厚生労働科学研究費補助金

がん臨床研究事業

早期前立腺がんにおける根治術後の再発に対する標準的治療法の確立に関する研究

平成20～22年度　総合研究報告書

研究代表者　内藤　誠二

平成23（2011）年　3月

厚生労働科学研究費補助金

がん臨床研究事業

早期前立腺がんにおける根治術後の再発に対する標準的治療法の確立に関する研究

平成20～22年度　総合研究報告書

研究代表者　内藤　誠二

平成23（2011）年　3月

研究報告書目次

目　　次

厚生労働科学研究費補助金（がん臨床研究事業）

Ⅰ　総合研究報告書

研究代表者　内藤誠二

早期前立腺がんにおける根治術後の再発に対する標準的治療法の確立に関する研究

研究要旨

限局性前立腺癌に対する標準的治療の一つとして根治的前立腺摘除術が広く行われている。しかし、術後、前立腺特異抗原（Prostate Specific Antigen ： PSA）の再上昇で発見される 、いわゆるPSA 再発が15-25%の患者にみられ、その治療法は未だ確立していない。本研究では、根治術後の PSA 再発患者を対象に、内分泌療法群と内分泌療法に先行して局所放射線療法を行う群によるランダム化比較試験を行い、PSA 再発に対する標準的治療法の確立を目指す。現在、「早期前立腺がんにおける根治術後の再発に対する標準的治療法の確立に関する研究」（phase III 試験）を継続している。

研究分担者

横溝　晃（九州大学大学院医学研究院泌尿器科　講師）、橋根　勝義（独立行政法人国立病院機構四国がんセンター泌尿器科　部長）、佐藤　威文（北里大学医学部泌尿器科　講師）、筧善行（香川大学医学部泌尿器科学　教授）、神波　大己（京都大学大学院医学研究科器官外科学講座泌尿器科学　講師）、平尾　佳彦（奈良県立医科大学医学部泌尿器科学　教授）、羽渕友則（秋田大学医学部生殖発達医学講座泌尿器科学分野　教授）、西澤　理（信州大学医学部泌尿器科学　教授）、篠原　信雄（北海道大学大学院医学研究科腎泌尿器外科　准教授）、川島　清隆（栃木県立がんセンター泌尿器科　部長）、頴川　晋（東京慈恵会医科大学泌尿器科教授）、後藤　百万（名古屋大学大学院医学系研究科泌尿器科　教授）、庭川　要（静岡県立静岡がんセンター泌尿器科　部長）、北村　康男（新潟県立がんセンター新潟病院泌尿器科部長）、野口　正典（久留米大学医学部　教授）、市川　智彦（千葉大学大学院医学研究院泌尿器科学　教授）、大家　基嗣（慶應義塾大学医学部泌尿器科　教授）、井川　幹夫（島根大学医学部泌尿器科学　教授）、栃木　達夫（宮城県立がんセンター泌尿器科　部長）大園 誠一郎（浜松医科大学医学部泌尿器科学）、山口　秋人（原三信病院　副院長）、塚本　泰司（札幌医科大学医学部泌尿器科　教授）、冨田　善彦（山形大学医学部腎泌尿器外科学分野　教授）、堀江 重郎（帝京大学医学部泌尿器科学　教授）、座光寺 秀典（山梨大学大学院医学工学総合研究部泌尿器科学　講師）、藤澤　正人（神戸大学大学院医学系研究科腎泌尿器科学教授）、寺井　章人（倉敷中央病院泌尿器科　部長）、荒井　陽一（東北大学大学院医学系研究科泌尿・生殖器科学　教授）、藤元　博行（国立がん研究センター中央病院泌尿器科　部長）、谷川　俊貴（新潟大学医歯学総合病院泌尿器科　講師）、杉村　芳樹（三重大学大学院医学系研究科腎泌尿器外科学　教授）、西村　和郎（大阪府立成人病センター泌尿器科　部長）、江藤　正俊（熊本大学大学院生命科学研究部泌尿器病態学分野　教授）、中川　昌之（鹿児島大学大学院医歯学総合研究科泌尿器科学　教授）、大山　力（弘前大学大学院医学研究科泌尿器科学　教授）、宮崎　淳（筑波大学大学院人間総合科学研究科腎泌尿器科学　講師）

A. 研究目的

根治的前立腺摘除術後の再発は、通常まずPSAの再上昇（PSA 再発）で発見されるが、その再発が局所か、遠隔転移か、さらには両者の合併かを画像的に同定することは困難である。一般的に局所再発であれば放射線療法、遠隔転移であれば内分泌療法が標準的な治療法になると思われるが、実際には再発部位の同定が困難であるため、明確な根拠もないままに治療法が選択され、現在までのところPSA再発患者に対する標準的治療法は確立されていない。そのため、限局性前立腺癌に対する根治的前立腺摘除術後のPSA再発患者に対して、内分泌療法前に放射線療法を行うことの臨床的有用性を、内分泌療法単独とのランダム化比較試験により評価する臨床試験を計画した。Primary endpointは抗アンドロゲン剤（ビカルタミド）の Time-to-Treatment Failure(TTF)とし、secondary endpointはプロトコール治療の TTF、全生存期間、臨床的無再発生存期間、放射線、抗アンドロゲン剤、LH-RH アナログの有害事象、QOLとした。

B. 研究方法

登録時に適格患者は以下のA群(内分泌療法群)かB 群(放射線療法群)にランダム割付される。治療開始後、臨床再発または PSA 再発を認めた場合、または有害事象や患者拒否により治療継続が困難となった場合、Time to treatment failure (TTF)と判断する。A群では抗アンドロゲン剤による治療とその後のTTFに対するLH-RHアナログ治療をもってプロトコール治療とし、B 群では前立腺床に対する 64.8Gy の外照射、その後 TTF を生じたらA群と同様の治療をもってプロトコール治療とする。登録期間は7年、追跡期間は登録終了後5年とし､総研究期間は12年を予定している。

C. 研究結果

1)当研究はJapan Clinical Oncology Group(JCOG)にプロトコール審査及び、データマネージメントを依頼し研究を行なっている。H23年3月1日現在、201名の登録が得られているが、重篤な有害事象は認めていない。放射線治療の品質管理も良好である。2) 本研究は、H14－効果（がん）-030、H17－がん臨床-003およびH20-がん臨床-一般-016として採択され、H16年5月からの4年間に各群100名を目標に患者登録を開始した。登録症例数が予定を下回っていたことが問題であったが、全施設での登録候補患者の調査、参加施設の入れ替え、患者への説明医の固定、コアメンバーによる縮小班会議の定期的開催、説明パンフレットの作成、配布、カルテ用シール等の配布などの対策を行った結果、登録数は増加し、直近の1年間であるH22年3月以降では40名の登録が得られている。H20年9月には、登録数が100例を超え、中間解析を行った。その結果、JCOG効果安全性評価委員会から「研究の続行」を承認され、登録期間の延長を指示された。そのため、プロトコール改訂を行い、登録期間を7年、追跡期間は登録終了後5年とし、総研究期間を12年とした。H22年12月の定期JCOGの施設監査の際、不適格登録の指摘を受け、調査した結果、合計9例の不適格登録があることが判明したため、登録数を10例増やし、計210例とした。登録期間の平成23年5月までに目標の患者登録を完遂できように、さらに積極的に患者登録推進に努める所存である。

（倫理面への配慮）参加患者の安全性確保については、適格条件やプロトコール治療の中止変更規準を厳しく設けており、試験参加による不利益は最小化される。また、「臨床研究に関する倫理指針」およびヘルシンキ宣言などの国際的倫理原則に従い以下を遵守する。

1) 研究実施計画書のIRB承認が得られた施設のみから患者登録を行う。

2) すべての患者について登録前に充分な説明と理解に基づく自発的同意を本人より文書で得る。

3) データの取り扱い上、患者氏名等直接個人が識別できる情報を用いず、かつデータベースのセキュリティを確保し、個人情報（プライバシー）保護を厳守する。研究の第三者的監視：JCOGは厚生労働省がん研究助成金指定研究5班（17指-1～5）を中心に、同計画研究班6班および厚生労働科学研究費がん臨床研究事業22研究班、計33班の任意の集合体であり、JCOGに所属する研究班は共同で、Peer reviewと外部委員審査を併用した第三者的監視機構としての各種委員会を組織し、科学性と倫理性の確保に努めている。本研究も、JCOGのプロトコール審査委員会、効果・安全性評価委員会、監査委員会、放射線治療委員会などによる第三者的監視を受けることを通じて、科学性と倫理性の確保に努める。

D. 考察

登録患者数が当初の予定を下回っているが、H19年以降登録数が急増しており、さらなる登録推進のための工夫、努力を積極的に行って、試験の円滑な進行を図り､目標症例数210例を達成したい。

E. 結論

H23年3月1日現在、201名の登録を行い、プロトコール治療を実施中であるが、両群ともに治療による重篤な有害事象は認めていない。

F. 健康危険情報

治療と関連するグレード3以上の有害事象は報告されていない。

G．研究発表

1. 論文発表

1. Yoshimitsu K, Kiyoshima K, Irie H, et al. Usefulness of apparent diffusion coefficient map in diagnosing prostate carcinoma: correlation with stepwise histopathology. J Magn Reson Imaging 27:132-139, 2008.

2. Kuroiwa K, Uchino H, Yokomizo A, Naito S. Impact of reporting rules of biopsy Gleason score for prostate cancer. J Clin Pathol 62:260-3, 2009.

3. Akaza H, Hinotsu S, Usami M, Arai Y, Kanetake H, Naito S, Hirao Y. Combined androgen blockade with bicalutamide for advanced prostate cancer: long-term follow-up of a phase 3, double-blind, randomized study for survival. Study Group for the Combined Androgen Blockade Therapy of Prostate Cancer. Cancer. 115:3437-45, 2009.

4. Shiota M, Yokomizo A, Tada Y, Inokuchi J, Kashiwagi E, Masubuchi D, Eto M, Uchiumi T, Naito S. Castration resistance of prostate cancer cells caused by castration-induced oxidative stress through Twist1 and androgen receptor overexpression. Oncogene. 29:237-50, 2010.

