厚生労働科学研究費補助金

がん臨床研究事業

高悪性度筋層非浸潤癌に対する経尿道的膀胱腫瘍切除後の治療方針の確立に関する研究

平成 22 年度 総括研究報告書

研究代表者　塚本　泰司

平成 23（2011）年3月

目　次

Ⅰ．総括研究報告

厚生労働科学研究費補助金（がん臨床研究事業）
総括研究報告書

高悪性度筋層非浸潤癌に対する経尿道的膀胱腫瘍切除後の治療方針の確立に関する研究

研究代表者　塚本　泰司　札幌医科大学医学部教授

研究要旨

近年、組織学的悪性度が high grade (G3)で膀胱粘膜下まで浸潤する T1（HG-T1）の癌については、臨床経過が良好ではないいことから、2nd TUR を行うことが標準治療となっている。2 nd TUR の結果、腫瘍が残存していた場合にはその所見によって治療法が決定される。しかし、T0（癌細胞がない）であった場合のその後の治療方針は明らかではない。2 nd TUR で T0 の症例は、T1 筋層非浸潤癌の中でも予後良好な群と考えられる。このような症例に対して、副作用の比較的多い BCG 膀注療法を避けることができれば、その利点は大きい。このように、本研究で計画した BCG 膀注療法と無治療経過観察とのランダム化比較試験は国内、国外とも実施されておらず、HG-T1 筋層非浸潤癌に対する 2nd TUR 後の治療に関して、唯一のランダム化比較試験によるエビデンスを発信することになる。本年度はこの考えに基づいて（1）臨床試験プロトコールを完成させること、（2）HG- T1 癌の臨床経過を検討することとした。その結果、本研究の臨床試験プロトコールの概要は、既に 2008 年 9 月に JCOG（日本臨床癌研究グループ）運営委員会の承認を得ており、現在プロトコールの最終版が完成し JCOG 運営委員会での承認申請を行い、現在審査中となっている。さらに、HG-T1 癌の臨床経過を検討する目的で初回の TUR で筋層が明らかに採取された HG-T1 癌の膀胱内非再発率（recurrence-free survival: RFS）、筋層非進展（浸潤）率（progression-free survival: PFS）を検討した。観察期間の中央値は 42 か月であった。初回の TUR で筋層が明らかに採取された 45 例の HG-T1 癌を対象にした。2 年および 3 年 RFS はそれぞれ 57%、54%（5 年目も同様）であった。TUR 後に BCG 膀胱内注療法を受けた患者の RFS が低い傾向にあった。一方、筋層浸潤への進展は 3 例（6.7%）に認められたのみで、3 年および 5 年 PFS はそれぞれ、91%であった。以上の結果から、初回の TUR で明らかに筋層が採取されていた場合の経過観察期間内での筋層浸潤への進展のリスクは少ないものの、再発のリスクは無視できないと思われた。現在計画中の上記の臨床試験の結果が今後の治療法の標準化に不可欠であると思われた。

分担研究者氏名・所属機関名及び所属機関における職名

北村　寛・札幌医科大学医学部講師、篠原信雄・北海道大学大学院医学研究科准教授、羽渕友則・秋田大学大学院医学研究科教授、冨田善彦・山形大学医学部教授、北村康男・新潟県立がんセンター新潟病院臨床部長、西沢　理・信州大学医学部教授、藤元博行・国立がん研究センター中央病院医長、川島清隆・栃木県立がんセンター泌尿器科

副部長、馬場志郎・北里大学医学部教授、武田正之・山梨大学大学院医学工学研究部教授、庭川　要・静岡がんセンター部長、杉村芳樹・三重大学大学院医学研究科教授、後藤百万・名古屋大学大学院医学研究科教授、西村和郎・大阪府立成人病センター泌尿器科部長、小川　修・京都大学大学院医学研究科教授、平尾佳彦・奈良医科大学教授、筧　善行・香川大学医学部教授、橋根勝義・四国がんセンター泌尿器科医長、荒井陽一・東北大学大学院医学系研究科教授、江藤正俊・熊本大学大学院生命科学部教授、松岡　啓・久留米大学医学部教授、中川昌之・鹿児島大学医歯学総合研究科教授、大家基嗣・慶応大学医学部教授、藤澤正人・神戸大学大学院医学研究科教授、井川幹夫・島根大学医学部教授、寺井章人・倉敷中央病院泌尿器科部長、大園誠一郎・浜松医科大学医学部教授、穎川　晋・東京慈恵会医科大学教授、堀江重郎・帝京大学医学部教授、山口秋人・原三信病院泌尿器科副院長、市川智彦・千葉大学大学院医学研究科教授、谷川俊貴・新潟大学大学院医歯学総合研究科講師、栃木達夫・宮城県立がんセンター泌尿器科部長、大山　力・弘前大学大学院医学研究科教授、浅野友彦・防衛医科大学校教授

A. 研究目的

筋層非浸潤癌の初発例では、多くの場合、経尿道的腫瘍切除(TUR)による膀胱温存治療が可能である。しかし、TUR後も経過中に膀胱内再発をみることが少なくなく、筋層非浸潤癌の10%程度が筋層浸潤癌として再発し、それに対しては膀胱全摘が行われる。筋層非浸潤癌の中で筋層浸潤をきたすリスクが最も高いのは、組織学的悪性度がhigh grade (G3)で膀胱粘膜下まで浸潤するT1（HG-T1）の癌であり、従来は、このような症例に対してはBCG(場合によってはマイトマイシン）の膀注あるいは全摘が選択されてきた。近年、HG-T1癌について、初回TURでは癌の深達度や残存の有無の診断精度が低く、T2（筋層浅層浸潤）の見落としや残存腫瘍があることが認識され、初回TURのおおむね6-8週後に再度TURすること（2nd TUR）が提唱され、本邦においても、現在はHG-T1 筋層非浸潤癌に対しては、標準治療となっている。2 nd TURの結果、腫瘍が残存していた場合にはその所見によって治療法が決定されるが、T0（癌細胞がない）であった場合のその後の治療方針は明らかではない。2 nd TURでT0の症例は、T1筋層非浸潤癌の中でも予後良好な群と考えられる。このような症例に対してBCG膀注療法を避けることができれば、その利点は大きい。

本年度はこの考えに基づいて（1）臨床試験プロトコールを完成させること、(2) HG-T1癌を含む筋層非浸潤癌の臨床研究検討することとした。

B. 研究方法

研究①　HG-T1 筋層非浸潤癌の2nd TUR後の治療に関するランダム化比較臨床試験

本臨床研究は日本臨床腫瘍研究グループ（JCOG）のデータセンター／運営事務局と各種委員会の支援と管理を受けJCOG泌尿器科腫瘍グループが実施する。

HG-T1筋層非浸潤癌患者を一次登録後、2nd TURを実施する。切除標本に癌が認められなかった（T0）患者を二次登録し補助治療としてBCG膀注を実施する群（BCG膀注群）と、無治療経過観察群（経過観察群）にランダム割付し、BCG膀注群に対して経過観察群が有効性において非劣性であることを検証する。Primary endpointは「T1以上の膀胱内再発および遠隔転移がない生存期間」とし、全生存期間、膀胱温存生存期間、無治療経過観察群の膀胱内非再発割合、無治療経過観察群のT2膀胱癌非再発割合、およびBCG膀注療法の有害事象をsecondary endpointとする。

