Int. J. Cancer: **125**, 345–352 (2009)

Identification of novel microRNA targets based on microRNA signatures in bladder cancer

Takahiro Ichimi[1], Hideki Enokida[1]*, Yasushi Okuno[2], Ryo Kunimoto[2], Takeshi Chiyomaru[1], Ken Kawamoto[1], Kazuya Kawahara[3], Kazuki Toki[1], Kazumori Kawakami[1], Kenryu Nishiyama[1], Gozoh Tsujimoto[4], Masayuki Nakagawa[1] and Naohiko Seki[5]

[1]*Department of Urology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan*
[2]*Department of Pharmacoinformatics, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan*
[3]*Kawahara Nephro-urology Clinic, Kagoshima, Japan*
[4]*Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan*
[5]*Department of Functional Genomics, Graduate School of Medicine, Chiba University, Chiba, Japan*

MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate protein-coding genes. To identify miRNAs that have a tumor suppressive function in bladder cancer (BC), 156 miRNAs were screened in 14 BCs, 5 normal bladder epithelium (NBE) samples and 3 BC cell lines. We identified a subset of 7 miRNAs (*miR-145*, *miR-30a-3p*, *miR-133a*, *miR-133b*, *miR-195*, *miR-125b* and *miR-199a) that were significantly downregulated in BCs. To confirm these results, 104 BCs and 31 NBEs were subjected to real-time RT-PCR-based experiments, and the expression levels of each miRNA were significantly downregulated in BCs ($p < 0.0001$ in all). Receiver-operating characteristic curve analysis revealed that the expression levels of these miRNAs had good sensitivity (>70%) and specificity (>75%) to distinguish BC from NBE. Our target search algorithm and gene-expression profiling in BCs (Kawakami *et al.*, Oncol Rep 2006;16:521–31) revealed that *Keratin7* (*KRT7*) mRNA was a common target of the downregulated miRNAs, and the mRNA expression levels of *KRT7* were significantly higher in BCs than in NBEs ($p = 0.0004$). Spearman rank correlation analysis revealed significant inverse correlations between *KRT7* mRNA expression and each downregulated miRNA ($p < 0.0001$ in all). Gain-of-function analysis revealed that *KRT7* mRNA was significantly reduced by transfection of 3 miRNAs (*miR-30-3p*, *miR-133a* and *miR-199a**) in the BC cell line (KK47). In addition, significant decreases in cell growth were observed after transfection of 3 miRNAs and si-KRT7 in KK47, suggesting that *miR-30-3p*, *miR-133a* and *miR-199a** may have a tumor suppressive function through the mechanism underlying transcriptional repression of *KRT7*.**

Key words: microRNA; *Keratin7*; bladder cancer

Bladder cancer (BC) is among the 5 most common malignancies worldwide, and it is the 2nd most common tumor of the genitourinary tract and the 2nd most common cause of death in patients with genitourinary tract malignancies.[1,2] Gene-expression profiling by microarray analysis is an excellent tool for screening candidate genes that have a tumor suppressive or oncogenic function in BC.[3,4] We previously identified that *SKP2* and *CKS1* contribute the progression and prognosis in BC by our microarray analysis.[5,6] However, the hundreds of candidate genes derived from microarray analysis may embarrass investigators to decide which gene should be studied. DNA hypermethylation is aberrant epigenetic event that negatively regulates the expression levels of tumor suppressor genes or oncogenes.[7] Hence, it seems to be a good strategy to find cancer-related genes by sorting methylated genes. Another major epigenetic event is caused by microRNA (miRNA). miRNAs are small noncoding RNA gene products about 22-nucleotide (nt) long, which are cleaved from 70- to 100-nt hairpin-shaped precursors (pre-miRNA).[8] Although their precise biology is not fully understood, miRNAs are found in diverse organisms and epigenetically function as negative regulators of gene expression through posttranslational mRNA degradation.[8,9] A more recent link between miRNA function and cancer pathogenesis is supported by studies examining the signatures of miRNA in clinical samples. miRNAs are aberrantly expressed in human cancer, indicating that they may have a novel oncogenic or tumor suppressive function.[10] The first evidence of involvement of miRNAs in human cancer came from molecular studies characterizing the 13q14 deletion in human chronic lymphocytic leukemia, which revealed 2 miRNAs, *miR-15a* and *miR-16-1*.[11] Subsequently, altered miRNA expression has been reported in lung cancer,[12] breast cancer,[13] glioblastoma,[14] hepatocellular carcinoma,[15] papillary thyroid carcinoma,[16] colorectal cancer,[17] pancreatic tumors[18] and, most recently, bladder and kidney cancer.[19] However, the target genes by these miRNAs were not fully elucidated especially in BC.

In our study, we determined the miRNA expression signatures specific to BC by evaluating 156 mature miRNAs expressions in 14 BCs, 5 normal bladder epithelium (NBE) specimens, and 3 BC cell lines. We identified a subset of 7 downregulated miRNAs that have a tumor suppressive function based on miRNA expression signatures of BC. Subsequently, we validated the expression levels of the miRNAs by using more than 100 clinical specimens. Our target search algorithm based on the gene-expression profiling in BCs[5] indicated that *Keratin7* (*KRT7*) transcript was a common target of the downregulated miRNAs. We transfected the downregulated miRNAs in a BC cell line (KK47) to examine their transcriptional repression of *KRT7*. Furthermore, we also examined the cell growth effect on KK47 cells transfected with the miRNAs to examine the functional roles of these miRNAs and *KRT7* in BC development.

Material and methods

Clinical BC specimens and BC cells culture

Tissue specimens were from 104 BC patients who had undergone cystectomy, or transurethral resection of BC at Kagoshima University Hospital and 3 affiliated hospitals between 2003 and 2007. The patients' backgrounds and clinicopathological characteristics are summarized in Table I. Our study was approved by the Bioethics Committee of Kagoshima University; written prior informed consent and approval were given by these patients. These samples were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer tumor-node-metastasis classification and histologically graded.[20] There was a significant difference in median age between BC and non-BC patients (76 *vs.* 70 years, respectively, $p < 0.001$). Of the

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ministry of Education, Science, Sports and Culture; Grant numbers: 20390427, 20591861.

***Correspondence to:** Associate Professor of Urology, Department of Urology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. Fax: +81-265-9727. E-mail: enokida@m.kufm.kagoshima-u.ac.jp

Received 4 September 2008; Accepted after revision 9 February 2009
DOI 10.1002/ijc.24390
Published online 23 February 2009 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – PATIENTS' CHARACTERISTICS

Bladder cancer (BC)	
Total number	104
Median age (range)	76 (33–100) years
Gender	
Male	74
Female	30
Stage	
Superficial (pTa)	71
Invasive (≥pT1)	33
Grade	
G1	6
G2	42
G3	56
Operation	
Cystectomy	16
TUR-Bt	88
Follow-up period (range)	476 (5–1440) days
Normal bladder epithelium (NBE)	
Total number	31
Median age (range)	70 (30–78)
Gender	
Male	27
Female	4

TABLE II – PATIENTS' CHARACTERISTICS FOR miRNA SCREENING TEST

No.	Age	Gender	Stage	Grade
1	55	F	Superficial	G2
2	61	M	Superficial	G2
3	68	M	Superficial	G2
4	68	M	Superficial	G2
5	88	M	Invasive	G3
6	80	M	Invasive	G3
7	53	F	Superficial	G2
8	85	M	Superficial	G2
9	53	M	Superficial	G2
10	78	M	Invasive	G3
11	83	M	Invasive	G2
12	74	F	Superficial	G2
13	89	M	Superficial	G3
14	70	M	Invasive	G3

104 BCs, 14 BCs were randomly selected for the screening test of 156 miRNAs that were available in November 2006 (Table II). We could not use the samples with G1 grade for the screening test, because these samples were obtained from transurethral resection, and they were too small to extract enough total RNA for screening 156 miRNAs.