5. Shiota M, Yokomizo A, Tada Y, Inokuchi J, Tatsugami K, Kuroiwa K, Uchiumi T, Fujimoto N, Seki N, Naito S. Peroxisome proliferator-activated receptor gamma coactivator-1alpha interacts with the androgen receptor (AR) and promotes prostate cancer cell growth by activating the AR. Mol Endocrinol. 24:114-27, 2010. Kuroiwa K, Shiraishi T, Naito S, et al. Clinicopathological Research Group for Localized Prostate Cancer Investigators: Gleason score correlation between biopsy and prostatectomy specimens and prediction of high-grade gleason patterns: significance of central pathologic review. Urology 77:407-11, 2011

6. Kuroiwa K, Shiraishi T, Ogawa O, et al. Clinicopathological Research Group for Localized Prostate Cancer Investigators: Discrepancy between local and central pathological review of radical prostatectomy specimens. J Urol 183: 952-957, 2010

7. Yokomizo A, Shiota M, Kashiwagi E, et al. Statins reduce the androgen sensitivity and cell proliferation by decreasing the androgen receptor protein in prostate cancer cells. Prostate. 71:298-304 2010

2. 学会発表

1. 横溝　晃　「前立腺全摘後の放射線治療：Salvage 放射線治療」2009年10月24日、第5回泌尿器腫瘍放射線研究会

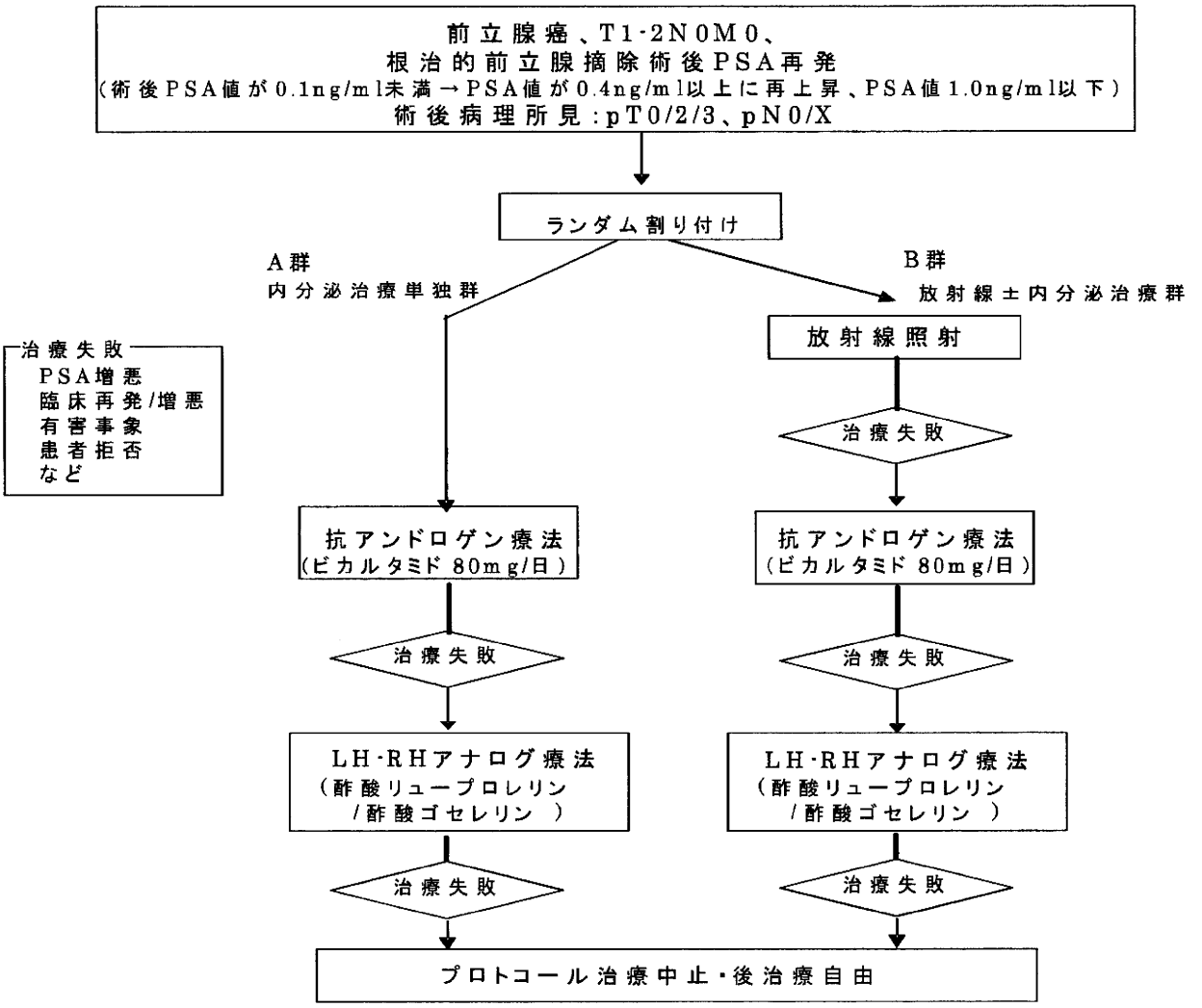
2. 横溝　晃、内藤誠二、鳶巣賢一.「根治術後の救済放射線療法の治療成績と予後因子」2010 年 7 月 17 日、尿路悪性腫瘍研究会

3. 横溝　晃、塩田真己、柏木英志、他.「スタチンによる前立腺癌のアンドロゲン受容体発現低下と増殖抑制効果」2010 年 12 月 11 日、前立腺シンポジウム

H. 知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし。

試験概要図


前立腺癌、T1-2N0M0、
根治的前立腺摘除術後PSA再発
(術後PSA値が0.1ng/ml未満→PSA値が0.4ng/ml以上に再上昇、PSA値1.0ng/ml以下)
術後病理所見:pT0/2/3、pN0/X
ランダム割り付け
A群
内分泌治療単独群
B群
放射線±内分泌治療群
治療失敗
PSA増悪
臨床再発/増悪
有害事象
患者拒否
など
放射線照射
治療失敗
抗アンドロゲン療法
(ビカルタミド 80mg/日)
抗アンドロゲン療法
(ビカルタミド 80mg/日)
治療失敗
治療失敗
LH-RHアナログ療法
(酢酸リュープロレリン
/酢酸ゴセレリン)
LH-RHアナログ療法
(酢酸リュープロレリン
/酢酸ゴセレリン)
治療失敗
治療失敗
プロトコール治療中止・後治療自由

III 研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saito S, Murayama Y, Pan Y, et al.	Haptglobin-β chain defined by monoclonal antibody RM2 as a novel Serum marker for prostate cancer.	Int J Cancer	123	633-640	2008
Ma Z, Tsuchiya N, Yuasa T, et al et al.	Polymorphisms of fibroblast growth factor receptor 4 have association with the development of prostate cancer and benign prostatic hyperplasia and the progression of prostate cancer in a Japanese population.	Int J Cancer	123	2574-2579	2008
Yoshimitsu K, Kiyoshima K, Irie H, et al.	Usefulness of apparent diffusion coefficient map in diagnosing prostate carcinoma: correlation with stepwise histopathology.	J Magn Reson Imaging	27	132-139	2008
Furuya R, Hisasue S, Furuya S, et al.	The fate of the seminal vesicle following medical castration: how long is the optimal duration of neoadjuvant treatment for prostate cancer before radiation.	Urology	72	417-421	2008
Miyajima N, Maruyama S, Bohgaki M, et al.	TRIM68 regulates ligand-dependent transcription of androgen receptor in prostate cancer cells.	Cancer Res	68	3486-3494	2008
Akaza H, Hinotsu S, Usami ,M et al.	Combined androgen blockade with bicalutamide for advanced prostate cancer: long-term follow-up of a phase 3, double-blind, randomized study for survival. Study Group for the Combined Androgen Blockade Therapy of Prostate Cancer.	Cancer	115	3437-45	2009
Satoh T, Kimura M, Matsumoto K, et al.	Single infusion of zoledronic acid to prevent androgen- deprivation therapy-induced bone loss in men with hormone-naïve prostate carcinoma.	Cancer	115	3468-74	2009

Kuruma H, Kamata Y, Takahashi H, et al.	Staphylococcal Nuclease Domain-Containing Protein 1 as a Potential Tissue Marker for Prostate Cancer.	Am J Pathol	174	2044-50	2009
Muramaki M, Miyake H, Kurahashi T, et al.	Characterization of the anatomical extension pattern of localized prostate cancer arising in the peripheral zone.	BJUI	105	1514-1518	2009
Hirata H, Hinoda Y, Kikuno N et al.	Bcl2 -938C/A polymorphism carries increased risk of biochemical recurrence after radical prostatectomy.	J Urol	181	1907-12	2009
Shiota M, Yokomizo A, Tada Y, et al.	Castration resistance of prostate cancer cells caused by castration-induced oxidative stress through Twist1 and androgen receptor overexpression.	Oncogene	29	237-50	2010
Shiota M, Yokomizo A, Tada Y, et al.	Peroxisome proliferator-activated receptor gamma coactivator-1alpha interacts with the androgen receptor (AR) and promotes prostate cancer cell growth by activating the AR.	Mol Endocrinol	24	114-27	2010
Kuroiwa K, Shiraishi T, Ogawa O, et al.	Discrepancy Between Local and Central Pathological Review of Radical Prostatectomy Specimens.	J Urol	183	952-957	2010
Terada N, Shimizu Y, Kamba T, et al.	Identification of EP4as a Potential Target for the Treatment of Castration-Resistant Prostate Cancer Using a Novel Xenograft Model.	Cancer Res	70	1606-1615	2010
Ma Z, Tsuchiya N, Yuasa T, et al.	Clinical Significance of Polymorphism and Expression of Chromogranin A and Endothelin-1 in Prostate Cancer.	J Urol	184	1182-1188	2010
Yokomizo A, Shiota M, Kashiwagi E, et al.	Statins Reduce the Androgen Sensitivity and Cell Proliferation by Decreasing the Androgen Receptor Proteinin ProstateCancer Cells.	Prostate	71	298-304	2011
Kuroiwa K, Shiraishi T, Naito S, et al.	Gleason Score Correlation Between Biopsy and Prostatectomy Specimens and Prediction of High-grade Gleason Patterns: Significance of Central Pathologic Review.	Urology	77	407-411	2011

Ⅳ．研究成果の刊行物・別刷

Int. J. Cancer: **123,** 633–640 (2008)

Haptoglobin-β chain defined by monoclonal antibody RM2 as a novel serum marker for prostate cancer

Seiichi Saito[1]*, Yasuko Murayama[1], Yuzhuo Pan[1], Takenobu Taima[1], Tsutomu Fujimura[2,3,4], Kimie Murayama[2,3], Martin Sadilek[5], Shin Egawa[6], Seiji Ueno[1], Akihiro Ito[1], Shigeto Ishidoya[1], Haruo Nakagawa[1], Masanori Kato[1], Makoto Satoh[7], Mareyuki Endoh[8] and Yoichi Arai[1]*

[1]*Department of Urology, Tohoku University, Graduate School of Medicine, Aoba-ku, Sendai, Japan*
[2]*Pacific Northwest Research Institute, Seattle, WA*
[3]*Departments of Pathobiology and Microbiology, University of Washington, Seattle, WA*
[4]*Biomedical Research Center, Division of Proteomics and Biomolecular Sciences, Juntendo University, Graduate School of Medicine, Bunkyo-ku, Tokyo, Japan*
[5]*Department of Chemistry, University of Washington, Seattle, WA*
[6]*Department of Urology, The Jikei University School of Medicine, Nishishinbashi, Minato-ku, Tokyo, Japan*
[7]*Department of Urology, Sen-en Hospital, Tagajo, Japan*
[8]*Department of Pathology, Tohoku University, Graduate School of Medicine, Aoba-ku, Sendai, Japan*

In our previous study, monoclonal antibody RM2, established toward the glycosyl epitope, reflected grade of malignancy of prostate cancer cells whereas RM2 reactivity to benign glands was negative or weak. RM2 reactivity was also detected in stroma, suggesting the glycoprotein RM2 recognizes could be released into the bloodstream. Then, we explored RM2 reactivity to sera of early prostate cancer. We compared RM2 reactivity to sera between 62 patients with early prostate cancer and 43 subjects with benign prostatic disease, and examined RM2 reactivity before and after radical prostatectomy in 15 patients by Western blotting. We also examined RM2 reactivity to sera of the other urogenital cancers. RM2 reactivity was significantly enhanced on a serum glycoprotein with molecular mass ~40 kDa, hereby termed GPX, in the patients with early prostate cancer when compared with those with benign prostatic disease ($p < 0.0001$). Setting an appropriate cutoff level, RM2 reactivity to GPX for detection of prostate cancer had sensitivity of 87% and specificity of 84%, respectively. Furthermore, the level of RM2 reactivity significantly decreased after radical prostatectomy ($p = 0.006$). However, increased RM2 reactivity to GPX was also observed in the other urogenital cancers. The proteomics approach identified GPX as haptoglobin-β chain and RM2 showed preferential reactivity toward haptoglobin-β chain derived from prostate cancer when compared with polyclonal anti-haptoglobin antibody. Haptoglobin-β chain defined by RM2 is a novel serum marker that may be useful for detection of early prostate cancer when coupled with prostate-specific antigen because it is not specific to prostate cancer.