併せて、本邦においては2nd TUR後の経過そのものが十分に明らかでないため、一次登録例全例について、二次登録ランダム化の有無にかかわらず、引き続いて実施された治療方法、生存期間、遠隔転移出現、膀胱全摘の有無、膀胱温存の場合の膀胱内

再発と筋層浸潤出現を追跡調査する。
研究②　HG-T1 癌を含む筋層非浸潤癌の臨床経過の検討
1) 初回 TUR で筋層が明らかに切除され HG-T1 癌と診断された症例の臨床経過の検討
2) Ta、T1、Tis 癌に対する BCG 維持療法の有効性の検討
3) 筋層非浸潤癌の晩期再発の検討

C. 研究結果

1）研究―1　臨床試験

実際の臨床試験計画は以下の通りである（図1）。初回 TUR で HG-T1 であり、本試験に同意の得られた患者を一次登録し、初回 TUR から 8 週以内に 2nd TUR を実施する。2nd TUR で T0 と診断され、術後の 2 回の尿細胞診がいずれも class II 以下であった患者を二次登録し、BCG 膀注群と経過観察群のいずれかにランダム割付を行う。BCG 膀注群で BCG の膀注を 2nd TUR から 2 週目以降 8 週以内に開始し、週 1 回 で 8 回行う。経過観察群では経過観察のみを行う。両群とも登録後 3 年間は 3 か月ごとの膀胱鏡および尿細胞診検査を行う。膀胱鏡で明らかな再発がなく、尿細胞診で class II 以下の場合は経過観察を継続する。上部尿路の検索は、試験期間中は 1 年に 1 回実施する。
膀胱内再発は以下のように定義する。
- 膀胱鏡での再発が確認された場合。
- 膀胱鏡では明らかな再発の所見はないが、自排尿の尿細胞診で class III 以上であり膀胱粘膜のランダム生検により病理組織学的に癌の再発（上皮内癌）が確認された場合。

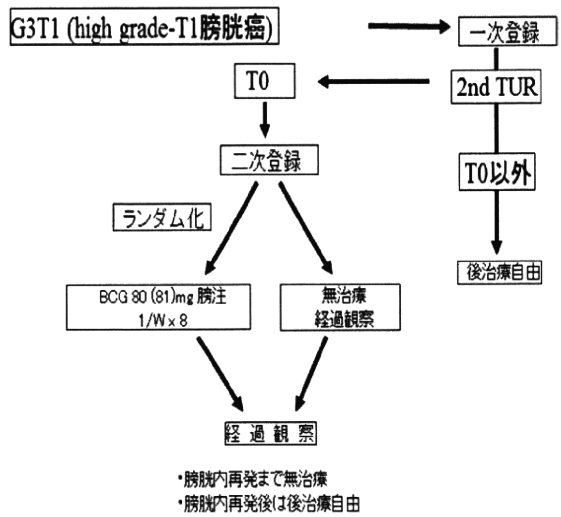

図1　臨床試験の治療プロトコール

【統計学的考察と症例集積見込み】

ランダム割付は、施設、初発あるいは再発、を調整因子とする最小化法にて行う。Primary endpoint である「T1 以上の膀胱内再発および遠隔転移がない生存期間」の 3 年の割合を 80%と仮定し、無治療経過観察群の非劣性の許容域を 10%（ハザード比で 1.6 に相当）、α片側 5%、検出力 70%、登録期間 5 年間、追跡期間 3 年とすると、Shoenfeld and Richter の方法による二次登録ランダム化に必要な症例数は 1 群 129 例、両群計 258 例となる。一次登録例中、2nd TUR で T0 となる患者の割合を 85%強と推測し、一次登録の予定登録数を 300 例とする。若干の不適格例を見込んで二次登録が 260 例のとなった時点で一次登録を終了する。JCOG 泌尿器腫瘍グループでの調査では 31 施設で HG-T1 症例が年間計 348 例あり、年間 60-70 例以上の登録が見込まれ、5 年間での集積が可能と思われる。

（倫理面への配慮）

本臨床試験では以下のような倫理面への配慮を行っている。
1) 本試験に参加可能な症例の各種条件（年齢、臨床病期、各種臓器機能など）を設定し、臨床試験プロトコールに明示している。

2) 本試験はヘルシンキ宣言を遵守し、試験参加の利益、不利益を「説明文書」に記載することで、参加者が容易に理解可能となるようにしている。
3) 本試験への参加はあくまでも本人の自由意志であることを明示し、文書で参加の意思を確認するようにしている。
4) 本臨床試験での治療費は通常の保険診療で行われることを明示している。
5) 本臨床試験に参加する施設での倫委員会あるいはそれに準ずる委員会の承認を求めている。
6) JCOG 臨床試験員会あるいは各施設の倫理員会で承認された後に実施される臨床試験であることを明示している。

2）研究―2　HG-T1 癌の臨床研究

1) 初回 TUR で筋層が明らかに切除され HG-T1 癌と診断された症例の臨床経過の検討 (Shindo et al, Jpn J Clin Oncol, 2010; 40: 153)

HG-T1 癌は筋層浸潤に関してはハイリスクであり、2nd TUR が推奨されている。一方、初回の TUR で十分に筋層を切除することがより重要であるとの考えもある。そこで、初回の TUR で十分な筋層切除が可能であった HG-T1 癌の臨床経過を検討した。42 か月（中央値）の経過観察期間で 21 例（47%）に膀胱内再発が見られた。この再発率は BCG の膀胱内注入を受けた症例のほうが低い傾向にあった。一方、筋層浸潤への進展は 3 例（7%）に認められた。全体の症例での 3 年および 5 年非再発率はいずれも 54%であり、3 年および 5 年非筋層浸潤進展率はいずれも 91%であった（図 2）。

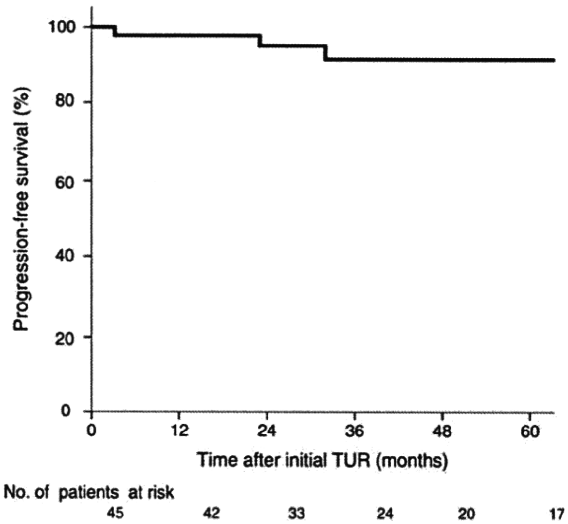

図 2　非筋層浸潤進展率

2) Ta、T1、Tis 癌に対する BCG 維持療法の有効性の検討 (Koga et al, Int J Urol, 2010; 17: 759)

BCG 膀胱内注入療法における維持療法の臨床的意義に関して 90 例の筋層非浸潤癌を対象として検討した。筋層非浸潤癌の確認後、BCG 80mg 膀胱内注入を週 1 回 8 回行った。導入療法で CR が得られ、かつ試験に同意の得られた 51 症例を最終的な対象として、維持療法（80mg を 3 か月ごと 4 回注入）と非維持療法群とに無作為化