The human BC cell lines (T24, KK47 and BOY[21]) were maintained in the recommended medium mixed with 10% fetal bovine serum, 50 μg/ml streptomycin and 50 U/ml penicillin in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Routine tests for mycoplasma infection were negative.

RNA isolation

Tissues and cells, freshly harvested and immediately frozen in liquid nitrogen and stored at −80°C until processing, were dissolved in ISOGEN (Nippon Gene, Tokyo, Japan); we followed the manufacturer's protocol for total RNA extraction. The concentrations of RNA were determined spectrophotometrically; integrity was checked by gel electrophoresis. The RNA quality was confirmed in an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA).

miRNA expression signature and data normalization

Each pooled sample in the 156 panel assay was analyzed in duplicate. Analysis of relative miRNA expression data was performed using GeneSpring GX version 7.3.1 software (Agilent Technologies) according to the manufacturer's instruction. We employed 2 different approaches for normalizing the miRNA expression data, global normalization and endogenous gene (*ACTB*) normalization. Briefly, the Ct values were transformed by the following formula: expression score = 2^ (40 − Ct), and the calculated data were uploaded onto GeneSpring software. We chose the following methods for global normalization: (*i*) Per Chip, normalize to 50th percentile and (*ii*) Per Gene, normalize to median; and for *ACTB* normalization: (*i*) Per Gene, normalize to median and (*ii*) Per Gene, normalize to positive control (*ACTB*) genes. To prepare a low-level filtered gene list, before analysis, we excluded miRNAs with Ct values of 35 cycles. The low-level filtered gene list was further processed to select only those genes with a fold change >2.0 or <0.5 in comparison with normal samples; these were considered as differential expressed genes. Furthermore, a Welch analysis of variance test (parametric test, with variances not assumed equal, cutoff p value of 0.001), using the Benjamini and Hochberg method to control the false discovery rate, was performed on the list of differentially expressed genes to finally generate a list of statistically differential expressed miRNAs. TaqMan® probe and primers for *ACTB* were assay-on-demand gene expression products (P/N: Hs99999903_m1, Applied).

The unsupervised clustering analysis was performed on the statistically differential expressed miRNAs using the condition tree, gene tree options and the Pearson correlation equation (GeneSpring software, Agilent Technologies).

miRNAs signatures in BC determined by stem-loop RT-PCR

Stem-loop RT-PCR (TaqMan® MicroRNA Assays, Applied Biosystems, Foster City, CA) was used to quantitate miRNAs according to the previously published conditions.[22] To prepare cDNA specific to the miRNAs, each 15 μl RT reaction contained 10 ng of purified total RNA, 50 nM stem-loop RT primer, 1× RT buffer, 0.25 mM each of dNTPs, 3.33 U/μl MultiScribe™ reverse transcriptase and 0.25 U/μl RNase inhibitor. The reactions were incubated in a 96-well plate for 30 min at 16°C, 30 min at 42°C, followed by 5 min at 85°C and then held at 4°C. Each stem-loop RT-PCR for each miRNA assay was carried out in triplicate, and each 20 μl reaction mixture included 1.33 μl of diluted RT product, 10 μl of 2×TaqMan® Universal PCR Master Mix and 1 μl of 20×TaqMan® MicroRNA Assay Mix. The reaction was incubated in a 7900HT Fast Real-Time PCR System in 384-well plates at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. RNA *U6B* small nuclear (*RNU6B*) (42 bp, a small RNA) served as an endogenous control according to the manufacturer's primary data across several human tissues and cell lines (P/N: 4373381, Applied).

Real-time quantitative RT-PCR

We synthesized first-strand cDNA with 1 μg of total RNA using random primers of the reverse transcription (RT) system (Promega, Tokyo, Japan). The initial PCR step was a 10-min hold at 95°C; the cycles (n = 40) consisted of a 15-sec denaturation step at 95°C followed by 1-min annealing/extension at 63°C. The TaqMan® probe and primers for *KRT7* (P/N: Hs00818825_m1) were assay-on-demand gene expression products (Applied). All reactions were performed in triplicate, and a negative control lacking cDNA was included.

Algorithm for target genes

Our previous study has demonstrated the original method for target.[23] Because many miRNAs cause degradation of their target mRNAs, expression patterns of miRNAs and their targets can be expected to indicate reverse relationships. Hence, our method identifies mRNAs as targets that not only contain complementary sequences but also show inverse expression patterns to the miRNA. To predict target mRNAs for the downregulated miRNAs, we identified the upregulated mRNAs that showed a more

TABLE III – TWENTY-SEVEN DIFFERENTIALLY EXPRESSED miRNAS IN BLADDER CANCER AFTER GLOBAL NORMALIZATION

miRNAs	*p*-value	Cancer score	Normal score	Fold change (cancer/normal)	Status in BC
miR-145	3.47E-08	0.764	23.838	0.03	Down
miR-96	1.50E-05	1.832	0.072	25.44	Up
miR-190	1.50E-05	1.233	0.098	12.58	Up
miR-183	4.43E-05	2.287	0.056	40.84	Up
miR-30a-3p	4.43E-05	0.756	19.267	0.04	Down
miR-100	5.19E-05	0.600	11.261	0.05	Down
miR-150	5.52E-05	0.912	5.928	0.15	Down
miR-133a	5.95E-05	0.940	61.245	0.02	Down
miR-320	1.69E-04	0.629	1.800	0.35	Down
miR-133b	3.84E-04	0.728	23.482	0.03	Down
miR-151	3.84E-04	0.706	1.690	0.42	Down
miR-195	3.84E-04	0.494	7.207	0.07	Down
miR-125b	9.00E-04	0.540	11.658	0.05	Down
miR-152	9.50E-04	0.758	4.315	0.18	Down
miR-218	1.05E-03	0.553	3.900	0.14	Down
miR-199-s	1.11E-03	1.009	8.689	0.12	Down
miR-99a	1.11E-03	0.295	4.870	0.06	Down
*miR-199a**	1.93E-03	0.742	6.469	0.11	Down
miR-223	2.15E-03	1.006	11.276	0.09	Down
miR-139	2.72E-03	0.589	9.017	0.07	Down
miR-130b	5.60E-03	1.353	0.476	2.84	Up
miR-9	7.92E-03	0.731	8.935	0.08	Down
miR-124b	7.96E-03	1.825	0.053	34.43	Up
miR-215	8.34E-03	2.266	0.150	15.11	Up
miR-140	8.42E-03	0.684	5.052	0.14	Down
miR-224	8.89E-03	0.956	0.034	28.12	Up
miR-106a	9.50E-03	0.801	0.126	6.36	Up

Underlined miRNAs are commonly detected with global- and beta-actin normalization.

than 2.0-fold increase in expression level in BCs relative to NBEs in our corresponding expression data.[5] Subsequently, the targets including the complementary sites to the miRNAs in the 3′-UTRs were selected from the upregulated mRNAs using the miRanda target prediction tool.[24]

Mature miRNA transfection

Mature miRNA molecules, Pre-miR™ (Applied), were incubated with Lipofectamine™ RNAiMAX (Invitrogen, Tokyo, Japan) before plating. Subsequently, the complex was just added to suspended 4×10^4 cells per well at plating (cotransfecting) in 24-well plates. We first tested the transfection efficacy of miRNA precursors in BC cell lines based on the downregulation of *PTK9* mRNA by overexpression of miR-1 (This method was recommended by the manufacturer).