Key words: prostate cancer; haptoglobin; serum marker; RM2

Prostate-specific antigen (PSA) has a problem especially with specificity, *i.e.*, PSA is not only elevated in prostate cancer but also in benign prostatic disease (BPD). Thus, only 25% of men with PSA value of 4 to 10 ng/mL will have diagnosis of cancer after prostate biopsy.[1–3] Furthermore, PSA alone cannot predict pathological stage of prostate cancer because PSA does not reflect the grade of malignancy.[1–4] Recently, it was pointed out that the prevalence of prostate cancer was 15% among men with PSA 4 ng/mL or less.[4] Therefore, a new serum marker to compensate for the problems of PSA is urgently required. However, there has been few serum markers reported, which have potential for clinical application.[5,6] We previously reported that monoclonal antibody (mAb) RM2 was established toward disialoganglioside and later found to recognize the glycosyl epitope (β1.4-GalNAcDSLc4).[7,8] In an attempt to find a new marker, we examined whether immunoreactivity of mAb RM2 was detected in radical prostatectomy specimens, and found that reactivity of mAb RM2 to prostate cancer cells was associated with grade of malignancy, whereas RM2 reactivity to benign glands was negative or weak.[9] RM2 immunoreactivity was also detected in stroma,[9] suggesting the glycoprotein RM2 recognizes may be shed from cancer cells into the surrounding stroma and then released into the bloodstream. In the current study, we explored RM2 reactivity to sera of early prostate cancer, and found that mAb RM2 also recognized haptoglobin-β chain and level of haptoglobin-β chain defined by RM2 significantly increased in sera of early prostate cancer.

Subjects and methods

Serum samples

Of serum samples obtained from Department of Urology, Tohoku University Hospital between June 2004 and May 2006, serum samples of 62 patients with early prostate cancer and those of 43 with BPD were randomly selected. All these subjects had biopsy proven histological diagnosis and PSA less than 10 ng/mL. Fifteen serum samples of the patients with various PSA values who underwent radical prostatectomy, 6 of the subjects with renal cell carcinoma (RCC), 8 of those with urothelial carcinoma and 8 of those with testicular germ cell tumors were also randomly selected. The Ethics Committees of both Tohoku University Graduate School of Medicine and Pacific Northwest Research Institute approved the present study, and informed consent was obtained from each patient. Clinical tumor-node metastasis staging was assigned using the 1997 tumor-node metastasis staging system.[10] Gleason scores of all slides were diagnosed by a single pathologist (M.E.).[11]

Cell lines

Prostate cancer cell lines PC3, LNCaP and DU145 were obtained from Human Science Research Resource Bank (Wako, Japan). Androgen-independent prostate cancer cell line AICaP1 was newly established from LNCaP in our laboratory (Taima

Abbreviations: BPD, benign prostatic disease; CBB, coomassie brilliant blue; IEF, isoelectric focusing; mAb, monoclonal antibody; PCa, prostate cancer; RCC, renal cell carcinoma; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Grant sponsor: Japan Society for the Promotion of Sciences; Grant number: 15390483; Grant sponsor: The Ministry of Education, Culture, Sports, Science and Technology; Grant number: 17659496 14th Research Grant from Japanese Urological Association; Grant sponsors: Tohoku University Hospital for the Promotion of Research for Clinical Application, and Japan Science and Technology Agency.

***Correspondence to:** Department of Urology Tohoku University Graduate School of Medicine, Sendai, Japan. Fax: 81-22-717-7283.
E-mail: ssaito@uro.med.tohoku.ac.jp or yarai@uro.med.tohoku.ac.jp

Received 22 August 2007; Accepted after revision 22 January 2008
DOI 10.1002/ijc.23490
Published online 7 May 2008 in Wiley InterScience (www.interscience.wiley.com).

et al., to be published elsewhere). PrEC, normal human prostatic epithelial cells were purchased from Cambrex Bioscience (Walkersville, MD). RCC cell line ACHN was purchased from Dainihonseiyaku. (Osaka, Japan) and TOS1 was previously established in our laboratory.[7]

Antibodies

mAb RM2 (isotype IgM) was established using RCC TOS1 cells as immunogen,[7] based on our earlier studies that indicated that degree of RCC malignancy was correlated with disialoganglioside expression in RCC cells.[12] RM2 is reactive with disialoganglioside but not with monosialoganglioside fraction, and the antigen was later identified as β1,4-GalNAc-disialyl-Lc4.[8] Polyclonal anti-haptoglobin antibody was purchased from Dako (Dako Cytomation Kyoto, Japan).

Western blotting of serum

After removing albumin and IgG using Aurum™ Serum Protein Mini Kit (Bio-Rad), a 20 μl aliquot of serum was electrophoresed on an 10% SDS-PAGE and transferred to Hybond P PVDF membrane (Amersham Biosciences, Uppsala, Sweden). Immunoblotting was performed as described previously.[9] Densitometric analysis of RM2 reactivity to a glycoprotein with molecular mass ~40 kDa (GPX) in serum was performed using Scion image (Scion Corp., Frederick, MD) and each value of ~40 kDa glycoprotein was normalized to that of a glycoprotein with molecular mass ~75 kDa from the same lane.

Pretreatment of sera by Agilent column, followed by 2D sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D SDS-PAGE)

Agilent Multiple Affinity Removal System 1.6 × 100 mm^2 column, designed to adsorb >98–99% of 6 abundant proteins (albumin, immunoglobulins IgG and IgA, transferrin, haptoglobin and antitrypsin) from human serum samples, was purchased from Agilent Technologies (Palo Alto, CA) together with solvents A and B used for adsorption and elution of the proteins.

Two-dimensional SDS gel electrophoresis, *in situ* alkylation, Western blotting and *in gel* digestion were performed as described previously.[13]

Identification of protein

The tryptic digest was analyzed using Agilent 110 capillary HPLC (Agilent Technologies) combined with LCQ ion trap mass spectrometer (Thermo Electron, Waltham, MA). Data were searched against NCBI human sequence database using TurboSEQUEST v.27, and in selected cases Mascot (Matrix Science).

mRNA levels of haptoglobin-β chain in prostate cancer cell lines

Total RNA was extracted from PC3, LNCaP, DU145 and PrEC using Trizol reagent (Gibco BRL, Grand Island, NY) following the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed into first-strand cDNA using ExScript reverse transcription kit (Takara Bio, Japan). PCR was performed using the primers of haptoglobin-β chain and those of β-actin.

Immunohistochemical staining

RM2 reactivity to prostate cancer was described in our previous study.[9] Twelve cases of benign prostatic hyperplasia were immunostained by mAb RM2 in this study. To examine haptoglobin expression in prostate cancer, polyclonal anti-human haptoglobin antibody was used because antibody specific to haptoglobin-β chain was not available. Twenty cases of radical prostatectomy specimens as previously described[9] were immunostained by polyclonal anti-haptoglobin antibody. Immunostaining intensity was graded as negative, weak and moderate to strong. Then, immunoreactivity of each slide was either classified as lower expression or higher expression based on staining intensity and percentage of cancer cells stained. When moderate to strong staining was observed in 10% or more of cancer cells, higher expression was assigned, and when moderate to strong staining was observed in less than 10% of cancer cells, lower expression was assigned.

Extraction of glycosphingolipids and thin-layer chromatography immunostaining

We investigated whether GalNAcDSLc4 as a ganglioside is also responsible for RM2 reactivity to prostate cancer cells. Briefly, glycosphingolipids were extracted from prostate cancer cell lines and separated from phospholipids by alkaline degradation of phospholipids, and glycosphingolipids were analyzed by thin-layer chromatography with immunostaining by mAb RM2 as described previously.[14] To examine whether GalNAcDSLc4 as a ganglioside was released into the culture media, glycosphingolipids were extracted from 10 ml of supernatant of cell lines cultured in serum-free media for 3 days by solid phase extraction using C18 Sep-Pak cartridge (Waters, Milford, MA) as described previously.[15] Monosialosyl globopentaosylceramide (MSGb5) from supernatant of ACHN cells was used as a positive control.

Treatment of protein extract from prostate cancer cells with the hemoglobin column and immunoblotting by mAb RM2

We examined whether RM2 reactivity to prostate cancer cells depends on haptoglobin-β chain because GalNAcDSLc4 as a ganglioside was not detected in prostate cancer cells as described in Results section. For this purpose, protein extract from prostate cancer cell line DU145 was treated with the hemoglobin column to adsorb haptoglobin. DEAE-purified human hemoglobin was purchased from Sigma and coupled to CNBr-activated Sepharose-4B (Sigma) according to the manufacturer's procedures. One hundred microliters of protein extract of DU145 was applied to the column and the eluate (termed fraction I) was immediately collected without incubation. Another 100 μl was reacted with the hemoglobin column beads at 4°C overnight, then the column was stood for 5 min, and the eluate (termed fraction II) passed through the column was collected. After collecting the fraction II, the column was washed with PBS and the washed fraction (termed wash I) was collected. RM2 reactivity to each fraction was examined by Western blotting.

Treatment of serum haptoglobin-β chain defined by mAb RM2 with glycosidase

Although our coworkers recently indicated that RM2 glycosyl epitope (β1.4-GalNAcDSLc4) was not found on haptoglobin-β chain,[16] we examined changes of RM2 reactivity after β-hexosaminidase and/or sialidase treatment on the assumption that RM2 reactivity to haptoglobin-β chain is based on the RM2 glycosyl epitope. After transferring proteins from the gel to the membrane, the membrane was blocked with 1% bovine serum albumin solution at room temperature for 2 hr and washed with phosphate buffered saline/0.05% Tween-20. The membrane was placed into a vinyl bag and treated with 2.0 U of β-hexosaminidase from jack beans (Sigma) at 37°C overnight or with 25 mU of sialidase from Newcastle disease virus (NDV; Glyko) at 37°C overnight. The membrane was washed with phosphate buffered saline/0.05% Tween-20 and subjected to Western blotting by mAb RM2. The control membrane was treated in the same way without glycosidase.

Statistical analysis

Statistical analysis was performed using the software from the SAS Institute (SAS Institute, Cary, NC).