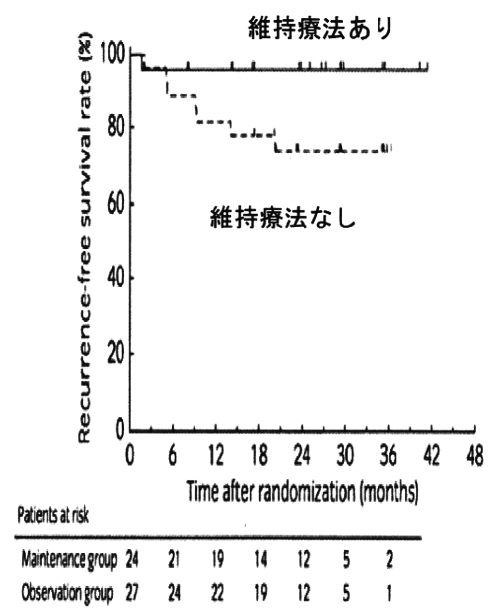

図 3　維持療法の有無による非再発率

を行い、非再発率を検討した。膀胱内非再発率は維持療法を行った群で高い傾向があったが、有意差は認められなかった（図３）。また、これまで報告されてきた維持療法の注入回数より少ない回数で再発防止に寄与する可能性が示唆された。なお、維持療法に特異的な有害事象はなかった。

3) 筋層非浸潤癌の晩期再発の検討
(Matsumoto et al, Urology, 2010; 75: 1385)

5年以上膀胱内再発がない筋層非浸潤癌は一般的にはその後の臨床経過も良好であると考えられるが、実際には5年以上の無再発期間後に再発し筋層浸潤をきたすことがある。そこで、このような症例の臨床経過を検討した。5年間以上再発のなかった262例を対象とした。再発のなかった5年後の時点を経過観察開始時点とすると、その後の5年および10年非再発率は82%、76%であった。低、中、高再発リスク別の非再発率に差はなかった。高再発リスク群の一部の症例は筋層浸潤あるいは上部尿路再発をきたした。

D. 考案

１）研究―１　臨床試験

既述のようにHG-T1筋層非浸潤癌に対する2 nd TURは現在では標準治療として位置づけられている。しかし、2 nd TURの結果、①T2と診断された場合は全摘が、②T1癌が残存していた場合には状況によって全摘か、BCG膀注療法が、③Tis（上皮内癌） あるいはTa（粘膜内に留まる癌）の残存していた場合はBCG膀注療法がそれぞれ標準的治療と考えられる。しかし、T0であった場合のその後の治療方針は確立されていない。このような症例の臨床経過は良好と考えられているため、２ケ月の通院治療が必要で、排尿痛や頻尿が必発で、稀ではあるが萎縮膀胱などの重篤な有害事象もある得るBCG膀注を一律に行うことがリスク／ベネフィットバランスの点で疑問視されている。

従って、本試験では、初回TURでHG-T1癌と診断された筋層非浸潤癌患者のうち、2 nd TURでT0と診断された患者を対象に、再発予防目的にBCG膀注を実施すべきか、無治療経過観察でも良いのかをランダム化比較試験で検討することにした。このようなランダム化比較試験は国内、国外とも実施されておらず、HG-T1 筋層非浸潤癌に対する2nd TUR後の治療に関して、唯一のランダム化比較試験によるエビデンスを発信することになると考えられる。

２）研究―２　HG-T1癌の臨床研究

1) 初回TURで筋層が明らかに切除されHG-T1癌と診断された症例の臨床経過の検討

HG-T1癌はその経過で高率に膀胱内再発をきたし筋層浸潤へと進展することが報告されてきた。HG-T1癌に対しては2nd TURを行うことが標準治療として位置づけられている理由でもある。しかし、初回のTURで膀胱筋層切除が明確ではない場合の筋層浸潤進展率は16-33%と報告されているが (Shindo, Jpn J Clin Oncol, 2010; 40: 153)、筋層切除が十分に確認された場合の筋層浸潤進展率はそれほど高くないのではないかという報告もある (Gohji et al, Urology, 1999; 53: 308)。今回の検討においても、初回のTURで膀胱筋層切除が十分になされ筋層浸潤が否定された場合には、その後の経過で筋層浸潤をきたす割合は決して高率ではないことが示された（5年非進展率：93%）。再発への影響は今後の検討課題であるが、初回TURで膀胱筋層を含めた十分な切除を行うことがHG-T1癌の診断と治療に不可欠であることが再確認された。

2) Ta、T1、Tis癌に対するBCG維持療法の有効性の検討

今回の検討結果は、ハイリスク筋層非浸潤癌に対するBCG膀胱注入の効果を再確認したと同時に、維持療法の実施方法にも示唆を与えるものと思われた。実際、これまでは、3回連続注入（3週間）のサイクルと再発とに関連があるとされ、この3回連続注入を3コース繰り返すこと（計9回

注入）が再発率の低下に寄与するとされてきた。しかし、今回の検討では4回の注入でも再発率を低下させることが示唆され、この点についての検討も今後必要であると考えられた。

3) 筋層非浸潤癌の晩期再発の検討

筋層非浸潤癌の再発および筋層浸潤進展は通常TUR後2-3年以内が多く、5年以上経過した後に再発、進展が出現することは比較的少ない。そのため、一般的には術後5年以内の集中的な経過観察が行われている。今回の検討では、5年以降も再発する傾向が継続し、5年以内に20%が再発を示した。この再発は、癌のリスク分類と無関係で再発のリスクが低い症例が実際に低い再発率ではなかった。さらに、頻度は低い（2%）ものの筋層浸潤癌へ進展する可能性も示唆された。したがって、5年以上にわたる経過観察の必要性が示唆された。

E. 結論

1）HG-T1 筋層非浸潤癌の2nd TUR後の治療に関するランダム化比較臨床試験のプロトコールを作成し、JCOG運営委員会での承認申請を行った。

2）HG-T1癌の臨床研究を行い以下の結果を得た。

1) HG-T1癌の初回TURで膀胱筋層を含めた十分な切除を行うことで、その後の筋層浸潤への進展率を抑えることができる。
2) BCG膀胱内注入療法では維持療法を行うことで再発率の低下が期待できるが、維持療法の回数は従来の回数より少なくともよい可能性がある。
3) 術後5年以上経過した後の再発、筋層浸潤進展の可能性は、これまで予想されていたよりは多い可能性がある。より長期の経過観察の必要性がある。

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

1) Shindo T, Masumori N, Fukta F, Miyamoto S, Tsukamoto T. Is T1G3 bladder cancer having a definite muscle layer in TUR specimens a highly progressive disease? Jpn J Clin Oncol, 2010; 40: 153-156, 2010.
2) Matsui Y, Watanabe J, Ding S, Nishizawa K, Kajita Y, Ichioka K, Saito R, Kobayashi T, Ogawa O, et al. Dicoumarol enhances doxorubicin-induced cytotoxicity in p53 wild-type urothelial cancer cells through p38 activation. BJU Int. 2010; 105:558-564.
3) Chiyomaru T, Enokida H, Tatarano S, Kawahara K, Uchida Y, Nisyiyama K, Fujimura L, Kikkawa N, Seki N, Nakagawa M. miR-145 and miR-133a function as tumour suppressors and directly regulate FSCN1 expression in bladder cancer. Br J Cancer. 102; 883-891, 2010.
4) Matsumoto M, Kawakami K, Enokida H, Toki K, Matsuda R, Chiyomaru T, Nishiyama K, Kawahara K, Seki N, Nakagawa M. CpG hypermethylation of human four-and a-half LIM domains 1 contributes to migration and invasion activity of human bladder cancer. Int J Mol Med. 2010; 26: 241-247.
5) Kakehi Y, Hirao Y, Kim WJ, Ozono S, et al. Bladder Cancer Working Group Reports. Jpn J Clin Oncol, 2010; 40 (supl. 1): i57-i64.
6) Behnsawy HM, Miyake H, Abdalla MA, Sayed MA, El-Fattah A, Fujisawa M, et al. Expression of integrin proteins in non-muscle invasive bladder cancer: significance of intravesical recurrence following transurethral resection. BJU International, 2010; 107: 240-246.
7) Koga H, Ozono S, Tsushima T, Tomita