Small interfering RNA treatment and XTT assay

After cotransfecting small interfering RNA (siRNA)-KRT7 (si-KRT7) (L-019277-00; Dharmacon, Lafayette, CO) or nonsilencing siRNA (si-control) (D-001810-10; Dharmacon), KK47 cells were seeded in 96-well plates at 3×10^3 cells per well. After 48 hr, cell viability was determined by an XTT assay following the manufacturer's protocol (Roche Applied Sciences, Tokyo, Japan).

Immunoblot

After 48-hr cotransfecting, the cells were harvested, and total protein lysate was prepared with common detergent lysis buffer in the presence of a protease inhibitor. The 20 μg protein lysate was separated by NuPAGE® on 4–12% bis-tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane. Immunoblot was done with diluted (1:300) monoclonal cytokeratin7 antibody (clone OV-TL 12/30; No. 7018, Dako, Tokyo, Japan) and GAPDH antibody (MAB374; Chemicon, Temecula, CA). The membrane was washed and then incubated with goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA). Specific complexes were visualized with an echochemiluminescence detection system (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

The relationship between the 2 variables and numerical values obtained by real-time PCR-based experiments was analyzed with the Mann-Whitney U-test. The relationship between downregulated miRNA expression and *KRT7* mRNA expression was analyzed by the Spearman rank correlation. All statistical values were age-adjusted because the mean ages of the BCs and NBEs were statistically different. The analysis software was Expert StatView (version 4, SAS Institute, Cary, NC); differences of $p < 0.05$ were considered statistically significant. The cutoff scores for the analysis were obtained by receiver-operating characteristic (ROC) curve analysis using MedCalc software (MedCalc Software, Mariakerke, Belgium).

Results

Identification of differentially expressed miRNAs between BC, NBE and BC cell lines

We evaluated 156 mature miRNA expression levels of 14 BCs, 5 NBE specimens and 3 BC cell lines. After global normalization of the raw data, we identified 27 differentially expressed miRNAs between BCs and NBEs when we employed a cutoff *p* value of <0.01 to narrow down the candidates (Table III). Among these miRNAs, 8 were up- and 19 were downregulated in BCs relative to NBEs. When we applied a Bon-ferroni correction ($p < 0.00032$), 9 miRNAs were still differentially expressed between BCs and NBEs with a statistical significance (Table III). After global normalization, hierarchical clustering analysis based on differentially expressed miRNAs generated a tree with clear distinction between BCs, BC cells and NBEs (Fig. 1).

When we employed another normalization using the internal reference, *ACTB*, 10 miRNAs still had a *p* value of <0.05, and all were downregulated in BCs. Seven miRNAs were commonly detected with the 2 different approaches; *miR-145*, *miR-30a-3p*, *miR-133a*, *miR-133b*, *miR-195*, *miR-125b* and *miR-199a** (Table III, underlined).

Verification of downregulated miRNAs expression in clinical specimens

To validate the results of our screening test, we subjected 6 miRNAs (*miR-145*, *miR-30a-3p*, *miR-133a*, *miR-195*, *miR-125* and *miR-199a**), which were selected as downregulated miRNAs by both different normalization methods to stem-loop RT-PCR on BCs ($n = 104$) and NBEs ($n = 31$). We did not examine *miR-133b* because it was considered to function similar to *miR-133a*, and they have very similar sequences (*miR-133a*: UUGGUCCCCUUCAACCAGCUGU, *miR-133b*: UUGGUCCCCUUCAACCAGCUA). The 6 miRNAs shown by our signature to be downregulated did indeed have a lower expression in BCs than in NBEs (*miR-145*: 0.079 ± 0.019 (BC) *vs.* 0.897 ± 0.197 (NBE), $p < 0.0001$; *miR-30a-3p*: 0.145 ± 0.036 (BC) *vs.* 0.747 ± 0.095 (NBE), $p < 0.0001$; *miR-133a*: 0.048 ± 0.016 (BC) *vs.* 0.960 ± 0.196 (NBE), $p < 0.0001$; *miR-195*: 0.124 ± 0.012 (BC) *vs.* 0.688 ± 0.088 (NBE), $p < 0.0001$; *miR-125b*: 0.069 ± 0.013 (BC) *vs.* 0.939 ± 0.171 (NBE), $p < 0.0001$; *miR-199a**: 0.099 ± 0.015 (BC) *vs.* 1.043 ± 0.194 (NBE), $p < 0.0001$) (Fig. 2*a*). Our cohort showed no significant relationship between the miRNA expression and clinicopathological parameters.

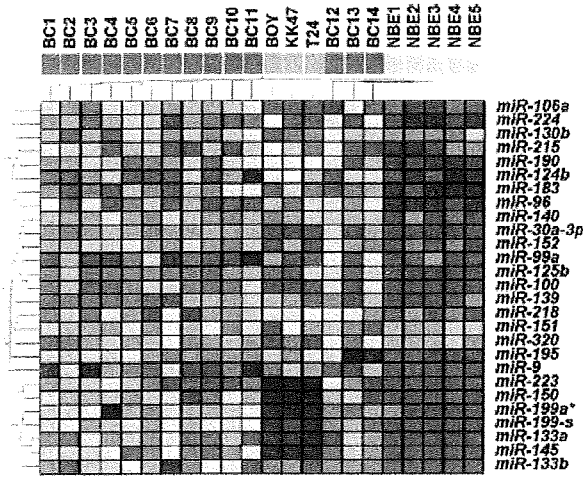

FIGURE 1 – Hierarchical clustering of BCs, NBEs and BC cells. 14 BCs, 5 NBEs and 3 BC cells were clustered according to the expression signature of 27 miRNAs differentially expressed between BCs and NBEs.

ROC curve analysis demonstrated that each miRNA had good sensitivity and specificity with optimal cutoff values as follows: *miR-145*: 90.5% (sensitivity), 77.4% (specificity), 0.173 (cutoff); *miR-30a-3p*: 94.3% (sensitivity), 76.7% (specificity), 0.310 (cutoff); *miR-133a*: 93.3% (sensitivity), 77.4% (specificity), 0.140 (cutoff); *miR-195*: 91.4% (sensitivity), 80.0% (specificity), 0.273 (cutoff); *miR-125b*: 89.4% (sensitivity), 77.4% (specificity), 0.206 (cutoff); *miR-199a**: 72.1% (sensitivity), 90.3% (specificity), 0.087 (cutoff). Each miRNA had a good area under curve (AUC) of >0.7 (Fig. 2*b*).

Identification of target genes of the downregulated miRNAs in our algorithm

We devised an original algorithm based on our BC mRNA profile to predict target genes for the miRNAs differentially expressed in BCs and NBEs. Table IV shows the predicted target genes for the downregulated miRNAs, which were sorted by number of predicted target sites. Interestingly, 12 genes were common targets among the 39 predicted target genes for the 6 miRNAs as follows: *KRT7* for all, *ALK* for *miR-145* and *miR-30a-3p*, *LOC400451* for *miR-145* and *miR-133a*, *PSMA7* for *miR-145* and *miR-133a*, *S100A14* for *miR-145* and *miR-125b*, *WWOX* for *miR-145* and *miR-125b*, *FLNB* for *miR-145*, *miR-30a-3p*, *miR-133a* and *miR-*