Results

RM2 reactivity to sera of prostate cancer and BPD

For comparison with RM2 reactivity to serum, examples of RM2 reactivity to prostate cancer cells and to benign prostatic

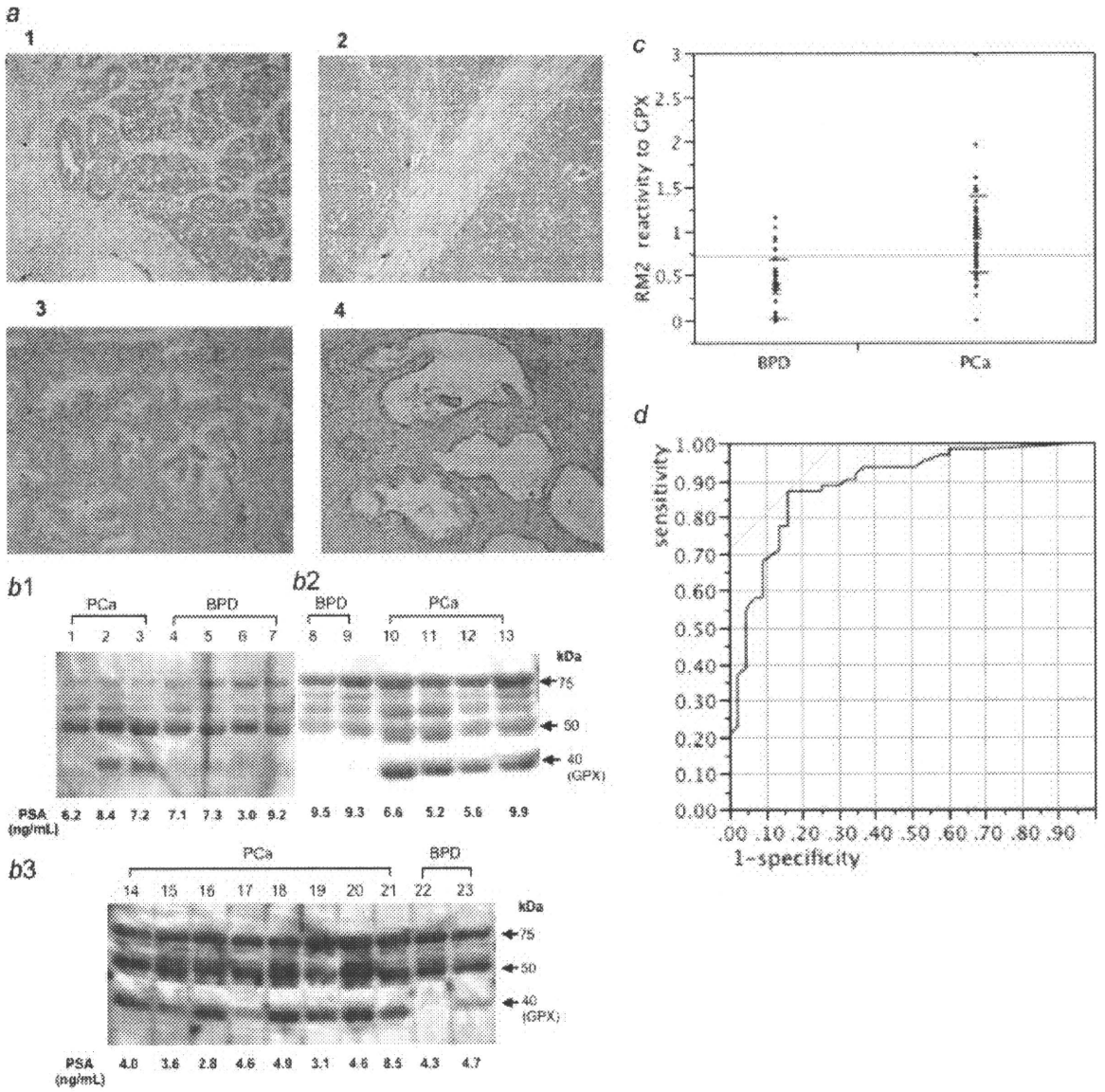


FIGURE 1 – Increased level of RM2 reactivity to GPX in sera of prostate cancer. (Panel *a*) Examples of immunohistochemical staining of prostate cancer cells (panels 1 and 2) and benign prostatic hyperplasia (panels 3 and 4). RM2 reactivity to stroma was also observed in prostate cancer tissues, but neither gland nor stroma in benign prostatic hyperplasia was stained by mAb RM2. (Panel *b*) Examples of serum Western blotting by mAb RM2. PCa: prostate cancer. BPD: benign prostatic disease. Arrow indicates position of size marker and GPX. In each panel, PSA level for each case was indicated at bottom. (Panel *c*) Comparison of RM2 reactivity to GPX between BPD and PCa. Large bars: standard deviation, Small bars: standard error of the mean. (Panel *d*) ROC curve of RM2 reactivity to GPX. The area under the ROC curve was 0.89. The difference between sensitivity and 1-specificity was highest at sensitivity of 87%.

hyperplasia were shown in Figures 1*a* and 1*b*, respectively. None of the 12 cases of benign prostatic hyperplasia examined was stained by mAb RM2. Level of RM2 reactivity to prostate cancer was associated with grade of malignancy as previously described.[9]

We found that RM2 reactivity was enhanced on a serum glycoprotein with molecular mass ~40 kDa, hereby termed "GPX," in the majority of 62 patients with early prostate cancer, when compared with 43 subjects with BPD (Figs. 1*b* and 4*b* right panel). No reactivity was observed for mouse IgM used as a negative control (data not shown). All these subjects had biopsy proven histological diagnosis and PSA less than 10 ng/mL (Tables I and II). There was no significant difference of age and PSA between the 2 groups. RM2 reactivity to GPX calculated by Scion image was normalized to the reactivity to ~75 kDa protein, which was used as an internal control because RM2 reactivity to ~75 kDa protein was relatively constant between prostate cancer and BPD. Level of RM2 reactivity to GPX in prostate cancer (0.96 ± 0.43) was significantly higher than that in BPD (0.35 ± 0.32) ($p < 0.0001$ by t test) (Fig. 1*c*). The receiver operating characteristic analysis was performed and the area under the receiver operating characteristic curve of RM2 reactivity to GPX was 0.89. Setting a cutoff level of RM2 reactivity to GPX as 0.59, RM2 reactivity to GPX for detection of prostate cancer had sensitivity of 87% and specificity of 84% (Fig. 1*d*). Then, we explored the variables predicting the level of RM2 reactivity to GPX by univariate analysis. Level of RM2 reactivity to GPX was not significantly associated with the pretreatment variables in 62 subjects with early prostate cancer (Table III). It was significantly associated with the origin of index (major) cancer among the postsurgical variables in 24 patients, who underwent radical prostatectomy (Table IV), *i.e.*, the index cancer of transition zone origin predicted lower level of RM2 reactivity when compared with that of peripheral zone origin.

TABLE I – CLINICAL CHARACTERISTICS OF THE PATIENTS WITH BIOPSY PROVEN HISTOLOGICAL DIAGNOSIS AND PSA VALUE LESS THAN 10 NG/ML

	PCa	BPD	*p*
Age	68.6 ± 6.4	66.8 ± 7.6	0.2
PSA	5.3 ± 2.1	5.0 ± 2.1	0.4
F/T	0.16 ± 0.1	0.22 ± 0.1	0.007

PCa, prostate cancer; BPD, benign prostatic disease; F/T, ratio of free to total PSA.

TABLE II – CLINICOPATHOLOGICAL CHARACTERISTICS OF THE PROSTATE CANCER PATIENTS WITH PSA VALUE LESS THAN 10 NG/ML

Parameters	No. Patients
cT	
lb	1
lc	45
2a	11
2b	4
3a	1
bGS	
6	8
7	43
8	5
9	6

cT, clinical T stage; bGS, biopsy Gleason score.

TABLE III – THE RELATIONSHIP BETWEEN THE PRETREATMENT VARIABLES AND RM2 REACTIVITY

Variables	*p*
Age	0.1769
PSA	0.0922
Bgs	0.4023
CT	0.8196
No. positive biopsy core	0.1429

cT, clinical T stage; bGS, biopsy Gleason score.

TABLE IV – THE RELATIONSHIP BETWEEN THE POSTSURGICAL VARIABLES AND RM2 REACTIVITY

Variables	*p*
Age	0.0980
PSA	0.9843
RPGS	0.3723
Index cancer origin	0.0117
Total cancer volume	0.3433
pT	0.6099

RPGS, radical prostatectomy Gleason score; pT, pathological T stage.

RM2 reactivity to GPX before and after radical prostatectomy was also examined in 15 patients with various preoperative PSA levels whose serum PSA level decreased to less than 0.1 ng/mL after radical prostatectomy, the value believed to be recurrence-free (Table V). Level of RM2 reactivity to GPX decreased in 13 of these 15 patients after radical prostatectomy, although the extent of decrease was varied (Figs. 2*a* and 2*b*). Level of RM2 reactivity significantly decreased after radical prostatectomy (pre- *vs.* post-operative value: 0.92 ± 0.52 *vs.* 0.60 ± 0.43; $p = 0.006$ by paired *t* test) (Fig. 2*b*). The profile of RM2 reactivity to sera of the other urogenital malignancies was almost the same as that to sera of prostate cancer, and level of RM2 reactivity to GPX 0.59 or more was observed in 5 of 6 subjects with RCC, 6 of 8 with urothelial carcinoma, and 2 of 8 with testicular germ cell tumor (data not shown), *i.e.*, RM2 reactivity to GPX was not specific to prostate cancer.

Identification of GPX as haptoglobin-β chain

In view of the clinical significance of GPX, we analyzed its molecular parameters. GPX was separated by Agilent column, followed by application of proteomics approach with 2D gel electrophoresis, in gel digestion, HPLC and electrospray ionization mass spectrometry.[13] The results showed clearly that GPX is haptoglobin-β chain (Fig. 3). In 2D SDS-PAGE, a clear difference was found in one fraction consisting of four contiguous spots (termed spot 1, 2, 3 and 4 from right to left), from malignant sera (specimen II-c: Fig. 3*a-1*, upper panel) *vs.* corresponding spots from nonmalignant sera (specimen II-b: Fig. 3*a-1*, lower panel). The difference between nonmalignant *vs.* malignant sera was more distinct when analysis was made by 2D SDS-PAGE with Western

TABLE V – CLINICOPATHOLOGICAL CHARACTERISTICS OF THE PATIENTS WHOSE RM2 REACTIVITY WAS COMPARED BEFORE AND AFTER RADICAL PROSTATECTOMY

Age (median)	55–75 yr (67)
Preop. PSA (median)	3.07–24.29 ng/ml (5.41)
Pathological parameters	No. patients
pT	
2a	1
2b	9
3a	5
RPGS	
5	1
7	13
8	1

Preop., preoperative; pT, pathological T stage; RPGS, radical prostatectomy Gleason score.