K, Horiguchi Y, Usami M, Hirao Y, Akaza H, Naito S, BCG Tokyo Strain Study Group. Maintenance intravesical bacillus Calmette-Guérin instillation for Ta, T1 cancer and carcinoma in situ of the bladder: randomized controlled trial by the BCG Tokyo Strain Study Group. Int J Urol, 2010; 17: 759-766.

8) Miyake M, Sugano K, Sugino H, Imai K, Matsumoto E, Maeda K, Fukuzono S, Ichikawa H, Kawashima K, Hirabayashi K, Kodama T, Fujimoto H, Kaskizoe T, Kanai Y, Fujimoto K, Hirao Y. Fibroblast growth factor receptor 3 mutation in voided urine is a useful diagnostic marker and significant indicator of tumor recurrence in non-muscle invasive bladder cancer. Cancer Sci, 2010; 101: 250- 258.
9) Matsumoto K, Kikuchi E, Horiguchi Y, Tanaka N, Miyajima A, Nakgawa K, Nakashima J, Oya M. Late recurrence and progression in non-muscle invasive bladder cancer after 5-year tumor-free periods. Urology, 2010; 75: 1385-1390.
10) Takeuchi A, Dejima T, Yamada H, Shibata K, Nakamura R, Eto M, Nakatani T, Naito S, et al. IL-17 production by gamma-delta T cells is important for the antitumor effect of Mycobacterium bovis bacillus Calmette-Guérin treatment against bladder tumor. Eur J immunol, 2011; 41: 246-251.
11) Furuse H, Ozono S. Transurethral resection of the bladder tumour (TURBT) for non-muscle invasive bladder cancer: Basic skills. Int J Urol, 2010; 17: 698-699.

Ⅱ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書 籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
		な			し		

雑 誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Shindo T, Masumori N, Fukuta F, Miyamoto S, Tsukmoto T	Is T1G3 bladder cancer having a definite muscle layer in TUR specimens a highly progressive disease?	Jpn J Clin Oncol	40	153-156	2010
Matsui Y, Watanabe J, Ding S, Nishizawa K Kajita Y, Ichioka K, Saito R, Kobayashi T, Ogawa O, Nishiyama H	Dicoumarol enhances doxorubicin-induced cytotoxicity in p53 wild-type urothelial cancer cells through p38 activation	BJU Int	105	558-564	2009
Chiyomaru T, Enokida H Tatarano S Kawahara K, Uchida Y Nishiyama K Fujimura L Kikkawa N Seki N Nakagawa M	miR-I45 and miR-I33a function as tumour suppressors and directly regulate FSCNI expression in bladder cancer	Brit J Cancer	102	883-891	2010
Matsumoto M, Kawakami K, Enokida H, Toki K, Matsuda R Chiyomaru T Nishiyama K Kawahara K, Seki N Nakagawa M	CpG hypermethylation of *human hour-and -a-half LIM domains 1* contributes to migration and invasion activity of human bladder cancer	Int J Mol Med	26	241-247	2010

Kakehi Y, Hirao Y Kim WJ Ozono S, et al.	Bladder cancer working group report	Jpn J Clin Oncol	40	i57-i64	2010
Behnsawy HM Miyake H, Abdalla MA, Sayed MA, Ahmed AE, Fujisawa M	Expression of integrin proteins in non-muscle-invasive bladder cancer: significance of intravesical recurrence after transurethral resection	BJU Int	107	240-246	2010
Koga H, Ozono S, Tsushima T, Tomita K, Horiguchi Y Usami M, Hirao Y, Akaza H, Naito S	Maintenance intravesical bacillus Calmette-Guerin instillation for Ta, T1 cancer and carcinoma *in situ* of the bladder: Randomized controlled trial by the BCG Tokyo Strain Study Group	Int J Urol	17	759-767	2010
Miyake M, Sugano K, Sugino H, Imai K, Matsumoto E, Maeda K, Fukuzono S, Ichikawa H, Kawashima K, Hirabayashi K, Kodama T, Fujimoto H, Hirao Y	Fibroblast growth factor receptor 3 mutation in voided urine is a useful diagnostic marker and significant indicastor of tumor recurrence in non-muscle invasive bladfer cancer	Cancer Sci	101	250-258	2010
Matsumoto K, Kikuchi E, Horiguchi Y, Tanaka N, Miyajima A, Nakagawa K, Nakashima J, Oya M	Late recurrence and progression in non-muscle-invasive bladder cancers after 5-year tumor-free periods	Urology	75	1385-1391	2010
Takeuchi A, Dejima T, Yamada H, Shibata K, Nakmura R, Eto M, Nakatani T, Naito S, et al.	IL-17 production by γδ T cells is important for the antitumor effect of Mycobacterium bovis bacillus Calmette-Guerin treatment against bladder cancedr	Eur J Imunol	41	246-251	2011
Furuse H, Ozono S	Transurethral resection of the bladder tumour (TURBT) for non-muscle invasive bladder cancer: Basic skills	Int J Urol	17	698-699	2010

Ⅲ. 研究成果の刊行物・別刷

JJCO Japanese Journal of Clinical Oncology

Jpn J Clin Oncol 2010;40(2)153–156
doi:10.1093/jjco/hyp132
Advance Access Publication 19 October 2009

Is T1G3 Bladder Cancer Having a Definite Muscle Layer in TUR Specimens a Highly Progressive Disease?

Tetsuya Shindo, Naoya Masumori, Fumimasa Fukuta, Shintaro Miyamoto and Taiji Tsukamoto

Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan

For reprints and all correspondence: Naoya Masumori, Department of Urology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan. E-mail: masumori@sapmed.ac.jp

Received June 10, 2009; accepted September 11, 2009

Objective: Patients with T1G3 bladder cancer are at high risk of progression to muscle-invasive cancer, and early cystectomy is considered as a treatment option in this particular situation. On the other hand, understaging of T1G3 bladder cancer has been gradually proven as second or repeat transurethral resection (TUR) has been widely applied. To evaluate the real rate of progression, we investigated the prognosis of T1G3 bladder cancer in which a muscle layer was histologically confirmed in the TUR specimens.

Methods: We retrospectively reviewed 48 patients with primary T1G3 bladder cancer in which a muscle layer in the TUR specimens was confirmed between 1990 and 2006 in our institute. We investigated recurrence and progression in 45 patients, excluding 3 who were immediately treated with radical cystectomy. Fifteen and 12 patients received intravesical treatment with bacillus Calmette–Guérin (BCG) and anticancer agents just after TUR, respectively. The remaining 18 did not have any such treatment.