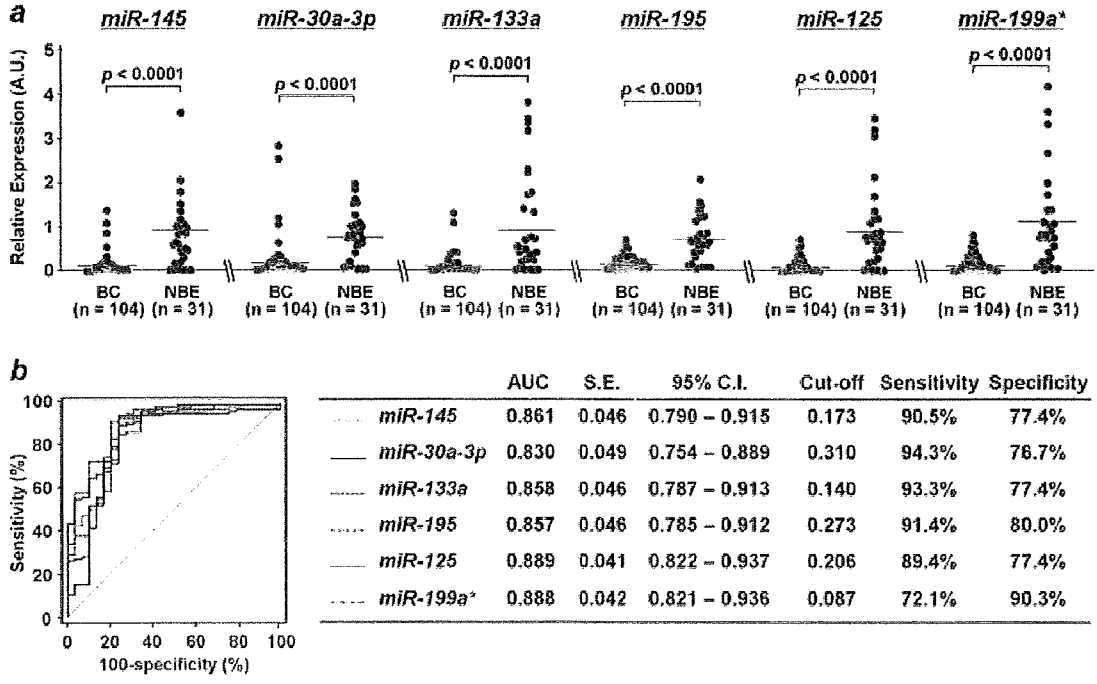

FIGURE 2 – (*a*) The 6 miRNAs that were commonly downregulated with 2 different normalizations in the screening test were significantly downregulated in BCs relative to NBEs ($p < 0.0001$). (*b*) ROC curve analysis showed each miRNA had good sensitivity and specificity to distinguish BC from NBE.

TABLE IV – ALGORITHM FOR PREDICTED TARGET GENES FOR THE DOWNREGULATED miRNAS IN BLADDER CANCER

miR-145			miR-30a-3p			miR-133a		
Unigene ID	Gene symbol	Target sites	Unigene ID	Gene symbol	Target sites	Unigene ID	Gene symbol	Target sites
Hs.411501	KRT7	17	Hs.445890	CNIH4	8	Hs.411501	KRT7	16
Hs.196534	ALK	10	Hs.196534	ALK	5	Hs.233952	PSMA7	7
Hs.27373	LOC400451	9	Hs.437656	LEPRE1	4	Hs.411573	GUCY1B2	7
Hs.233952	PSMA7	7	Hs.491912	COPS5	4	Hs.476448	FLNB	7
Hs.288998	S100A14	7	Hs.2233	CSF3	3	Hs.520967	MDH2	7
Hs.461453	WWOX	7	Hs.411501	KRT7	3	Hs.27373	LOC400451	6
Hs.259412	C1orf63	6	Hs.440961	CAST	3	Hs.533782	KRT8	6
Hs.476448	FLNB	6	Hs.476448	FLNB	3	Hs.129673	EIF4A1	4
Hs.2785	KRT17	5	Hs.504609	ID1	3	Hs.2233	CSF3	4
Hs.356794	RPS24	5	Hs.534169	HSPA14	3	Hs.27475	CYB5D1	4
	miR-195			miR-125b			miR-199a*	
Unigene ID	Gene symbol	Target sites	Unigene ID	Gene symbol	Target sites	Unigene ID	Gene symbol	Target sites
Hs.90093	HSPA4	1	Hs.411501	KRT7	12	Hs.90073	CSE1L	2
Hs.533782	KRT8	1	Hs.437656	LEPRE1	5	Hs.534169	HSPA14	2
Hs.529989	RNASET2	1	Hs.461453	WWOX	4	Hs.48029	SNAI1	2
Hs.524219	TPI1	1	Hs.288998	S100A14	3	Hs.445890	CNIH4	2
Hs.498397	SCCPDH	1	Hs.489287	CPSF4	3	Hs.356794	RPS24	2
Hs.490394	TAS2R5	1	Hs.495728	PIR	3	Hs.213470	PSMB7	2
Hs.487774	HNRPA2B1	1	Hs.519756	STK10	3	Hs.197071	NEK6	2
Hs.476448	FLNB	1				Hs.159918	PHF14	2
Hs.443831	PDCD5	1				Hs.80545	RPL37	1
Hs.411501	KRT7	1				Hs.411501	KRT7	1

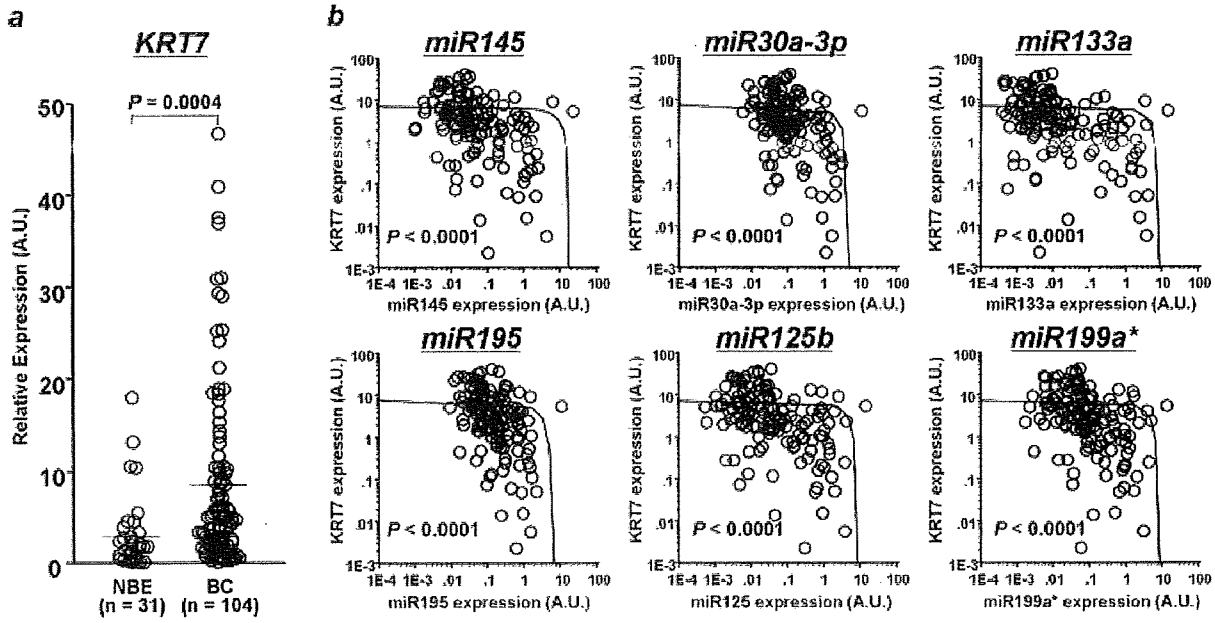

FIGURE 3 – (*a*) *KRT7* mRNA expression was significantly higher in BCs than in NBEs. (*b*) There were significant inverse correlations between *KRT7* mRNA expression and the downregulated miRNAs.