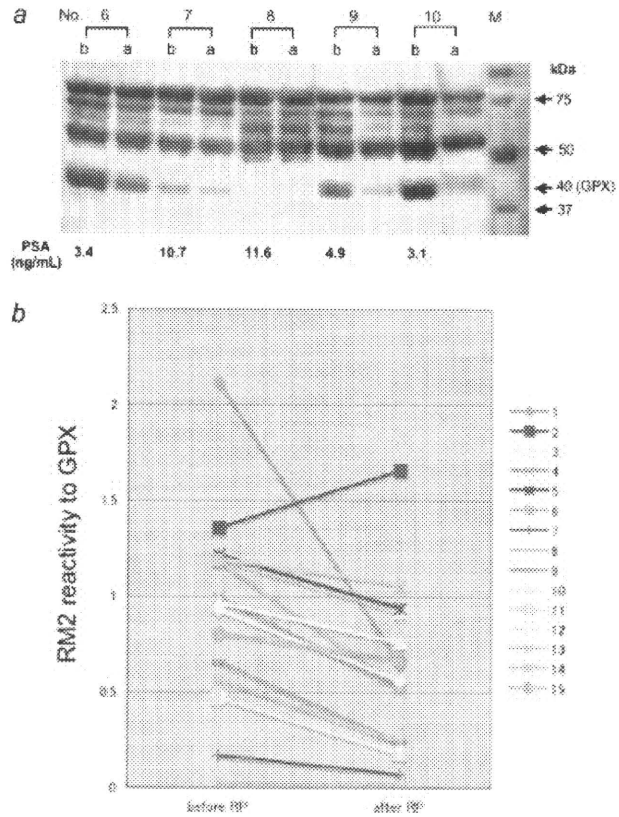
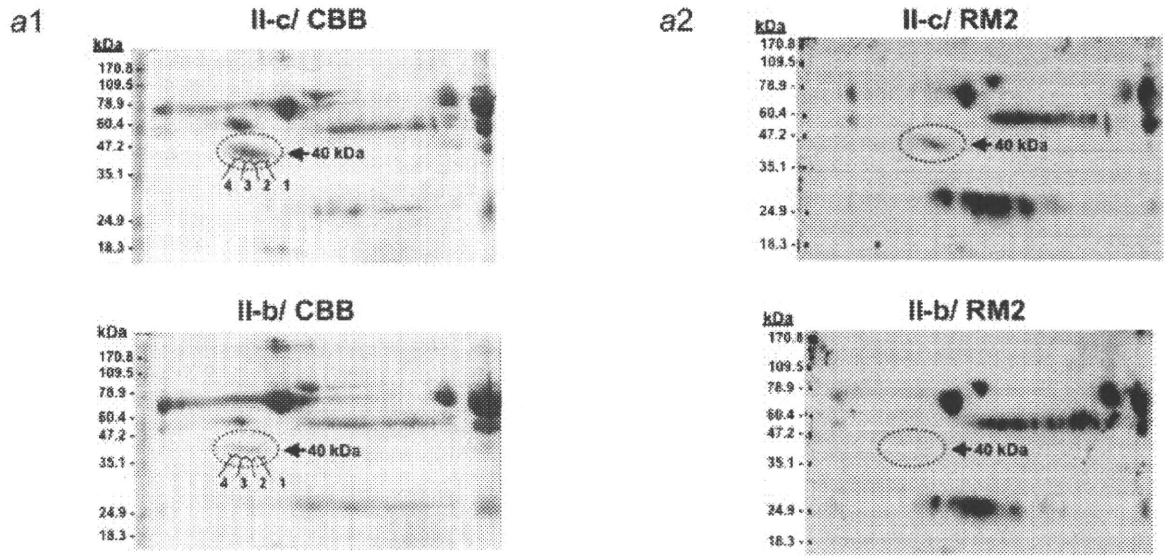
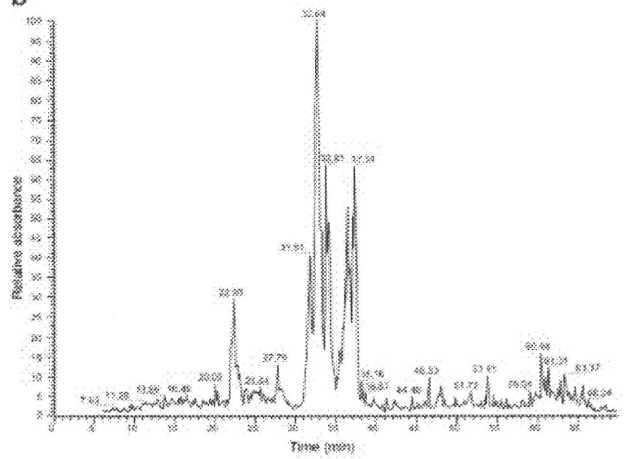


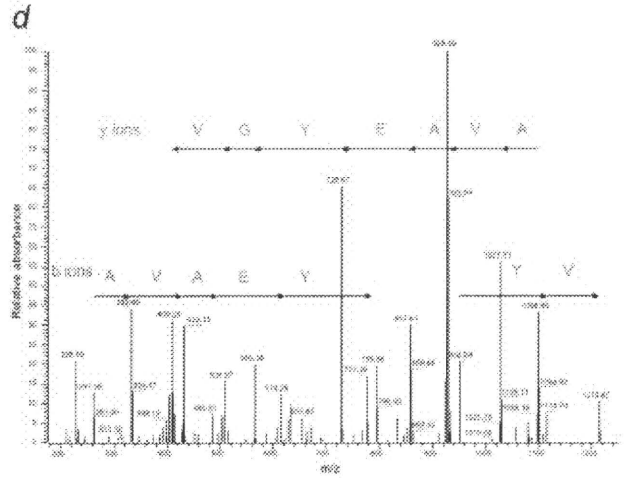
FIGURE 2 – Changes of RM2 reactivity to GPX after radical prostatectomy. (Panel *a*) Examples of RM2 reactivity to sera before and after radical prostatectomy. Preoperative PSA level in each case was indicated at bottom. In all five cases radical prostatectomy Gleason scores were 7 and pT were 2b but No. 10 (3a). b, Before radical prostatectomy; a, after radical prostatectomy; M, size marker. (Panel *b*) Changes of RM2 reactivity to GPX after radical prostatectomy in 15 patients. Longitudinal axis: RM2 reactivity to GPX, RP: radical prostatectomy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]





c

File No.	Sequence
1	R.ILGGHLDAK.G
2	K.GSFPWQAK.M
3	K.DIAPTLTLYVGK.K
4	K.DIAPTLTLYVGKK.Q
5	K.QLVEIEK.V
6	R.VGYVSGWGR.N
7	K.YVMLPVADQDQC*IR.H
8	K.YVMLPVADQDQC*IR.H
9	K.SPVGVQPILNEHTFC*AGMSK.Y
10	K.SC*AVAEYGVYVK.V
11	K.VTSIQDWVQK.T



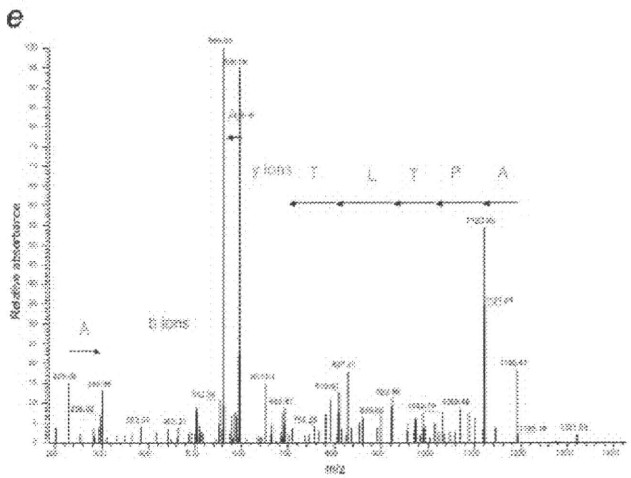


FIGURE 3 – Identification of GPX as haptoglobin-β chain. (Panel *a-1*) Protein components separated by 2D SDS-PAGE: Staining by Coomassie Brilliant Blue (CBB). Agilent column-adsorbed fractions (fraction II) from nonmalignant serum and from prostate cancer serum were analyzed by 2D SDS-PAGE. *Upper*: The pattern from sample II-c (fraction II from serum of malignant subject c). *Lower*: The pattern from sample II-b (fraction II from serum of nonmalignant subject b). Horizontal direction: isoelectric focusing (IEF). Vertical direction: SDS-PAGE. Area circled by dotted line on both left and right: GPX. Note that sample II-c (malignant) has higher CBB staining than sample II-b (nonmalignant). For each sample, GPX is separated into four contiguous spots with different IEF, termed 1, 2, 3 and 4 as indicated. (Panel *a-2*) Upper and Lower. Same as in upper and lower in panel a-1, but stained by mAb RM2. Note that RM2-stained bands are clearly seen for sample II-c but not for sample II-b. (Panel *b*) LC-MS chromatogram of spot 2 on the 2D SDS-PAGE in Panel a after *in gel* trypsin digestion and extraction of the tryptic peptides. Abscissa: LC retention time. Ordinate: MS1 of total ion chromatogram. (Panel *c*) Identification of haptoglobin. (Panel *d*) MS/MS spectrum of *m/z* 680 at the $[M + 2H]^{2+}$ ion. (Panel *e*) MS/MS spectrum of *m/z* 710 at the $[M + 2H]^{2+}$ ion. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

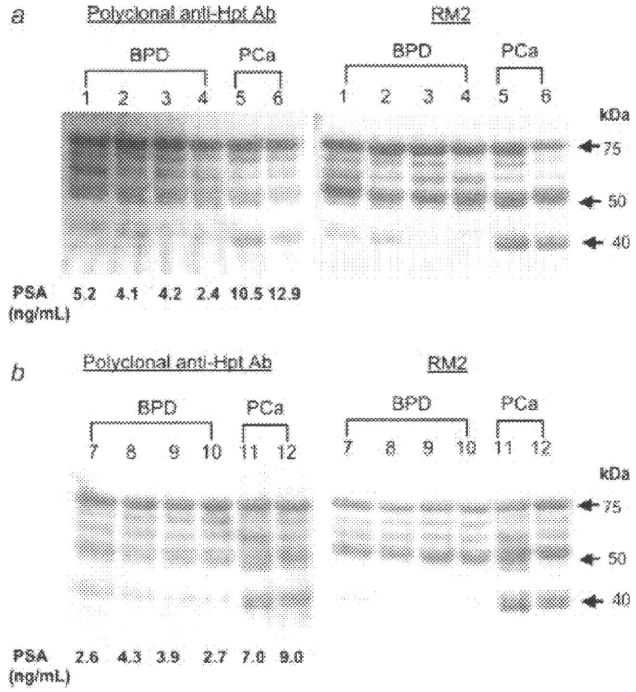


FIGURE 4 – Preferential reactivity of mAb RM2 toward haptoglobin-β chain from prostate cancer. (Panel *a* and *b*) Examples of reactivity to sera of BPD and PCa by polyclonal anti-haptoglobin antibody and mAb RM2, respectively. Left and right panels are from the same patients. *Left panel*: Reactivity to sera by polyclonal anti-haptoglobin antibody. *Right panel*: Reactivity to sera by mAb RM2. PSA level in each case was indicated at bottom. Hpt: haptoglobin, Ab: antibody.

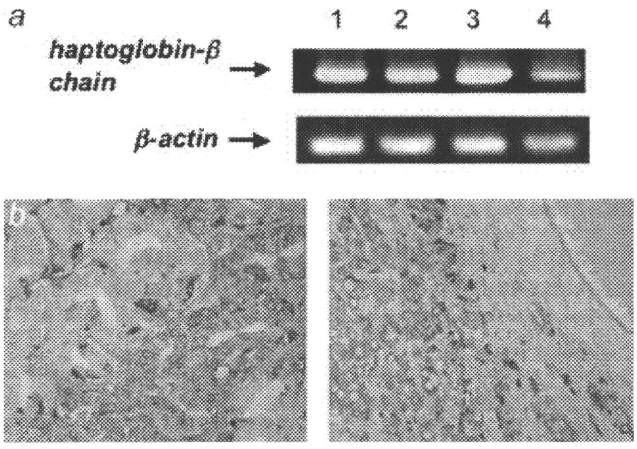


FIGURE 5 – Increased expression of haptoglobin in prostate cancer cells. (Panel *a*) Level of haptoglobin-β mRNA in prostate cancer cell line. 1: LNCaP, 2: PC3, 3: DU145, 4: PrEC. (Panel *b*) Examples of immunohistochemical staining of prostate cancer by polyclonal anti-haptoglobin antibody. Left: Gleason pattern 3, Right: Gleason pattern 4.

blotting by mAb RM2. RM2-blotted spots were strong for prostate cancer specimen II-c but absent for nonmalignant specimen II-b (Fig. 3*a-2*, upper and lower panels). The contiguous spots 1, 2, 3 and 4 as above from specimen II-c were excised from the gel, and subjected to *in gel* digestion by trypsin, followed by LC-MS/MS for protein identification. An example of a base peak chromatogram for tryptic peptides of spot 2 of GPX is shown in Figure 3*b*. The results of the TurboSEQUEST search on the data were displayed in Figure 3*c*, showing human haptoglobin-2 precursors

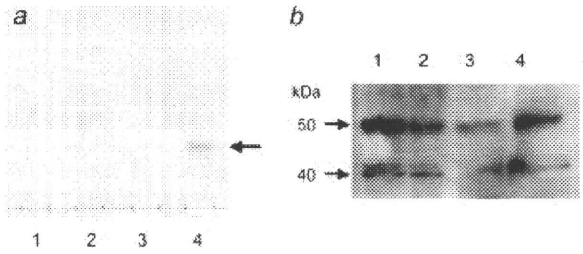


FIGURE 6 – RM2 reactivity is largely due to haptoglobin-β chain in prostate cancer cells. (Panel *a*) TLC immunostaining by mAb RM2. Total glycolipid equivalent to 15 mg of wet weight cell pellet was applied per lane. Lane 1: PC3, lane 2: LNCaP, lane 3: AICaP1, lane 4: TOS1. Arrow: GalNAcDSLc4. (Panel *b*) Comparison of Western blotting of the protein extract of DU145 before and after treatment with the hemoglobin column. The major glycoprotein with approximate molecular mass of 50 kDa is thought to be a form of haptoglobin-α and -β chain complex. Lane 1: protein extract untreated, lane 2: fraction I (the eluate of protein extract passed through the column without incubation), lane 3: fraction II (the eluate of protein extract passed through the column 24 hr after incubation with the column beads), lane 4: wash I (the washed fraction after collecting fraction II).