Results: Recurrence and progression were observed in 21 (47%) and 3 patients (6.7%), respectively, during a median follow-up period of 42.1 months. The 3-year recurrence-free and progression-free survival rates were 54% and 91%, respectively. No significant differences were observed in the rates between the patients with and without BCG treatment in the study.

Conclusions: There is a possibility that the progression rate in patients with T1G3 bladder cancer is not as high as previously reported when only patients whose muscle layer was histologically confirmed were analyzed. An adequate technique for TUR that unmistakably collects the muscle layer may be important to predict the outcome accurately.

Key words: T1G3 – bladder cancer – progression – understaging – muscle layer

INTRODUCTION

Approximately 70% of bladder tumors are diagnosed as superficial cancer without invasion to the muscle layer at initial presentation (1). Although the prognosis of superficial bladder cancer is generally favorable, it is known that T1G3 bladder cancer is a distinct clinical entity from the remaining superficial cancer since it has been reported that the disease eventually progresses in approximately half of the patients with T1G3 cancer during follow-up (2,3). Because of its high risk for progression, early radical cystectomy is sometimes considered as an initial treatment, although it is controversial (4).

As second or repeat transurethral resection (TUR) for T1 bladder cancer is becoming widely used, it is known that understaging of T1 cancer frequently occurs (5). Although T1 cancer is defined as a tumor with invasion to the lamina propria but no invasion to the detrusor muscle, it seems to be clinically composed of two types. One is genuine T1 cancer in which cancer cells really exist up to the lamina propria. The other is T2 or higher cancer misdiagnosed as T1 cancer because the detrusor muscle is not included in the TUR specimens.

As previously noted, it has been believed that patients with T1G3 cancer have a high progression rate. If there is contamination by T2 or higher cancer, the prognosis of T1G3 cancer must be worse than the true one. However, only a few reports analyzing the prognosis of T1G3 cancer demonstrate whether the TUR specimens included the

detrusor muscle (6). In the present study, we retrospectively evaluated recurrence and progression in patients with T1G3 bladder cancer whose detrusor muscle was histologically confirmed.

PATIENTS AND METHODS

In our institute, TUR of bladder cancer was done in the standard manner. Once all visible tumors were resected, the base of the main tumor was resected again until the perivesical fat tissue became visible. Care had to be taken not to extensively penetrate the bladder wall. Between 1990 and 2006, we treated 48 patients with initially diagnosed T1G3 bladder cancer in our institute. It was histologically confirmed that TUR specimens derived from all patients included enough of the muscle layer to adequately determine the T stage. On the other hand, there were another 11 patients diagnosed with T1G3 bladder cancer at the same period in our institute who had no evaluable muscle layer in the TUR specimens. Patients who had findings suggestive of muscle-invasive bladder cancer on radiographic studies and a history of upper urinary tract cancer were excluded. In addition, patients who did not receive regular cystoscopic examination every 3 months after TUR were excluded.

Of the 48 patients, 3 patients underwent immediate radical cystectomy because of the patients' decisions, based on concomitant localized prostate cancer, severe hematuria and storage symptoms caused by bladder cancer. In the remaining 45 patients with T1G3 cancer, the bladder was preserved. Eighteen patients did not have any bladder instillation therapy after TUR. Of the remaining 27 patients, 15 and 12 received intravesical treatment with bacillus Calmette–Guérin (BCG, 80 mg of Tokyo strain for 8 patients and 81 mg of Connaught strain for 7 patients) and an anticancer agent (anthracyclines for 8 patients and mitomycin C for 4 patients), respectively. BCG was intravesically instillated every week for 6–8 weeks as one course and every patient received at least one course of BCG. None received maintenance BCG therapy. Because of the retrospective nature of the present study, indications for additional therapy and criteria for drug selection were not uniform. However, patients who had carcinoma *in situ* or persistent positive urine cytology after TUR were exclusively treated with BCG (Table 1).

The patients were followed by cystoscopic examination and urine cytology every 3 months. If necessary for suspicious symptoms and findings of metastatic development, radiographic examination using computed tomography and chest X-rays was conducted. When bladder tumors were found on cystoscopic examination, TUR was performed to pathologically confirm recurrence. Invasion of the muscle layer, as well as development of distant metastasis, was defined as progression.

The Kaplan–Meier method and log-rank test were used for statistical analysis of recurrence and progression. A value of $P < 0.05$ was defined as statistically significant.

Table 1. Backgrounds of patients treated with BCG and an anticancer agent

	BCG ($n = 15$)	Non-BCG ($n = 30$)
Carcinoma *in situ*	3 (20.0%)	0 (0%)
Positive cytology[a] (after TUR)	2 (13.3%)	0 (0%)
Tumor size (mm)		
<10	6 (40.0%)	4 (13.3%)
≥10	9 (60.0%)	26 (86.7%)
Tumor number		
Solitary	3 (20.0%)	9 (30.0%)
Multiple	12 (80.0%)	21 (70.0%)
Repeat TUR	1 (6.7%)	0 (0%)

BCG, bacillus Calmette–Guérin; TUR, transurethral resection.
[a]Patients who represented positive urine cytology after TUR were different from patients who had carcinoma *in situ*.

RESULTS

The median age of the 45 patients (42 men and 3 women) at initial TUR was 68 years, ranging from 29 to 97. The median follow-up period was 42.1 months (range, 9.5–131.4).

Recurrence was observed in 21 (47%) patients. The recurrence-free survival rates at 2 and 3 years were 57% and 54%, respectively (Fig. 1). Most recurrences occurred within 2 years after TUR. The recurrence-free survival rate in the 15 patients treated with BCG was 73% at 2 and 3 years (Fig. 2). Although the 50% 2-year and 44% 3-year recurrence-free survival rates in 30 patients without BCG were lower than those with BCG, there was no significant difference in recurrence-free survival between them ($P = 0.097$, log-rank test). The 3-year progression-free survival rate of the 45 patients was 91% (Fig. 3).

Progression was observed in three patients (6.7%). None of the three patients received BCG therapy or had distant metastasis at the initial presentation of progression. After progression, two patients (4.4%) eventually died of bladder cancer.

Pathological comparison between the TUR and cystectomy specimens in the three patients treated with immediate radical cystectomy revealed understaging for one patient (pT3pN0). The patient developed distant metastasis 19 months after surgery and finally died of bladder cancer in spite of treatment by several courses of systemic chemotherapy.

Of the 11 patients without a muscle layer in the TUR specimens, 6 (54.5%) and 3 (27.3%) patients showed recurrence and progression, respectively. Eventually, two patients died of bladder cancer.

DISCUSSION

One of the biggest problems of T1G3 bladder cancer is progression to muscle-invasive cancer. Since it is obvious that

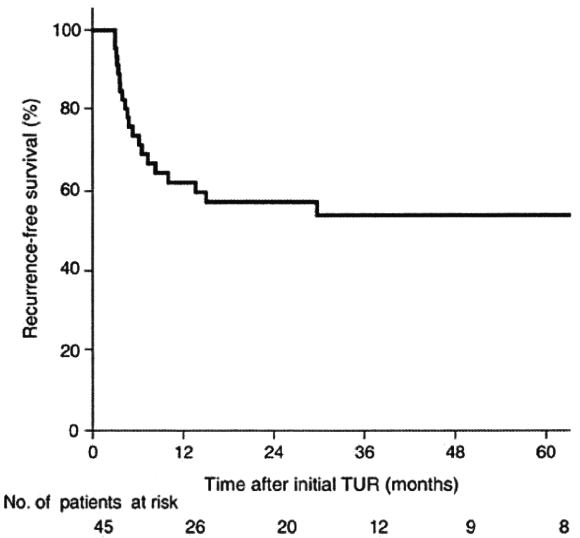

Figure 1. Recurrence-free survival in 45 patients with primary T1G3 bladder cancer for which the muscle layer was histologically confirmed. TUR, transurethral resection.