195, *CNIH4* for *miR-30a-3p* and *miR-199a**, *LEPRE1* for *miR-30a-3p* and *miR-125b*, *CSF3* for *miR-30a-3p* and *miR-133a*, *HSPA14* for *miR-30a-3p* and *miR-199a** and *KRT8* for *miR-133a* and *miR-195* (Table IV and Supporting Information Table SI).

KRT7 mRNA expression and its correlation with each miRNA expression

We focused on *KRT7* because it was the most frequent target of all miRNAs and was listed at the top in 3 of 6 miRNAs in our algorithm (Table IV, Supporting Information Table SI). Real-time RT-PCR revealed that the mRNA expression levels of *KRT7* were significantly higher in BCs than in NBEs (8.220 ± 0.973 (BC) *vs.* 3.119 ± 0.762 (NBE), $p = 0.0004$) (Fig. 3*a*). Spearman's rank correlation test indicated that there were significant inverse correlations between *KRT7* mRNA expression and the downregulated miRNAs (*miR-145*, *miR-30a-3p*, *miR-133a*, *miR-195*, *miR-125* and *miR-199a**) ($p < 0.0001$ for all miRNAs examined) (Fig. 3*b*).

Effects of miRNA transfection on KRT7 expression in BC cell lines

To determine whether *KRT7* expression is actually downregulated by miRNAs from our algorithm, we transfected the 6 miRNAs (mature miRNAs; Pre-miR™) to KK47 cells, which overwhelmingly expressed *KRT7* mRNA compared to T24 or BOY (Fig. 4*a*). After 24- or 48-hr transfection, *KRT7* mRNA expression

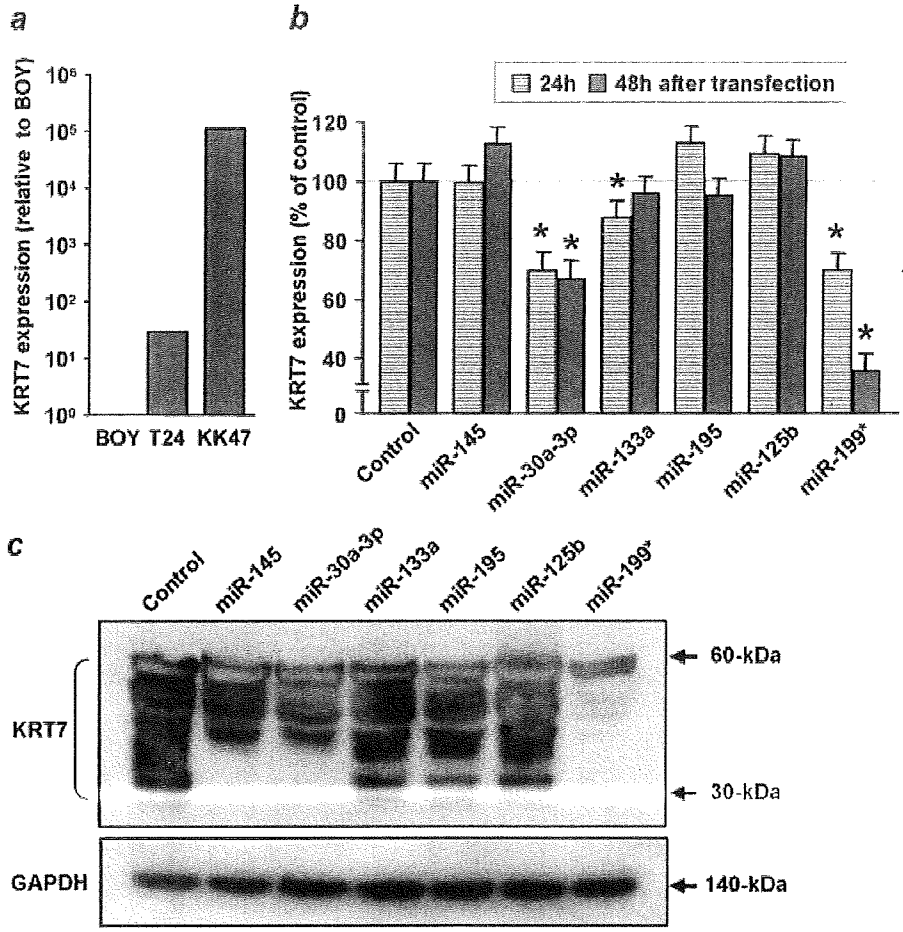

FIGURE 4 – (*a*) *KRT7* mRNA expression in 3 BC cells (BOY, T24 and KK47) by real-time RT-PCR. (*b*) *KRT7* mRNA expression after 24- and 48-hr transfection of miRNAs (*miR-145*, *miR-30a-3p*, *miR-133a*, *miR-195*, *miR-125* and *miR-199a**) (right). *$p < 0.05$. (*c*) Immunoblot revealed that various KRT7 isotypes were markedly decreased in *miR-199a** transfectant and slightly decreased in *miR-145*- and *miR-30a-3p* transfectant.

was significantly repressed ($p < 0.05$) by *miR-30a-3p*, *miR-133a* and *miR-199a** (Fig. 4*b*). Particularly, *miR-199a** transfection caused *KRT7* mRNA expression to decrease by 35% of the control (Fig. 4*b*). Immunoblot also showed that the protein expression of various KRT7 isotypes was markedly decreased in *miR-199a** transfection and slightly decreased in *miR-145*- and *miR-30a-3p* transfection (Fig. 4*c*).

Effects of si-KRT7 and miRNA transfection on the cell growth

We then determined whether *KRT7* and the 6 miRNAs contribute to cell viability. After 48-hr transfection, an XTT assay demonstrated that cell growth was significantly decreased in si-KRT7-transfected KK47 cells in comparison with the si-control-transfectant or moc (lipofectamine only) (80.3% of the moc, $p < 0.001$) (Fig. 5*a*). In addition, significant decreases in cell growth ($p < 0.05$) occurred in *miR-30-3p*-, *miR-133a*-, *miR-195*- and *miR-199a** transfectant (88, 86, 82 and 88% of the si-control-transfectant, respectively) (Fig. 5*b*).

Discussion

The screening test identified 27 miRNAs that were up- or downregulated in BCs differently from those in NBEs. Among them, 7 miRNAs (*miR-145*, *miR-30a-3p*, *miR-133a*, *miR-133b*, *miR-195*, *miR-125b* and *miR-199a**) were persistently remained as the downregulated miRNAs in BC after 2 different normalizations were applied. Stem-loop RT-PCR confirmed that these miRNAs were actually downregulated in 104 BCs when compared to 31 NBEs with a significant p value. Recent investigations detected several up- or downregulated miRNAs in breast cancer (*miR-145* and *miR-125b*), hepatocellular cancer (*miR-199a*, *miR-195* and *miR-224*) and colorectal cancer (*miR-145*, *miR-133a*, *miR-133b*, *miR-195*, *miR-125b*, *miR-199a*, *miR-96* and *miR-215*), which were identical to our results.[13,15,17] These miRNAs might be part of the underlying mechanism of carcinogenesis in various cancers. Recently, Gottardo *et al.* identified 10 miRNAs that were significantly upregulated in 25 BCs in comparison with only 2 NBEs, by using their custom oligonucleotide microchip for miRNA.[19] However, our study and theirs had no miRNAs in common, and they did not detect any miRNAs that were downregulated in BCs.[19] We found that both up- and downregulated miRNAs in BCs in the stem-loop RT-PCR experiment, which carries the advantages of accuracy, specificity and reproducibility for miRNA-detection.[22] ROC curve analysis demonstrated that the miRNAs showed a good AUC of >0.7 with good sensitivity and specificity (>70%) to distinguish BC from NBE when an appropriate cutoff value was employed. These results suggest that miRNA can be used as an