(P00738). There were 9 tryptic peptides found, which corresponded to 25% coverage of the haptoglobin precursor. However, the protein precursor consists of signal peptide (1–18 residue), α-chain (19–160 residue) and β-chain (162–406 residue).[17–20] All tryptic peptides identified were from β-chain. The corresponding peptide coverage for haptoglobin β-chain (40 kDa) was 38.8% for spot 2. The peptides found for the other 3 spots were similar, also identifying GPX as the haptoglobin β-chain protein. The coverage for spots 1, 3 and 4 was 35.5, 20.0 and 35.5%, respectively. Figure 3*d* shows the MS/MS spectrum of a doubly charged precursor ion at *m/z* 680 identifying SC(PAM)AVAEYGVYVK peptide with annotated amino acid sequence. Figure 3*e* shows the MS/MS spectrum of $[M + 2H]^{2+}$ at *m/z* 710 of peptide DIAPTLTLYVGKK. The amino acid sequences in Figures 3*d* and 3*e* correspond to the residues 380–391 and 216–228 of the haptoglobin precursor.

Preferential reactivity of mAb RM2 toward haptoglobin-β chain from prostate cancer when compared with polyclonal anti-haptoglobin antibody

The profile of reactivity to serum with mAb RM2 was very similar to that with polyclonal anti-haptoglobin antibody (Figs. 4*a* and 4*b*). This finding may support the results of the proteomics analysis described earlier, and also suggests that the 4 bands above haptoglobin-β chain may be various forms of haptoglobin-α and -β chain complex because haptoglobin occurs *in vivo* as polymers of an α and β chain complex.[21] In addition, RM2 showed preferential reactivity toward haptoglobin-β chain from prostate cancer rather than that from BPD when compared with polyclonal anti-haptoglobin antibody (Figs. 4*a* and 4*b*).

Increased expression of haptoglobin in prostate cancer cells

Increased level of haptoglobin-β mRNA was observed in prostate cancer cell lines, LNCaP, PC3 and DU145, when compared with PrEC, normal human prostate epithelial cells (Fig. 5*a*). In immunohistochemical staining, negative to weak staining of polyclonal anti-haptoglobin antibody was observed in benign prostatic glands or stroma. Higher expression of polyclonal anti-haptoglobin antibody in prostate cancer cells was observed in 9 of 20 cases (Fig. 5*b*), whereas expression level was not clearly different between cancer cells and benign glands or stroma in 11 cases. There was no significant association between Gleason score and expression level of polyclonal anti-haptoglobin antibody in prostate cancer cells (data not shown).

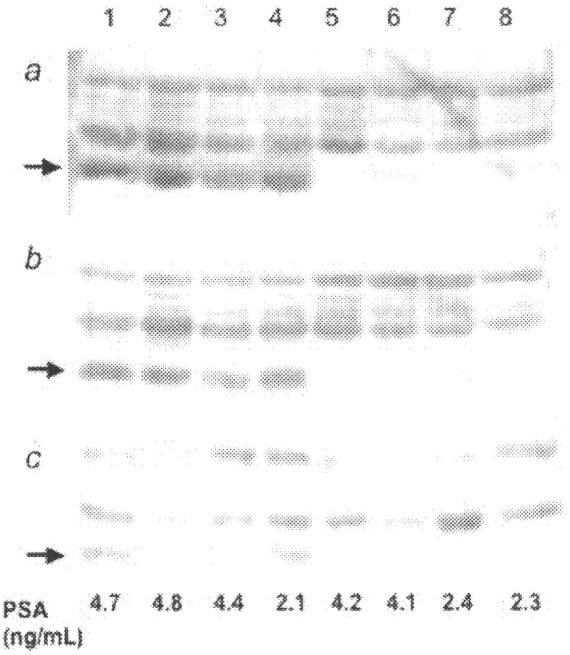


FIGURE 7 – Involvement of glycosylation in RM2 reactivity to serum haptoglobin-β chain. (Panel *a*) No treatment, (Panel *b*) treatment with β-hexosaminidase and (Panel c) treatment with β-hexosaminidase followed by NDV sialidase. Arrow: haptoglobin-β chain. 1–4: sera from prostate cancer patients, 5–8: sera from BPD patients. PSA level for each case was indicated at bottom.

RM2 reactivity is largely due to haptoglobin-β chain in prostate cancer cells

Thin-layer chromatography immunostaining of glycolipids by mAb RM2 showed that expression of GalNAcDSLc4 was not observed in prostate cancer cell lines, whereas it was detected in RCC cell line TOS1 as a positive control (Fig. 6*a*). Expression of GalNAcDSLc4 was neither detected in culture media of prostate cancer cells nor in that of TOS1 cells, whereas MSGb5 as a positive control was detected in supernatant of ACHN cells (data not shown). Most of RM2 reactivity disappeared after treatment with the hemoglobin column (Fig. 6*b*, lane 3). These results indicate that RM2 reactivity in prostate cancer cells is largely due to haptoglobin-β chain.

Involvement of glycosylation in RM2 reactivity to serum haptoglobin-β chain

RM2 reactivity to serum haptoglobin-β chain was little changed after treatment with β-hexosaminidase (Fig. 7*b*) or sialidase from NDV (data not shown). However, most of RM2 reactivity to serum haptoglobin-β chain disappeared after treatment with β-hexosaminidase followed by NDV sialidase (Fig. 7*c*).

Discussion

In the current study, we found that haptoglobin-β chain defined by RM2 is a novel marker, which significantly increased in sera of early prostate cancer. Furthermore, RM2 showed preferential reactivity toward haptoglobin-β chain derived from sera of prostate cancer, suggesting haptoglobin-β chain is qualitatively different between prostate cancer and BPD, and haptoglobin-β chain defined by RM2 is more associated with prostate cancer than BPD. Qualitative change of haptoglobin was also suggested in head and neck cancer since haptoglobin derived from cancer sera was immunosuppressive whereas that from normal sera was not.[22] Thus, both quantitative and qualitative changes of haptoglobin-β chain may be the basis of significant difference of RM2 reactivity to haptoglobin-β chain between prostate cancer and BPD. We also examined whether GalNAcDSLc4 as a ganglioside recognized by mAb RM2 was observed in prostate cancer cells. However, GalNAcDSLc4 was not detected in cell pellets or culture supernatants of prostate cancer cell lines. Furthermore, most of RM2 reactivity to protein extract from prostate cancer cells disappeared after incubation with the hemoglobin column. These results indicate RM2 reactivity to prostate cancer cells largely depends on haptoglobin-β chain.

Because haptoglobin-β chain defined by RM2 also increased in the other urogenital cancers, *i.e.*, it is not specific to prostate cancer, it may be useful for detection of early prostate cancer when coupled with PSA as organ-specific marker. For clinical application, men with lower level of haptoglobin-β chain defined by RM2 may be a problem. Although the number of cases examined was small, transition zone cancer predicted lower level of RM2 reactivity when compared with peripheral zone cancer. Considering that transition zone cancer demonstrates more favorable pathologic features,[23] the method that among men with increased PSA (4–10 ng/mL), those with increased level of haptoglobin-β chain defined by RM2 undergo prostate biopsy, and those without increase in haptoglobin-β chain undergo periodical PSA check and have prostate biopsy when rising PSA is observed may be one of the options for cancer screening. However, a large number of cases are necessary to confirm the relationship between haptoglobin-β chain defined by RM2 and the origin of index cancer.

mAb RM2 was originally established toward disialoganglioside and later found to recognize the glycosyl epitope (β1.4-GalNAcDSLc4).[7,8] In the present study, the proteomics approach showed that mAb RM2 also reacted with haptoglobin-β chain. Based on the findings of the current study, glycosylation status of serum haptoglobin-β chain was examined.[16] Fujimura found that haptoglobin-β chain has minor O-glycosylation site in addition to 4 *N*-glycosylation sites,[19,20] and glycosylation status of serum haptoglobin-β chain was different between prostate cancer and BPD.[16] However, the glycosyl epitope (β1,4-GalNAcDSLc4) recognized by mAb RM2 was not detected on haptoglobin-β chain.[16] Nevertheless, large amount of serum is necessary for thorough analysis of glycosylation to conclude whether the RM2 glycosyl epitope exists or not on haptoglobin-β chain (personal communication with Dr. Fujimura). In the current study, RM2 reactivity to haptoglobin-β chain was little changed after treatment with β-hexosaminidase or NDV sialidase, but most of it disappeared after treatment with β-hexosaminidase followed by NDV sialidase. Because mAb RM2 showed reactivity to DSLc4 in addition to GalNAcDSLc4,[8] changes of RM2 reactivity to haptoglobin-β chain after β-hexosaminidase/sialidase treatment may be compatible with RM2 reactivity to the glycosyl epitope (β1.4-GalNAcDSLc4). However, the existence of RM2 glycosyl epitope has not been verified on haptoglobin-β chain. Therefore, these results only indicate that glycosylation is involved in RM2 reactivity to haptoglobin-β chain, although it remains to be answered whether RM2 reacts directly with a glycosyl epitope or with possible conformational changes induced by glycosylation.

As to the production site, haptoglobin also known as an acute-phase protein has been reported to be mainly produced by liver and secreted into the bloodstream.[24] In the patients with cancer, haptoglobin could be either produced by the tumor cells[25] or the normal cells in response to the stimuli such as IL-6 produced from the tumor cells.[26] Increased expression of haptoglobin in prostate cancer cells as suggested by RT-PCR and immunohistochemistry in the current study indicates that haptoglobin could be produced by cancer cells. In addition to quantitative increase, qualitative change of molecule also indicates haptoglobin is produced from cancer cells since modification of proteins such as aberrant glycosylation could be induced with carcinogenesis. Thus, the previous and the current studies indicate that haptoglobin could be produced from prostate cancer cells. Because haptoglobin occurs *in vivo* as polymers of an α and β chain complex,[27] elevation of

haptoglobin-β chain may be explained by dissociation of α and β chain from haptoglobin.[27] However, although cleavage of haptoglobin by protease is assumed to be responsible for elevation of haptoglobin-α and -β chains, the exact mechanism of elevation of these chains remains to be clarified.[27]

There has been accumulating evidence of haptoglobin expression in sera of various cancers. Haptoglobin-α or -β chain is upregulated in sera of ovarian cancer, breast cancer, acute myeloid leukemia, hepatocellular carcinoma, small cell lung cancer and RCC.[27–32] Furthermore, haptoglobin-β chain carrying α1 Fuc residue, blotted by *Aleuria aurantia* lectin, is significantly enhanced in sera of gastric cancer, colon cancer, hepatocellular carcinoma and pancreatic cancer.[25] In the present study, increase in haptoglobin-β chain defined by RM2 was observed in sera of RCC, urothelial carcinoma and testicular germ cell tumors as well as prostate cancer. Because of this universal increase in haptoglobin-α or -β chain in various malignancies, haptoglobin-β chain defined by RM2 may have potential of exploiting a new approach to serum diagnosis of cancer other than the urogenital malignancies.