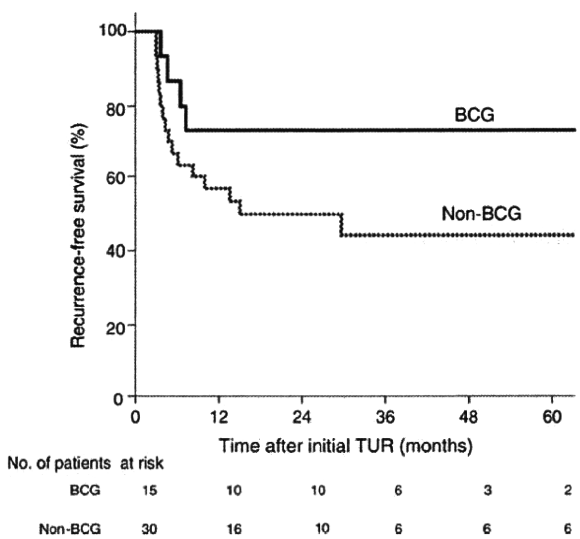

Figure 2. Recurrence-free survival in patients with BCG ($n = 15$) and without BCG treatment ($n = 30$). No significant difference was observed ($P = 0.097$, log-rank test). BCG, bacillus Calmette–Guérin.

the prognosis of muscle-invasive cancer is not promising, to accurately predict the probability of progression in patients with T1G3 cancer is clinically important to make treatment decisions, especially considering whether immediate radical cystectomy should be offered as the initial treatment.

Previous studies have demonstrated that the recurrence and progression rates in patients with T1G3 cancer vary widely from 27% to 70% and 4% to 33%, respectively (Table 2) (3,6,13,14). Such variation may indicate that T1G3 cancer is composed of heterogeneous groups. Since it is known that understaging of T1 cancers frequently occurs (4), the degree of contamination by muscle-invasive cancer in

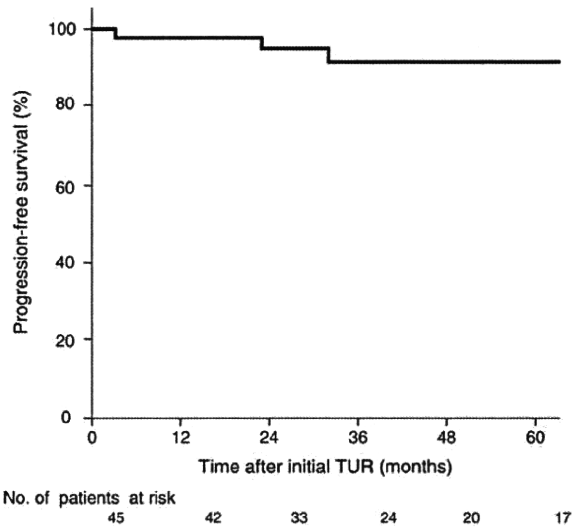

Figure 3. Progression-free survival in 45 patients with primary T1G3 bladder cancer for which the muscle layer was histologically confirmed.

Table 2. Recurrence and progression rates of T1G3 bladder cancer

Author (reference)	No. of patients	Median follow-up period (months)	Recurrence rate (%)	Progression rate (%)
Gohji et al. (6)[a]	45	63	36	4
Brake et al. (13)	44	43	27	16
Patard et al. (14)	50	65	52	22
Shahin et al. (3)	92	64	70	33
Present study[a]	45	42	47	6.7

[a]Studies analyzed patients whose muscle layers were histologically included in the transurethral specimens.

T1G3 cancer may be related to the differences in progression rates among the reports.

Dutta et al. (7) performed radical cystectomy for 63 patients who were diagnosed as having T1 bladder cancer. The muscle layer was confirmed in the TUR specimens from 37 patients, whereas it was not included for 26 patients. Pathological evaluation of cystectomy specimens indicated that 30% and 62% of the patients with and without the muscle layer in the TUR specimens showed pT2 or higher diseases, respectively.

Herr (5) reported a similar result. They performed repeat TUR for 58 patients who were considered to have T1 bladder cancer in the initial TUR. Repeat TUR found muscle invasion in 5 patients (14%) with the muscle layer and in 11 patients (49%) without the muscle layer in the initial TUR. Thus, it is apparent that understaging of T1 cancer is likely to occur if the muscle layer is not included in the TUR specimen.

In the present study, the progression rate of T1G3 bladder cancer during 42 months of follow-up was 6.7% when only

patients with a histologically confirmed muscle layer in the TUR specimens were analyzed. The 3-year progression-free survival rate was 91%. On the other hand, the 27.3% of the progression rate in the patients without a muscle layer was much higher, although it is hard to compare the rates of the two groups because of the small number of the patients. Gohji et al. (6) reported the lowest progression rate to our knowledge (Table 2). Their study consisted of 45 patients with T1G3 cancer whose TUR specimens included a definite muscle layer. Thus, the progression rate in patients with T1G3 cancer was not so high if we analyzed only patients whose muscle layer was histologically confirmed, which suggests less possibility of contamination by muscle-invasive bladder cancer. In the present study, of the three patients treated with immediate radical cystectomy, one was proven to be understaged because muscle invasion was found in the cystectomy specimen. If this patient is followed without immediate radical cystectomy, progression should be observed. However, even though we assume the patient to be a subject with progression, the progression rate, 8.7% (4 of the 46 patients), was still lower than those in the previous reports, although it is hard to draw a solid conclusion because of the small sample size in this study.

The second clinical problem of T1G3 cancer is its high recurrence rate. Similar to the previous reports, recurrence was observed in 47% in the present study (Table 2). Recurrence occurred within 2 years in most patients. To prevent early recurrence in T1G3 cancer, BCG or anticancer agents are indicated (8,9). Although there was no significant difference in the recurrence-free survival rate between patients with and without BCG therapy in our study, the lack of uniform criteria for intravesical therapy and small sample size may have influenced the result. There was a 29% difference at 3 years after TUR between the two groups and no recurrence was observed after 1 year in the BCG group. Several large studies have indicated that intravesical BCG therapy makes a contribution to prevent recurrence for patients with superficial bladder cancer superior to those of intravesical anticancer agents (10–12).

