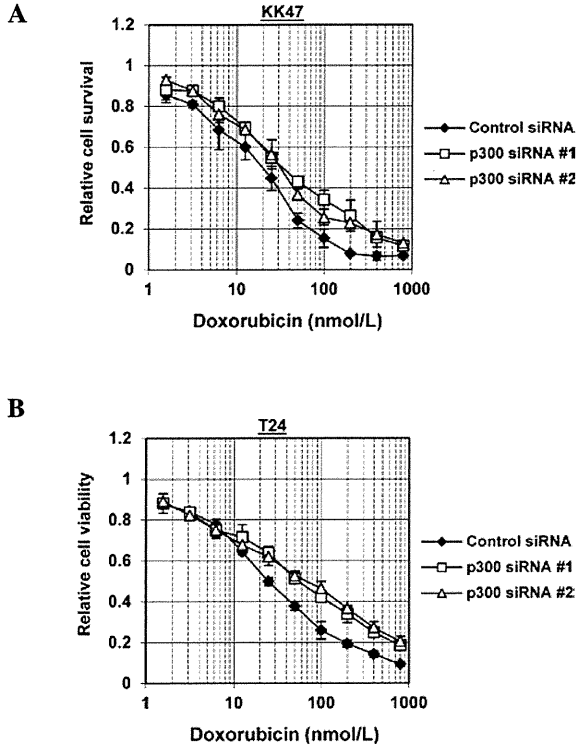

Figure 3. Suppression of p300 renders bladder cancer cells resistant to doxorubicin. KK47 (A) and T24 (B) cells were transfected with 40 nM of the indicated siRNA. On the following day, various concentrations of doxorubicin were added. After 48 h, cell survival rates were analyzed by cytotoxicity analyses. Cell survival in the absence of doxorubicin was set as 1. Data represent the means ± SD.

Discussion

In this study, p300 expression was reduced in doxorubicin-resistant bladder cancer cells. Although the mechanism of this downregulation remains unclear, it may result from the direct effect of the doxorubicin treatment, as exposure to doxorubicin also reduced p300 expression. Doxorubicin is known to exert oxidative stress in various cell systems (17,18). We previously reported that p300 expression was also suppressed by exposure to cisplatin, which can also exert oxidative stress (15,19,20). Therefore, oxidative stress may be involved in the regulation of p300 expression.

We previously reported that PCAF suppression reduced the expression of YB-1, resulting in retarded cell growth and vulnerability to cisplatin and doxorubicin in bladder cancer cells (14). Inversely, p300 suppression induced YB-1 expression, resulting in augmented cell growth and cellular resistance to cisplatin (14,16). However, the involvement of p300 in doxorubicin resistance remained unknown. This study reveals that p300 suppression renders bladder cancer cells resistant to doxorubicin. This finding is compatible with the result that p300 was downregulated in doxorubicin-resistant cells. p300 may confer resistance to doxorubicin by modulating the expression levels of target genes. YB-1 is known to be associated with doxorubicin resistance (14). We previously showed that YB-1 expression was shown to be affected by p300 in KK47 cells but less so in T24 cells which have a low YB-1 expression (16). p300 affected chemosensitivity to doxorubicin in both cells. Thus, p300 may be involved in drug resistance through molecules other than YB-1 as p300 interacts with a number of proteins that may affect drug sensitivity.

Somatic missense mutations of *EP300*, the gene encoding p300, which is thought to be a tumor-suppressor gene, occur in a number of malignancies. Chromosome translocations targeting *EP300* have been reported in acute myeloid leukemia and treatment-related hematological disorders (21). *EP300* gene mutations that result in truncated p300 protein products or amino-acid substitutions in critical protein domains have also been shown in solid tumors (21). Thus, p300 activity may be downregulated in broad range of cancers. Therefore, such cancers with low p300 activity may be resistant to doxorubicin chemotherapy regimens. Hense, the strategy to restore the activity of p300 in such cancers seems to be promising because it is predicted to suppress cell growth as well as augment the chemosensitivity to cisplatin and doxorubicin.

In summary, this study reveals that p300 is downregulated in doxorubicin-resistant bladder cancer cells and by doxorubicin treatment. Consistent with this, p300 suppression promoted drug resistance to doxorubicin in bladder cancer cells. Taken together, this study reveals that p300 may be a promising molecular therapeutic target through modulating chemosensitivity to doxorubicin in bladder cancer.

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