In conclusion, haptoglobin-β chain defined by RM2 is a novel serum marker that may be useful for detection of early prostate cancer when coupled with PSA as organ-specific marker because it is not specific to prostate cancer. However, larger trials are necessary to confirm the findings in the current study. It is also important to examine whether haptoglobin-β chain defined by RM2 can be detected in sera of various malignancies other than the urogenital cancers.

Acknowledgements

The authors thank Dr. Sen-itiroh Hakomori and Dr. Taeko Miyagi for helpful advice to this study, Dr. Stephen Anderson for preparation and arrangement of the manuscript and figures and Ms. Emiko Idutsu for assisting serum sample collection.

References

1. Carter HB, Partin AW. Diagnosis and staging of prostate cancer. In: Walsh PC, Retik AB, Vaughan ED, Wein AJ, eds. Campbell's urology, 8th edn. Philadelphia: Saunders, 2002.3209–226.
2. Gretzer MB, Partin AW. PSA markers in prostate cancer detection. Urol Clin North Am 2003;30:677–86.
3. Canto EI, Shariat SF, Slawin KM. Biochemical staging of prostate cancer. Urol Clin North Am 2003;30:263–77.
4. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, Minasian LM, Ford LG, Lippman SM, Crawford ED, Crowley JJ, Clotman CA. Prevalence of prostate cancer among men with a prostate-specific antigen level < or = 4.0 ng per milliliter. N Engl J Med 2004;350:2239–46.
5. Adam B-L, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, Semmes OJ, Schellhammer PF, Yasui Y, Feng Z, Wright GL, Jr. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostatic hyperplasia. Cancer Res 2002;62:3609–14.
6. Lehman ES, Cannon GW, Trock BJ, Sokoll LJ, Chan DW, Mangold L, Partin AW, Getzenberg RH. EPCA-2: a highly specific serum marker for prostate cancer. Urology 2007;69:714–20.
7. Saito S, Levery SB, Salyan MEK, Goldberg RI, Hakomori S. Common tetrasaccharide epitope NeuAcalpha2–3Galbeta1–3(NeuAcalpha2–6)GalNAc, presented by different carrier glycosylceramides or *O*-linked peptides, is recognized by different antibodies and ligands having distinct specificities. J Biol Chem 1994;269:5644–52.
8. Ito A, Levery SB, Saito S, Satoh M, Hakomori S. A novel ganglioside isolated from renal cell carcinoma. J Biol Chem 2001;276:16695–703.
9. Saito S, Egawa S, Endoh M, Ueno S, Ito A, Numahata K, Satoh M, Kuwao S, Baba S, Hakomori S, Arai Y. RM2 antigen (β1,4-GalNAc-disialyl-Lc_4) as a new marker for prostate cancer. Int J Cancer 2005;115:105–13.
10. Flemming ID, Cooper JS, Henson DE, Hutter RVP, Kennedy BJ, eds. American Joint Committee on Cancer Staging Manual, 5th edn. Philadelphia: Lippincott; 1997:219.
11. Eble JN, Sauter G, Epstein JI, Sesterhenn IA, eds. Pathology and genetics of tumours of the urinary system and male genital organs. Lyon (France): IARC Press, 2004:179.
12. Saito S, Orikasa S, Ohyama C, Satoh M, Fukushi Y. Changes in glycolipids in human renal-cell carcinoma and their clinical significance. Int J Cancer 1991;49:329–34.
13. Mineki R, Taka H, Fujimura T, Kikkawa M, Shindo N, Murayama K. In situ alkylation with acrylamide for identification of cysteinyl residues in proteins during one- and two-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteomics 2002;2:1672–81.
14. Aoki H, Satoh M, Mitsuzuka K, Ito A, Saito S, Funato T, Endoh M, Takahashi T, Arai Y. Inhibition of motility and invasiveness of renal cell carcinoma induced by short interference RNA transfection of β 1,4 GalNAc transferase. FEBS Lett 2004;567:203–8.
15. Saito S, Aoki H, Ito A, Ueno S, Wada T, Mitsuzuka K, Satoh M, Arai Y, Miyagi T. Human α 2,3-sialyltransferase (ST3Gal II) is a stage-specific embryonic antigen-4 synthase. J Biol Chem 2003;278:26474–79.
16. Fujimura T, Shinohara Y, Tissot B, Pang P-C, Kurogochi M, Saito S, Aarai Y, Sadilek M, Murayama K, Dell A, Nishimura S, Hakomori S. Glycosylation status of haptoglobin in sera of patients with prostate cancer *vs.* benign prostate disease or normal subjects. Int J Cancer 2008:122:39–49.
17. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. Clin Chem 1996;42:1589–1600.
18. Kurosky A, Barnett DR, Lee TH, Touchstone B, Hay RE, Arnott MS, Bowman BH, Fitch WM. Covalent structure of human haptoglobin: a serine protease homolog. Proc Natl Acad Sci USA 1980;77:3388–92.
19. Zhang H, Li XJ, Martin DB, Aebersold R. Identification and quantification of N-linked glycoproteins using hydrazide chemistry, stable isotope labeling and mass spectrometry. Nat Biotechnol 2003;21:660–66.
20. Liu T, Qian WJ, Gritsenko MA, Camp DG, II, Monroe ME, Moore RJ, Smith RD. Human plasma *N*-glycoproteome analysis by immunoaffinity subtraction, hydrazide chemistry, and mass spectrometry. J Proteome Res 2005;4:2070–80.
21. Tolson J, Bogumil R, Brunst E, Beck H, Elsner R, Humeny A, Kratzin H, Deeg M, Kuczyk M, Mueller GA, Mueller CA, Flad T. Serum protein profiling by SELDI mass spectrometry: detection of multiple variants of serum amyloid alfa in renal cancer patients. Lab Invest 2004;84:845–56.
22. Oh S-K, Kim S-H, Walker JE. Interference with immune response at the level of generating effector cells by tumor-associated haptoglobin. J Natl Cancer Inst 1990;82:934–40.
23. McNeal JE, Redwine EA, Freiha ES, Stamey TA. Zonal distribution of prostate adenocarcinoma. Correlation with histologic pattern and distribution of spread. Am J Surg Pathol 1988;12:897–906.
24. Wang Y, Kinzie E, Berger FG, Lim S-K, Baumann H. Haptoglobin, an inflammation-inducible plasma protein. Redox Rep 2001;6:379–85.
25. Okuyama N, Ide Y, Nakano M, Nakagawa K, Yamanaka K, Moriwaki K, Murata K, Ohigashi H, Yokoyama S, Eguchi H, Ishikawa O, Ito T, et al. Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation. Int J Cancer 2006;118:2803–8.
26. Oliviero S, Cortese R. The human haptoglobin gene promoter: interleukin-6-responsive elements interact with a DNA-binding protein induced by interleukin-6. EMBO J 1989;8:1145–51.
27. Ye B, Cramer DW, Skates SJ, Gygi SP, Pratomo V, Fu L, Horick NK, Licklider LJ, Schorge JO, Berkowitz RS, Mok SC. Haptoglobin-α subunit as potential serum biomarker in ovarian cancer: identification and characterization using proteomic profiling and mass spectrometry. Clin Cancer Res 2003;9:2904–11.
28. Ahmed N, Oliva KT, Barker G, Hoffmann P, Reeve S, Smith IA, Quinn MA, Rice GE. Proteomic tracking of serum protein isoforms as screening biomarkers of ovarian cancer. Proteomics 2005;5:4625–36.
29. Gonçalves A, Esterni B, Bertucci F, Sauvan R, Chabannon C, Cubizolles M, Bardou VJ, Houvenaegel G, Jacquemier J, Granjeaud S, Meng X-Y, Fung ET, et al. Postoperative serum proteomic profiles may predict metastatic relapse in high-risk primary breast cancer patients receiving adjuvant chemotherapy. Oncogene 2006;25:981–9.
30. Kwak J-Y, Ma T-Z, Yoo M-J, Choi BH, Kim H-G, Kim S-R, Yim C-Y, Kwak Y-G. The comparative analysis of serum proteomes for the discovery of biomarkers for acute myeloid leukemia. Exp Hematol 2004;32:836–42.
31. Ang IL, Poon TCW, Lai PBS, Chan ATC, Ngai S-M, Hui AY, Johnson PJ, Sung JJ. Study of serum haptoglobin and its glycoforms in the diagnosis of hepatocellular carcinoma: a glycoproteomic approach. J Proteome Res 2006;5:2691–700.
32. Bharti A, Ma PC, Maulik G, Singh R, Khan E, Skarin AT, Salgia R. Haptoglobin alpha-subunit and hepatocyte growth factor can potentially serve as serum tumor biomarkers in small cell lung cancer. Anticancer Res 2004;24:1031–8.

Int. J. Cancer: **123**, 2574–2579 (2008)

Polymorphisms of fibroblast growth factor receptor 4 have association with the development of prostate cancer and benign prostatic hyperplasia and the progression of prostate cancer in a Japanese population

Zhiyong Ma[1], Norihiko Tsuchiya[1]*, Takeshi Yuasa[1], Takamitsu Inoue[1], Teruaki Kumazawa[1], Shintaro Narita[1], Yohei Horikawa[1], Hiroshi Tsuruta[1], Takashi Obara[1], Mitsuru Saito[1], Shigeru Satoh[1], Osamu Ogawa[2] and Tomonori Habuchi[1]

[1]*Department of Urology, Akita University School of Medicine, Akita, Japan*
[2]*Department of Urology, Graduate School of Medicine, Kyoto University, Kyoto, Japan*

Fibroblast growth factor receptor 4 (FGFR4) is a member of a family of transmembrane receptors with ligand-induced tyrosine kinase activity. The Glycine (Gly) to Arginine (Arg) polymorphism at codon 388 (Gly388Arg), which encodes an amino acid in the transmembrane part of the FGFR4 gene, was reported to be associated with an increased risk in some carcinomas. We investigated the association between the Gly388Arg polymorphism or the G or A polymorphism at intron 11 (rs2011077) of FGFR4, which was located 1,213 base pairs apart from the Gly388Arg polymorphism, and the risk of prostate cancer or benign prostate hyperplasia (BPH), and the prostate cancer disease status in Japanese men. Genotypes of Gly388Arg and rs2011077 polymorphisms of FGFR4 were determined in 492 patients with prostate cancer, 165 patients with BPH and 179 male controls. Regarding the Gly388Arg polymorphism, individuals with the ArgArg genotype had a 2.207- and 1.958-fold increased risk of prostate cancer and BPH, and a 1.804-fold increased risk of metastatic prostate cancer compared with those with the GlyGly genotype. Regarding the rs2011077 polymorphism, individuals with the GG genotype had a 6.260- and 3.033-fold increased risk of prostate cancer and BPH, and a 5.550-fold increased risk of metastatic prostate cancer compared with those with the AA genotype. Our results indicate that the FGFR4 Arg allele of the Gly388Arg polymorphism and the G allele of the rs2011077 polymorphism have a significant impact on the development of prostate cancer and BPH, and the progression of prostate cancer in a Japanese population.