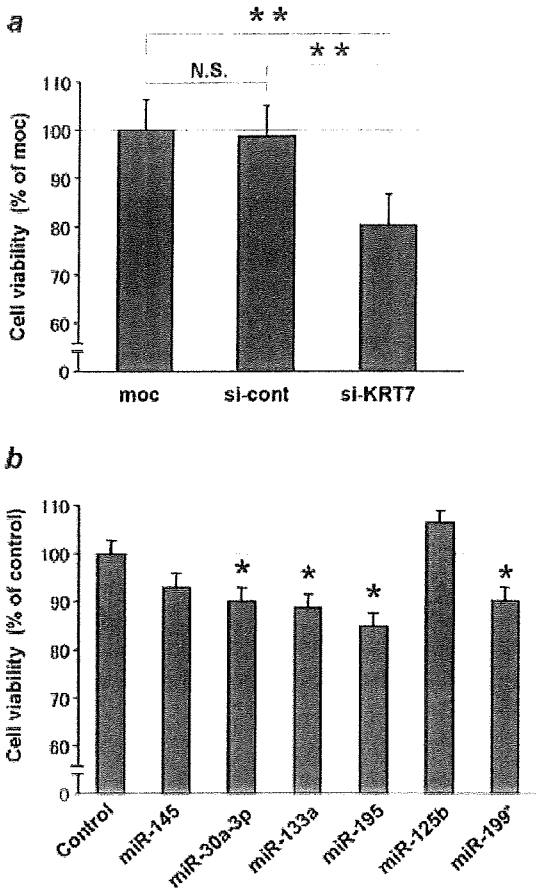

FIGURE 5 – (*a*) Effects of si-KRT7 transfection on the cell growth determined by XTT assay. si-KRT7-transfected KK47 cells exhibited a significant decrease in cell growth in comparison with si-control-transfectant or moc (lipofectamine only). (*b*) The cell growth was also significantly reduced in comparison with the control by transfection of the downregulated miRNAs (*miR-30a-3p*, *miR-133a*, *miR-195* and *miR-199a**). $*p < 0.05$, $**p < 0.001$.

outstanding diagnostic biomarker for BC if it could be detected in serum or urine samples. These ideas are still speculative, and studies of miRNAs as diagnostic biomarkers are now underway in our laboratory.

We devised an algorithm based on our BC mRNA profile[5] to predict target genes for the miRNAs differentially expressed in BCs and NBEs. Twelve genes were the common targets among the 39 predicted target genes, implying that one target gene is not regulated by one miRNA, and one miRNA regulates several target genes. Namely, there are one-to-many and many-to-one relationships between miRNAs and the target genes. To verify our algorithm's results, we focused on *KRT7*, which was the most frequent target of all miRNAs and was at the top of most miRNA lists. We found significant inverse correlations between *KRT7* mRNA expression and each miRNA expression in clinical BCs. Moreover, significant decreases in *KRT7* mRNA expression were observed in some miRNA transfectants (*miR-30a-3p*, *miR-133a* and *miR-199a**). Unexpectedly, *miR-145* transfection did not decrease the *KRT7* mRNA expression, regardless of the fact that *miR-145* has the most predicted targeting sites[17] on *KRT7*, while *miR-199a** has only one target site in our algorithm (Table IV, Supporting Information Table SI). Our data suggest that, for mRNA cleavage of the target genes, the specific region targeted by the corresponding miRNA may have an advantage over the number of the targeting sites. This hypothesis is consistent with a previous report demonstrating that an arrangement of miRNA recognition site through interaction with other miRNAs as well as the number of the targeting sites can influence the degree and specificity of miRNA-mediated gene repression.[25] Considering this phenomenon from another aspect, immunoblot revealed that *miR-145* transfection decreased small KRT7 isoformes (30–40 kDa). TaqMan® probes and primers for *KRT7* transcript (assay-on-demand, Applied) were designed to cover the last exon-junction (around 4,000 bp from 5′ region) but unfortunately, uncovered small transcript variants (for example, accession No: BC107082.1 or BC107083.1), suggesting that *miR-145* may target these small transcript variants of *KRT7*. Thus, our algorithm is incomplete, and some targets might be regulated by secondary effects of the miRNAs. However, the algorithm could provide novel candidates of miRNA targets based on miRNA signatures in human BC. In our study, miR-195 transfectant showed significant cell growth inhibition, even though it had no effect on KRT7 expression in the BC cell line. Other targets of *miR-195* may be involved in cell growth inhibition. Further investigations are necessary to validate the interactions between the miRNAs and predicting miRNA-targeted genes.

KRT7 is a member of the cytokeratin gene family and encodes type II cytokeratin, which is a useful biomarker for detecting BC or making a differential diagnosis of the origin of a tumor.[26] However, the functional role of *KRT7* in cancer development has not been elucidated. In our study, cell proliferation assays showed that a significant decrease in cell growth occurred in si-KRT7-transfectants in comparison with the controls. This result suggests that the *KRT7* gene may have an oncogenic function in human BC and that these miRNAs may play a role of tumor suppressive function. In our cohort, there was no significant relationship between the miRNA expressions and clinicopathological parameters including tumor stage, tumor grade or patient's prognosis. Altered expression of the miRNAs is a frequent event, but it may function in the early step of BC carcinogenesis.

Ours is the first study demonstrating that *miR-30-3p*, *miR-133a* and *miR-199a** have a tumor suppressive function in human BC. These miRNAs from our algorithm lead us to a new strategy to find novel oncogenes, such as *KRT7*, and we believe that they are promising candidates for biomarkers and gene therapy of human BC.

Acknowledgements

The authors thank Ms. M. Miyazaki for her excellent laboratory assistance.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008;58:71–96.
2. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
3. Schulz WA. Understanding urothelial carcinoma through cancer pathways. Int J Cancer 2006;119:1513–8.
4. Mitra AP, Datar RH, Cote RJ. Molecular pathways in invasive bladder cancer: new insights into mechanisms, progression, and target identification. J Clin Oncol 2006;24:5552–64.
5. Kawakami K, Enokida H, Tachiwada T, Gotanda T, Tsuneyoshi K, Kubo H, Nishiyama K, Takiguchi M, Nakagawa M, Seki N. Identification of differentially expressed genes in human bladder cancer through genome-wide gene expression profiling. Oncol Rep 2006;16:521–31.
6. Kawakami K, Enokida H, Tachiwada T, Nishiyama K, Seki N, Nakagawa M. Increased SKP2 and CKS1 gene expression contributes to the progression of human urothelial carcinoma. J Urol 2007;178:301–7.