As previously discussed, it is likely that understaging is more frequent in T1G3 bladder cancer in which the muscle layer is not included. On the other hand, understaging was sometimes observed even in patients whose muscle layer was sufficiently collected in the TUR specimens. In the present study, one of the three patients with muscle-layer-confirmed T1G3 who underwent immediate cystectomy had pT3 bladder cancer. Herr (5) reported that repeat TUR for 35 patients with T1 bladder cancer having a definite muscle layer in the initial TUR showed residual cancer in 26 (74%) and muscle-invasive cancer in 5 (14%). A recent randomized study done by Divrik et al. (15) indicated that T1 patients with repeat TUR plus intravesical mitomycin C had significantly lower recurrence (26%) than those with only initial TUR plus intravesical mitomycin C (63%). In addition, the progression rate was lower in patients with repeat TUR (4%) than in those without it (12%), although the difference did not reach statistical significance ($P = 0.097$). Thus, repeat TUR may contribute to not only more accurate diagnosis and a more precise prognosis but also an improved outcome for patients with T1G3 cancer whose muscle layer is confirmed in the initial TUR specimen.

In conclusion, there is a possibility that the progression rate in patients with T1G3 bladder cancer is not as high as previously reported when only patients whose muscle layer was histologically confirmed were analyzed. An adequate technique for TUR that clearly collects the muscle layer may be important to predict the outcome accurately.

Conflict of interest statement

None declared.

References

1. Herr HW, Donat SM, Dalbagni G. Can restaging transurethral resection of T1 bladder cancer select patients for immediate cystectomy. *J Urol* 2007;177:75.
2. Andius P, Holmang S. Bacillus Calmette Guerin therapy in Stage Ta/T1 bladder cancer: prognostic factor for time to recurrence and progression. *BJU Int* 2003;93:980.
3. Shahin O, Thalman GN, Rentsch C, Mazzuchelli L, Studer UE. A retrospective analysis of 153 patients treated with or without intravesical BCG for primary stage T1 grade 3 bladder cancer: recurrence, progression and survival. *J Urol* 2003;169:96.
4. Stockle M. Radical cystectomy—often too late? *Eur Urol* 1987;13:361.
5. Herr HW. The value of a second transurethral resection in evaluating patients with bladder tumors. *J Urol* 1999;162:74.
6. Gohji K, Nomi M, Okamoto M, Takenaka A, Hara I, Okada H, et al. Conservative therapy for stage T1b, grade 3 transitional cell carcinoma of bladder. *Urology* 1999;53:308.
7. Dutta SC, Smith JA Jr, Shappell SB, Coffey CS, Chang SS, Cookson MS. Clinical understaging of high risk nonmuscle invasive urothelial carcinoma treated with radical cystectomy. *J Urol* 2001;166:490.
8. Bono AV, Benvenuti C, Damiano G, Lovisolo J. Results of transurethral resection and intravesical doxorubicin prophylaxis in patients with T1G3 bladder cancer. *Urology* 1994;44:329.
9. Serretta V, Piazza S, Pavone C, Piazza B, Pavone-Macaluso M. Results of conservative treatment (transurethral resection plus adjuvant intravesical chemotherapy) in patients with primary T1G3 transitional cell carcinoma of the bladder. *Urology* 1996;47:647.
10. Hara I, Miyake H, Takechi Y, Eto H, Gotoh A, Fujisawa M, et al. Clinical outcome of conservative therapy for stage T1, grade 3 transitional cell carcinoma of the bladder. *Int J Urol* 2003;10:19.
11. Han RF, Pan JG. Can intravesical bacillus Calmette Gurein reduce recurrence in patients with superficial bladder cancer? A meta analysis of randomized trials. *Urology* 2006;67:1216.
12. Shelley MD, Wilt TJ, Court J, Coles B, Kynaston H, Mason MD. Intravesical bacillus Calmette Gurein is superior to mitomycin C in reducing tumor recurrence in high risk superficial bladder cancer: a meta analysis of randomized trials. *BJU Int* 2004;93:485.
13. Brake M, Loertzer H, Horsch R, Keller H. Recurrence and progression of stage T1, grade 3 transitional cell carcinoma of the bladder following intravesical immunotherapy with bacillus Calmette-Guerin. *J Urol* 2000;163:169.
14. Patard J, Moudouni S, Saint F, Rioux-Leclercq N, Manunta A, Guy L, et al. Tumor progression and survival in patients with T1G3 bladder tumors: multicentric retrospective study comparing 94 patients treated during 17 years. *Urology* 2001;58:551.
15. Divrik RT, Yildirim U, Zorlu F, Ozen H. The effect of repeat transurethral resection on recurrence and progression rates in patients with T1 tumors of the bladder who received intravesical mitomycin: a prospective, randomized clinical trial. *J Urol* 2006;175:1641.

BJUI
BJU INTERNATIONAL

Dicoumarol enhances doxorubicin-induced cytotoxicity in p53 wild-type urothelial cancer cells through p38 activation

Yoshiyuki Matsui, Jun Watanabe, Sentai Ding, Koji Nishizawa, Yoichiro Kajita, Kentaro Ichioka, Ryoichi Saito, Takashi Kobayashi, Osamu Ogawa and Hiroyuki Nishiyama

Department of Urology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Accepted for publication 23 April 2009

OBJECTIVE

To investigate the effectiveness of a combined treatment of 3-30-methylene-bis[4-hydroxycoumarin] (dicoumarol) with doxorubicin for the treatment of urothelial cancer, as doxorubicin is a common chemotherapeutic agent but its therapeutic efficacy is limited.

MATERIALS AND METHODS

The synergistic effect of dicoumarol with chemotherapeutic agents such as cisplatin, doxorubicin and paclitaxel was evaluated in RT112 urothelial cancer cells. Then, dicoumarol-mediated enhancement of doxorubicin-induced cytotoxicity was screened in urothelial cancer cell lines with different p53 statuses or RT112 stable transfectants with a dominant-negative mutant of p53 (p53DN). To clarify the importance of the modification of p53 function by dicoumarol to enhance doxorubicin toxicity, the change in the p53-p21 pathway and mitogen-activated protein kinase (MAPK)-mitochondria pathway by the combined treatment were elucidated by Western blot analysis. Finally, the effect of p21 knockdown in the susceptibility to doxorubicin was examined with RT112 stable transfectants with short hairpin RNA (shRNA) of p21.

RESULTS

Dicoumarol significantly increased the susceptibility of RT112 cells to cisplatin and doxorubicin, but not to paclitaxel in RT112 cells. Dicoumarol (100 μM) also enhanced the cytotoxicity of doxorubicin in other bladder cancer cell lines with wild-type p53 (wt-p53; three times in 253J and 13 times in KK47), but not in those with mutant-type p53 (TCCsup, J82 and EJ) or in RT112 p53DN. The combined treatment with dicoumarol suppressed p53/p21 induction by doxorubicin and resulted in sequential p38 MAPK activation, myeloid cell leukaemia 1 suppression and caspase cleavage. The synergistic effect of doxorubicin/dicoumarol was suppressed by the p38 MAPK inhibitor SB202190 and, furthermore, p21 knockdown with shRNA transfection made RT112 cells six times more susceptible to doxorubicin with p38 MAPK activation.

CONCLUSION

These results suggest that concomitant use of dicoumarol could enhance the cytotoxicity of doxorubicin in urothelial cancer cells with wt-p53 through the p53/p21/p38 MAPK pathways. This combined treatment may provide a new therapeutic option to overcome chemoresistance in bladder cancer.

KEYWORDS

urothelial cancer, dicoumarol, doxorubicin, p53, p38

INTRODUCTION

More than 70% of bladder cancers present as moderately-to-well differentiated, superficial papillary TCC, treated with endoscopic transurethral resection of the bladder tumour. Although progression to muscle-invasive cancer is relatively infrequent, >60% of patients have metachronous intravesical recurrence one or more times, and these have a serious impact on patients' quality of life [1]. To prevent intravesical recurrence, prophylactic and therapeutic intravesical instillation of various chemotherapeutic agents has been tried but its efficacy is still limited [2], thus new molecular targets for therapy to enhance its efficacy are being explored.