Key words: prostate cancer; polymorphism; fibroblast growth factor receptor; benign prostatic hyperplasia

Prostate cancer is the second common cancer among men in most western populations.[1] There are striking differences in the age-adjusted incidence of prostate cancer between different racial groups and between different geographic regions of the world.[2] Rates among African-Americans are the highest in the world (275.8 per 100,000 person-years), followed by Caucasian-Americans (171.9 per 100,000 person-years).[3] The incidence of 19.9 per 100,000 person-years in Japanese men is among the lowest in the world.[4] On the other hand, Japanese immigrants in the United States have experienced a marked increase in prostate cancer incidence, although the rates in Japanese men in Los Angeles and the San Francisco Bay Area are still less than in whites.[5,6] These epidemiological data demonstrate that both genetic and environmental factors play a significant role in the incidence of prostate cancer.

Fibroblast growth factor receptor 4 (FGFR4) is a member of a tyrosine kinase receptor (TKR) family that displays multiple biological activities, including mitogenic and angiogenic activity, with a consequent crucial role in cell differentiation, development, hormonal and proliferative signaling,[7] in response to more than 20 known ligands. A few studies demonstrated that it was expressed in normal human prostate, PIN, prostate cancer, prostate cancer cell lines and the immortalized human prostate epithelial cell line.[8–10] Potent activators of FGFR4 (FGF2, FGF6 and FGF8) and FGFR4 itself are frequently overexpressed in human prostate cancer.[8,11] Recently, Bange *et al.*[12] reported a Gly to Arg polymorphism at codon 388 (Gly388Arg) in the FGFR4 gene (*FGFR4*) and found that the presence of the *FGFR4* Arg388 allele had a substantial negative impact on disease-free survival in patients with breast cancer with lymph node metastasis, although other groups have not observed a similar effect in their patients with breast cancer.[13] Bange *et al.*[12] also reported that the presence of the *FGFR4* Arg388 allele was associated with metastasis and poor prognosis in colon cancer. In head and neck squamous cell carcinoma, an association was demonstrated between the high expression of *FGFR4* Arg388 allele and poor clinical outcome.[14] In prostate cancer, Wang *et al.*[10] demonstrated that the presence of *FGFR4* Arg388 allele was associated with clinical aggressiveness. These findings were supported by independent groups with similar results in soft tissue sarcoma and lung adenocarcinoma,[15,16] although contradictory results have also been presented.[17–19] Benign prostate hyperplasia (BPH) has an inheritable genetic component,[20] and Boget *et al.*[21] reported a close relationship between FGFR and BPH. Therefore, an analysis that includes men with BPH in a control group may mask the role of *FGFR4* polymorphism in prostate cancer. The present study was conducted to explore the association of the Gly388Arg or the *G* to *A* polymorphism in *FGFR4* intron 11 (rs2011077) with the development of prostate cancer and BPH, and the progression of prostate cancer in a Japanese population. In addition, we measured linkage disequilibrium (LD) between two polymorphisms.

Material and methods

Subjects

A total of 836 subjects, consisting of 492 patients with prostate cancer, 165 patients with BPH and 179 male controls treated at Akita University Medical Center and related community hospitals were enrolled in this study. Medical records were used to obtain clinical data, with approval from the institute's ethics committee and with informed consent. All patients with prostate cancer were pathologically diagnosed from specimens obtained from transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms. The clinical or pathological stage of prostate cancer at the time of diagnosis was determined by reviewing the medical records based on the Tumor-Node-Metastasis system. Prostate cancers were classified into Stage A ($T_{1a-b}N_0M_0$), Stage B ($T_{1c-2}N_0M_0$), Stage C ($T_{3-4}N_0M_0$) and Stage D ($T_{1-4}N_1M_{0-1}$ or $T_{1-4}N_{0-1}M_1$) by the modified Whitmore–Jewett system. In patients who underwent radical prostatectomy, the final pathological stage was applied; and in patients without radical prostatectomy, the

Abbreviations: FGFR, fibroblast growth factor receptor; TKR, tyrosine kinase receptor; BPH, benign prostate hyperplasia; LD, linkage disequilibrium; LPA, lysophosphatidic acid; ERK, extracellular signal-regulated kinase; OR, odds ratios; CI, confidence intervals; aOR, age-adjusted OR.

***Correspondence to:** Department of Urology, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. Fax: +81-18-8362619. E-mail: tsuchiya@med.akita-u.ac.jp

Received 30 October 2007; Accepted after revision 28 February 2008
DOI 10.1002/ijc.23578
Published online 28 August 2008 in Wiley InterScience (www.interscience.wiley.com).

clinical stage was applied. Pathological grading of prostate cancer was determined according to the General Rules for Clinical and Pathological Studies on prostate cancer by the Japanese Urological Association and the Japanese Society of Pathology, which is based on the WHO criteria and according to the Gleason score. All pathological grading was based on needle biopsy specimens in patients of Stages B–D and surgical specimens in patients of Stage A. Well-, moderately and poorly differentiated carcinoma generally corresponded to Gleason scores of 2–4, 5–7 and 8–10, respectively. In the present study, because the two grading systems were used by local pathologists, the tumor grade system was newly categorized as follows: (*i*) low-grade cancer including well-differentiated or Gleason 2–4 carcinomas; (*ii*) intermediate-grade cancer including moderately differentiated or Gleason 5–7 carcinomas; and (*iii*) high-grade cancer including poorly differentiated or Gleason 8–10 carcinomas. All patients with BPH had various lower urinary tract symptoms of different degrees and enlarged prostates measured with transabdominal ultrasound. The serum PSA levels of all patients with BPH were measured by the Tandem-R assay (Hybritech, San Diego, CA). Patients who had elevated serum PSA levels (4.0 ng/ml or more) had transrectal sextant biopsies to eliminate the possibility of prostate cancer. The 179 male controls were over 60 years old with no voiding symptoms, selected from admitted patients in nonurological departments in community hospitals. They all had serum PSA levels less than 4.0 ng/ml and showed no signs of prostate cancer or prostatic enlargement by digital transrectal ultrasound. Serum PSA was measured using the Tandem-R assay in most cases. When serum PSA was measured by kits other than the Tandem-R assay, the measured PSA level was adjusted to that of the Tandem-R assay using a formula published elsewhere.[22]

Genotyping of FGFR4 polymorphisms

We analyzed 2 single nucleotide polymorphisms of *FGFR4*, Gly388Arg and rs2011077. The 2 polymorphisms are located 1,213 base pairs apart on the chromosome. DNA was extracted from blood samples collected from each patient and control using a QIAamp Blood Kit (QIAGEN, Hilden, Germany) or by the standard method with proteinase K digestion followed by phenol/chloroform extraction. Polymerase chain reaction (PCR) amplification of fragments encompassing polymorphic sites was performed in a 15-μl PCR reaction mix containing ~20 ng genomic DNA, 1× PCR buffer supplied by the manufacturer, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$, 50 pmol of each primer and 1.0 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). For the Gly388Arg polymorphism, after the 10-min step of initial denaturation at 94°C, PCR was carried out at 94°C for 1 min, at 64°C for 1 min and at 72°C for 1 min for a total of 35 cycles, followed by a 10-min final extension step at 72°C in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer, Waltham, MA). The 168-bp production containing the *BstN* I polymorphic site was PCR-amplified using forward primer 5'-GACCGCGACGACGCCCGGAGCCGA-3' and reverse primer 5'-AGAGGGAAGCGGGAGAGCTTCTGC-3'. For the rs2011077 polymorphism, the reaction situation was the same as that of Gly388Arg except that the annealing temperature was 55°C. The 102-bp production containing the *Bfa* I polymorphic site was PCR-amplified with forward primer 5'-AGAGAGGTAGAGGGCCTGTGGAGCTGACTA-3' and reverse primer 5'-GAAGAATTTGGTGTGACAGGCTTG-3'. After confirmation of successful PCR amplification by loading 5 μl of the reaction products on 2.5% agarose gel electrophoresis, each PCR product was digested overnight with 5 U of restriction enzyme *BstN* I (60°C) or *Bfa* I (37°C) (New England Biolabs, Beverly, MA). For the Gly388Arg polymorphism, restriction fragments were 109-, 37- and 22-bp for the Gly allele and 82-, 37-, 27- and 22-bp for the Arg allele. For the rs2011077 polymorphism, restriction fragments were 72- and 30-bp for the *G* allele and 102-bp for the *A* allele. Restriction enzyme-treated PCR products were electrophoresed on 3% agarose gels and visualized by ethidium bromide staining for analyses of *FGFR4* polymorphisms. To avoid genotyping errors caused by DNA contamination, incomplete digestion or other technical failures, we repeated the experiment at least twice for all samples.

Statistical analyses

All data were entered into an access database and analyzed using Excel 2003 (Microsoft, Richmond, WA and Washington, DC) and SPSS, version 15.0J (SPSS, Chicago, IL) software. The mean age of the subjects in the three groups was examined using the independent *t* test. Hardy–Weinberg equilibrium analyses were performed to compare observed and expected genotype frequencies using the Pearson χ^2 test. Case–control data (allelic frequencies and genotype distributions) were analyzed using the Pearson χ^2 test. A multivariate logistic regression model was used to assess the association between the disease risk and genotypes by calculating odds ratios (OR) and 95% confidence intervals (CI) adjusted by age as a confounding factor. We hypothesized the Arg allele of the Gly388Arg polymorphism as an inherent genetic risk factor for prostate cancer, BPH and prostate cancer progression. Statistical modeling was performed on the relative risk of the ArgArg or GlyArg genotype against the GlyGly genotype for Gly388Arg independently using the logistic regression model adjusted by age. In addition, the gene dosage effect of the Arg risk allele was assessed by modeling the linear effect on the log odds scale for each risk allele in multivariate logistic regression, such as the genotypes ArgArg, GlyArg and GlyGly, which were valued as "2," "1" and "0," respectively. To rs2011077, the *G* allele was hypothesized as the inherent genetic risk factor for prostate cancer, BPH and prostate cancer progression. We also performed statistical modeling analysis for the *G* allele as mentioned above. The disease-free interval was defined as the period from the date of radical retropubic prostatectomy to the date when PSA was more than 0.4 ng/mL. Cancer-specific death was defined as death from prostate cancer or from other causes strongly associated with the progression of prostate cancer. Survival time was calculated from the date of prostate cancer diagnosis to the day of death due to cancer-specific death, with deaths from other causes censored. The relationship between the polymorphisms and the disease-free survival in prostate cancer patients of Stages A–D1 or cancer-specific survival in patients with prostate cancer of Stage D2 was estimated by the Kaplan–Meier method and evaluated by the log-rank test. LD was measured between two polymorphisms in 3 groups. All statistical tests and *p* values were 2-tailed and results were considered significant at $p < 0.05$.

Results

Subject characteristics

The present study included 492 cases of pathologically confirmed prostate cancer, 165 cases of BPH with lower urinary tract symptoms and 179 male controls. The mean age ± SD was 70.34 ± 7.43, 70.53 ± 9.41 and 70.98 ± 7.38 years, respectively. No significant difference in the mean age was found between patients with prostate cancer and controls ($p = 0.322$) or between patients with BPH and controls ($p = 0.621$).

Genotype and allelic frequencies of FGFR4 Gly388Arg and rs2011077 polymorphisms

The genotype distributions of *FGFR4* Gly388Arg and rs2011077 polymorphisms are presented in Table I. The distribution of genotypes for the two polymorphisms in the control group (for Gly388Arg, $\chi^2 = 0.147$, $df = 2$, $p = 0.701$; for rs2011077, $\chi^2 = 3.168$, $df = 2$, $p = 0.075$) were consistent with Hardy–Weinberg equilibrium. Statistical analyses of genotype frequency for the polymorphisms showed a significant difference between the prostate cancer group and the control group (Gly388Arg: $p = 0.002$, rs2011077: $p < 0.001$), and between the BPH group and the control group (Gly388Arg: $p = 0.023$, rs2011077: $p = 0.009$). The allelic frequencies of *FGFR4* Gly388Arg and rs2011077