7. Enokida H, Nakagawa M. Epigenetics in bladder cancer. Int J Clin Oncol 2008;13:298–307.
8. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001; 294:853–8.
9. Huang JC, Babak T, Corson TW, Chua G, Khan S, Gallie BL, Hughes TR, Blencowe BJ, Frey BJ, Morris QD. Using expression profiling data to identify human microRNA targets. Nature Methods 2007;4: 1045–9.
10. Wu W, Sun M, Zou GM, Chen J. MicroRNA and cancer: current status and prospective. Int J Cancer 2007;120:953–60.
11. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, et al. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA 2002;99:15524–9.
12. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 2004;64:3753–6.
13. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Ménard S, Palazzo JP, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005;65:7065–70.
14. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005;65:6029–33.
15. Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 2006;25:2537–45.
16. He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S, Kloos RT, Croce CM, et al. The role of microRNA genes in papillary thyroid carcinoma. Proc Natl Acad Sci USA 2005;102:19075–80.
17. Bandrés E, Cubedo E, Agirre X, Malumbres R, Zárate R, Ramirez N, Abajo A, Navarro A, Moreno I, Monzó M, García-Foncillas J. Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. Mol Cancer 2006;5:29.
18. Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer 2007;120:1046–54.
19. Gottardo F, Liu CG, Ferracin M, Calin GA, Fassan M, Bassi P, Sevignani C, Byrne D, Negrini M, Pagano F, Gomella LG, Croce CM, et al. Micro-RNA profiling in kidney and bladder cancers. Urol Oncol 2007;25:387–92.
20. Sobin LH, Wittekind C. TNM Classification of Malignant Tumours, 6th ed. International Union Against Cancer (UICC). New York: Wiley-Liss, 2002. 199–202.
21. Takemoto M, Shirahama T, Miyauchi T, Matsusako T, Kaneda N, Muramatsu H, Ozawa M, Ohi Y, Muramatsu T. Metanestin, a glycoprotein with metastasis-associated expression in transitional cell carcinoma of the urinary bladder. Int J Cancer 1997;74:7–14.
22. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res 2005;33:e179.
23. Ruike Y, Ichimura A, Tsuchiya S, Shimizu K, Kunimoto R, Okuno Y, Tsujimoto G. Global correlation analysis for micro-RNA and mRNA expression profiles in human cell lines. J Hum Genet 2008;53:515–23.
24. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in Drosophila. Genome Biol 2003;5:R1.
25. Hon LS, Zhang Z. The roles of binding site arrangement and combinatorial targeting in microRNA repression of gene expression. Genome Biol 2007;8:R166.
26. Schroeder GL, Lorenzo-Gomez MF, Hautmann SH, Friedrich MG, Ekici S, Huland H, Lokeshwar V. A side by side comparison of cytology and biomarkers for bladder cancer detection. J Urol 2004;172: 1123–6.

Japanese Journal of Clinical Oncology Advance Access published December 3, 2009

Jpn J Clin Oncol 2009
doi:10.1093/jjco/hyp157

Original Article

Clinical Safety and Feasibility of a Newly Developed, Simple Algorithm for Decision-making on Neurovascular Bundle Preservation in Radical Prostatectomy

Kohei Hashimoto[1], Shin-ichi Hisasue[1], Naoya Masumori[1]*, Ko Kobayashi[1], Ryuichi Kato[1], Fumimasa Fukuta[1], Atsushi Takahashi[2], Tadashi Hasegawa[3] and Taiji Tsukamoto[1]

[1]Department of Urology, Sapporo Medical University School of Medicine, [2]Department of Urology, Hakodate Goryokaku Hospital, and [3]Department of Surgical Pathology, Sapporo Medical University School of Medicine, Hokkaido, Japan

* For reprints and all correspondence: Naoya Masumori, Department of Urology, Sapporo Medical University School of Medicine, S1, W16, Chuo-ku, Sapporo, Hokkaido 060-8543, Japan. E-mail: masumori@sapmed.ac.jp

Received July 21, 2009; accepted October 28, 2009

Objective: We investigated the clinical safety and feasibility of an algorithm we developed for the decision-making on neurovascular bundle preservation in radical prostatectomy to decrease the incidence of positive surgical margins.
Methods: We prospectively applied our algorithm to 82 patients (164 prostate sides) with clinically localized prostate cancer who underwent radical prostatectomy at our institution between October 2004 and September 2006. The algorithm was developed using the apical core characteristics, clinical T stage, preoperative prostate-specific antigen level and Gleason sum. All prostate sides were divided into two groups by the algorithm: 115 sides (70.1%) were qualified for neurovascular bundle preservation (favorable algorithm side group) and 49 sides (29.9%) for non-neurovascular bundle preservation (unfavorable algorithm side group).
Results: Median patient age was 66 years (range: 52–77) and median prostate-specific antigen was 7.1 ng/ml (range: 1.4–29.6). Overall, a positive surgical margin was observed in 23 sides (14.0%). The incidence of positive surgical margins at the apex was significantly correlated with the maximal diameter of the tumor in the apex ($P < 0.001$). The incidence of positive surgical margins was 8.7% in the favorable algorithm group, whereas it was 26.5% in the unfavorable algorithm group ($P = 0.003$). When this algorithm was combined with surgeons' intraoperative assessments, the incidence of positive surgical margins was 2.1% in neurovascular bundle preservation sides, compared with 25.0% in non-neurovascular bundle preservation sides ($P = 0.002$).
Conclusions: This simple algorithm is safe and feasible for the decision-making on neurovascular bundle preservation from the aspect of cancer control in radical prostatectomy patients.

Key words: radical prostatectomy – nerve sparing – indication

INTRODUCTION

Nerve-sparing radical prostatectomy (RP) is an important procedure for the recovery of the erectile function after surgery, which leads to the improvement in the quality of life of patients with organ-confined prostate cancer (1). However, it is still controversial whether this procedure compromises surgical margin (SM) status. The neurovascular bundles (NVBs) are located outside of the prostatic capsule,

and positive SMs can occur as a result of either incising into prostate cancer that extends beyond the prostatic capsule or the anterior side of the apex lacking the prostate capsule or incising into an intracapsular cancer via an inadequate procedure (2). Many studies have indicated that a positive SM is an independent risk factor for disease progression and mortality (2–4). Therefore, NVBs should be preserved appropriately without compromising SM status, and the decision for NVB preservation should be made carefully.

In this study, we validated an algorithm we developed as a decision-making tool for NVB preservation in RP to confirm its clinical safety and feasibility for SM status.

PATIENTS AND METHOD

Between October 2004 and September 2006, 95 patients with clinically localized prostate cancer underwent RP at our institution. A total of 13 patients (11 with neoadjuvant hormonal treatment and 2 with an insufficient total number of biopsy cores of less than 6) were excluded from the study. Finally, 82 patients were enrolled in this study.

All patients had serum prostate-specific antigen (PSA) determination (ECLusys PSA II assay) before prostate biopsy. Systematic prostate biopsy was performed by taking 14 cores from each of 16 patients in our institution, and a median of 8 (range: 6–12) biopsy cores in 66 patients who underwent biopsy in other clinics. Clinical stage was determined by digital rectal examination, transrectal ultrasound, abdominal computed tomography, chest X-ray and bone scanning. Magnetic resonance imaging was performed when prostatic capsular penetration was suspected. The preoperative erectile function was evaluated by the International Index of Erectile Function Questionnaire (IIEF5) and the penile circumferential change by nocturnal penile tumescence (5).

RP was done through a standard retropubic approach with limited pelvic lymph node dissection. The nerve-sparing procedure was performed as described by Walsh (6). An optical loupe was not routinely used during the operation in the series of this study. Fourteen surgeons and 7 staff members participated in the radical prostatectomies in this academic-institution setting.

The algorithm for the decision-making on NVB preservation consisted of four elements, including the clinical T stage, preoperative PSA, Gleason sum on biopsy and cores in the apex (Fig. 1a and b). These parameters have been previously reported to predict SM status (2,7–12). If 6–10 biopsy cores incorporating a sextant biopsy and 1–4 lateral sides were obtained, a core in the apex was defined as a distal core on sextant biopsy (Fig. 1a). If 12 biopsy cores incorporating a sextant biopsy and 6 lateral sides were obtained, a core in the apex was defined as a distal or lateral-distal core. If apical anterior core biopsy was performed, a positive core in the apex was defined as an apical anterior core in addition to a distal and lateral-distal core. Of all the prostate sides, 32 (19.5%) were in the 6-core pattern, 54 (32.9%) in the 8-core pattern, 44 (26.9%) in the 10-core pattern, 2 (1.2%) in the 12-core and 32 (19.5%) in the 14-core and apical anterior core pattern. The cutoff values for the other parameters were 10 ng/ml for PSA and 7 for the Gleason sum on biopsy.