NADPH: quinone oxidoreductase 1 (NQO1) is a ubiquitous flavoprotein that functions as an antioxidant enzyme [3]. The expression levels of NQO1 are elevated in various cancers compared with surrounding normal tissue [4]. NQO1 was reported to protect cancer cells against anticancer agents through detoxification of intracellular oxidative stress [4]. Furthermore, we recently reported that NQO1 contributed to inhibition of apoptosis through maintenance of p53-p21 expression in urogenital cancer cells with wild-type p53 (wt-p53), and 3-30-methylene-bis[4-hydroxycoumarin] (dicoumarol), which inhibited enzymatic activity of NQO1, promoted cisplatin-induced apoptosis through the attenuation of the p53-p21 pathway [5].

In urothelial cancer, most superficial bladder cancers retain wt-p53 [6], and therefore, we hypothesized that dicoumarol might be appropriate as a modulator of anticancer

JOURNAL COMPILATION © 2009 BJU INTERNATIONAL | 105, 558–564 | doi:10.1111/j.1464-410X.2009.08732.x

agents for preventing recurrence of superficial bladder cancer and for improving patients' quality of life. In the present study, to assess the potential of dicoumarol in treating superficial bladder cancer cells, we investigated the cytotoxic effects of dicoumarol combined with doxorubicin (a commonly instilled intravesical agent).

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Antibodies were obtained as follows: anti-p53 (Ab-2) from Calbiochem (San Diego, CA, USA), p21 and NQO1 from Santa Cruz (Santa Cruz, CA, USA), anti-β-actin from Abcam (Cambridge, MA, USA), and antibodies against cleaved poly(ADP-ribose) polymerase (PARP), caspase 8, caspase 9, cleaved caspase 3, c-Jun amino-terminal kinase (JNK), pJNK, pp38, p38, pERK, ERK, pHsp27, Bax, Bcl-2, Bcl-xL and myeloid cell leukaemia 1 (Mcl-1) from Cell Signalling Technology (Beverly, MA, USA). SB202190 as a p38 inhibitor, Z-IETD-FMK as a caspase-8 inhibitor, Z-LEHD-FMK as a caspase-9 inhibitor and Z-VAD(OMe)-FMK as a pan-capase inhibitor were obtained from Calbiochem. Cis-diamminedichloroplatinum (cisplatin) and doxorubicin (adriamycin) were from WAKO (Osaka, Japan), and paclitaxel and dicoumarol were from Sigma (St Louis, MO, USA).

CELL CULTURE

Six urothelial cancer cell lines (RT112, 253J, KK47, TCCsup, EJ and J82) were used in the present study. Sequencing analysis and genotyping of p53 confirmed that RT112, 253J and KK47 had wt-p53 whereas the three other cell lines harboured mutant p53 (mt-p53); J82 harboured double missense mutations (codon 271, CAG→AAG and codon 320, AAG→AAC), TCCsup harboured a nonsense mutation (codon 349, GAA→TAA), and EJ contained a missense mutation (codon 175, CGC→CAC). All six bladder cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

GENOTYPING OF NQO1

DNA was extracted from bladder cancer cells using the QIAamp Blood Kit (QIAGEN, Hilden, Germany). To detect Pro187Ser (C to T at position 609 of the cDNA) NQO1 polymorphism in bladder cancer cell lines, the PCR primers used were 5'-TCCTCAGAGTGGCATTCTGC-3' and 5'-TCTCCTCATCCTGTACCTCT-3'. PCR conditions were 10 min at 95 °C (one cycle) and 30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C (35 cycles) and 5 min at 72 °C (one cycle). Each PCR product was digested with HinfI for Pro187Ser NQO1 polymorphism. DNA samples were concomitantly amplified and digested together with previously examined DNA samples serving as quality controls. For the Pro187Ser NQO1 polymorphism, restriction fragments were 195 and 35 bp for the Pro allele, and 151, 44, and 35 bp for the Ser allele.

PLASMIDS AND TRANSFECTION

pCMV-neo was a gift from Dr K. Cho (University of Michigan Medical School). Dominant-negative mutant of p53 (p53DN) containing one missense mutation at codon 135 (TGC→TAC), which were purchased from BD Bioscience, were ligated into pCMVneo. psiRNA-h7SKhp21 vectors containing short hairpin RNA (shRNA) targeting p21 were purchased from InvivoGen (San Diego, CA, USA). Cells were transfected with the indicated vectors using Lipofectamine 2000 (Life Technologies, Inc., Grand Island, NY, USA) following the manufacturer's instructions. Stable transfectants were selected by appropriate selection antibiotics, and were confirmed by sequencing, reverse transcription-PCR or Western blot analysis.

CELL VIABILITY ASSAY AND DETECTION OF APOPTOSIS

Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously [5]. Briefly, 1×10^4 cancer cells with 100 μL suspension were grown in each well of 96-well plates. After 24 h incubation, cells were treated with or without different concentrations of drugs for another 24 h. Then, 20 μL of MTT working solution (5 mg/mL; Sigma) was added to each culture well, and incubated for 4 h. The formazan crystal was dissolved with 100 μL dimethyl sulphoxide. The absorbance (*A*) of each well was measured by a microculture plate reader (Immunoreader; Japan Intermed Co., Ltd, Tokyo, Japan) at 540 nm. The percentage of cytotoxicity = [1 – (*A* of experimental wells/*A* of control wells)] × 100.

To detect the ratio of viable cells or apoptotic cells to total cells, Trypan blue staining or Hoechst 33342 nuclear staining was used. For Trypan blue staining, cells were stained with 0.3% Trypan blue, and counted on haemocytometer. The percentage of viable cells was calculated as the ratio of unstained cells (not blue) to total cells counted. For nuclear staining, cells were stained with 1 mM Hoechst 33342 solution (Wako, Osaka, Japan), and analysed with a fluorescence microscope. Apoptotic cells were identified by morphology and by condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted.

IMMUNOBLOTTING

After drug treatments, cells were washed with PBS and lysed in an appropriate volume of ice-cold RIPA buffer composed of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS containing 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulphonyl fluoride and protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). Cellular lysates were clarified by centrifugation at 13 000***g*** for 15 min and the protein concentrations of the lysates were determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Aliquots of 30–50 μg of the lysates were boiled for 5 min in SDS sample buffer and separated by SDS-PAGE on a 10–15% Tris-HCl minigel, and transferred onto a polyvinylidene difluoride membrane using standard methods. Membranes were probed with appropriate dilutions of primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, proteins were visualized by a chemiluminescent detection system (GE Healthcare, Buckinghamshire, UK).

RESULTS

DICOUMAROL ENHANCES DOXORUBICIN- OR CISPLATIN-INDUCED CYTOTOXICITY BUT NOT PACLITAXEL-INDUCED CYTOTOXICITY

We have previously reported that dicoumarol enhanced cisplatin-induced cytotoxicity via suppression of wt-p53 [5]. In the present study, we explored the further potential of dicoumarol to enhance the cytotoxicity of doxorubicin, which is commonly used for treating bladder cancer and paclitaxel, which