The algorithm we used in this study is shown in Fig. 1b. In this algorithm, nerve-sparing RP was not offered to patients with T2c because it has been shown to independently predict the likelihood of a positive SM (2,13). In patients with T1c, T2a or T2b, the presence of a positive core in the apex was evaluated for each prostate side. If a core in the apex was negative, or positive with a preoperative PSA level of <10 ng/ml and Gleason sum of 6 or less, the NVB on that side was preserved. The decision-making for NVB preservation was generally done based on this algorithm for all sides. However, in some cases, the NVB was resected due to the patient's preference or surgeons' assessment of cancer biology; for example, a positive core rate on biopsy, or palpable induration or adhesion of the prostate during the operation.

Both biopsy specimens and prostatectomy specimens were histologically examined by several pathologists. All biopsy specimens from other clinics were reviewed by pathologists in our institution. The prostatectomy specimens were horizontally sectioned at 5 mm intervals. In addition, apical specimens were longitudinally sectioned at 5 mm intervals. Stage and histological grade were assigned using the 2002 UICC-American Joint Committee on Cancer TNM system and the Gleason system, respectively. A positive SM was defined as cancer tissue present at the inked specimen edge. Extraprostatic extension (EPE) was defined as cancer extension over the prostatic capsule. If the NVB was preserved, EPE in the NVB was defined as EPE in the posterolateral region of the prostate. A tumor in the apex was defined as a tumor in a 5 mm section of the apex in a prostatectomy specimen, and the maximal diameter of the tumor in the apex as the long diameter of the tumor estimated from all sections of the apex.

All prostate sides were divided into two groups by the algorithm: the favorable algorithm side group qualified for NVB preservation, and the unfavorable algorithm side group for non-NVB preservation. The chi-square test, Fisher's exact test and Mann–Whitney *U*-test were carried out to compare various clinical and pathological parameters between the groups. A P value of <0.05 was considered to be statistically significant. We used the computer program StatView 5.0 for Windows (SAS Institute, Cary, NC, USA) for statistical analyses.

RESULTS

The characteristics of patients are shown in Table 1. Of the 82 patients, bilateral NVB preservation was done for 47 (57.3%), unilateral NVB preservation for 21 (25.6%) and non-NVB preservation for 14 (17.1%) according to our

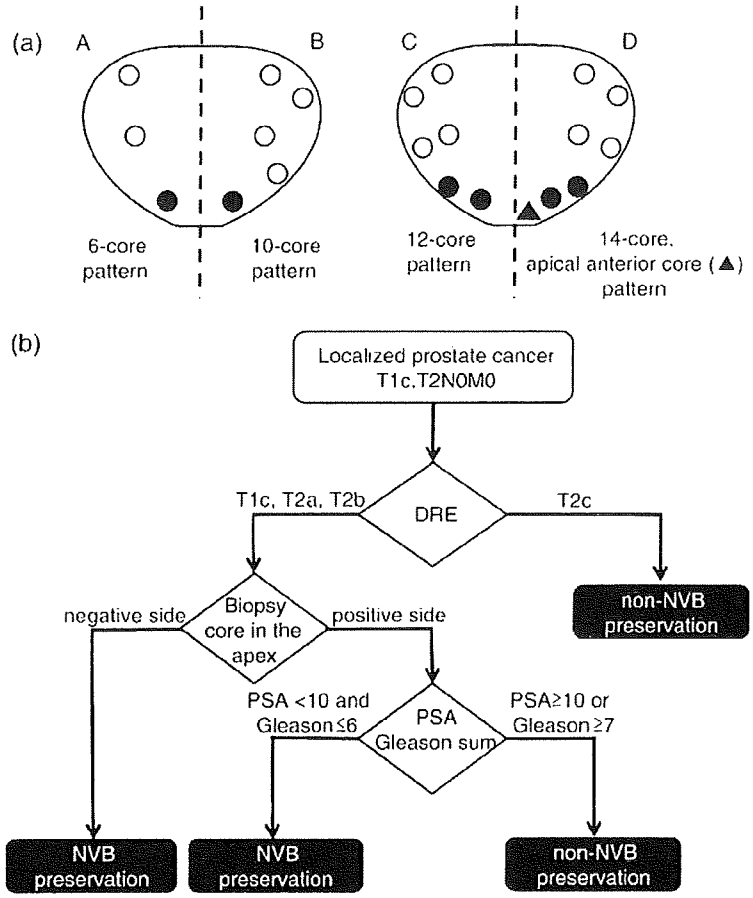

Figure 1. (a) Schematic drawing of a 6–14 core (circles and triangle) biopsy site on the posterior surface of the unilateral prostate. A core in the apex is drawn as a black circle or black triangle. If 6–10 biopsy cores (A and B) incorporating sextant biopsy and 1–4 lateral sides were obtained, a core in the apex was defined as a distal core on sextant biopsy. If 12 biopsy cores (C) incorporating a sextant biopsy and 6 lateral sides were obtained, a core in the apex was defined as a distal and lateral-distal core. If apical anterior core biopsy (D) was performed with 14 biopsy cores, a core in the apex was defined as an apical anterior core (black triangle) in addition to a distal and lateral-distal core. (b) The algorithm for the decision-making on neurovascular bundle (NVB) preservation. First, the clinical stage was determined by digital rectal examination (DRE). If the clinical stage was T2c, bilateral NVB were not preserved. Next, the presence of a positive core in the apex was evaluated in each prostate side. If cores in the apex were negative, ipsilateral NVB were preserved. If a core in the apex was positive with a PSA level of <10 ng/ml and a Gleason sum of 6 or less, ipsilateral NVB were preserved.

algorithm. Of all the prostates sides, 115 (70.1%) were in the favorable algorithm side group and 49 (29.9%) in the unfavorable algorithm side group.

The incidence of pathologically confirmed organ-confined disease (OCD) was significantly higher in the favorable algorithm side group than in the unfavorable algorithm side group (91.3% vs. 57.1%, respectively, $P < 0.001$) (Table 2). A tumor in the apex was observed in 79 sides (48.2%), including 47 sides of the favorable algorithm side group and 32 sides of the unfavorable algorithm side group. The median maximal diameter of the tumor in the apex was 5.0 mm (range: 1.0–17.0) in the favorable algorithm side group compared with 7.0 mm (range: 2.0–20.0) in the unfavorable algorithm side group ($P = 0.019$).

Overall, positive SMs were observed in 23 sides (14.0%), with 11 sides at the apex. The rate was 6.0% in sides with pathologically confirmed OCD, but increased to 48.4% in those with non-OCD ($P < 0.001$). The incidence of positive SMs at the apex was correlated with the maximal diameter of the tumor in the apex (Fig. 2). The overall incidence of positive SMs was significantly lower in the favorable algorithm side group than in the unfavorable algorithm side group (8.7% vs. 26.5%, $P = 0.003$) (Table 3). In the favorable algorithm side group, 48 sides (41.7%) actually had NVB preservation, and 32 sides (27.8%) had non-NVB preservation based on the surgeons' assessments of biological risk. In these 32 sides, the positive core rate was significantly higher than that of the other sides in the favorable algorithm side group (median: 0% vs. 31.0%, $P < 0.001$). When this algorithm was combined with the surgeons' assessments, a positive SM and EPE were observed in one side (2.1%) and zero in NVB-preservation sides, compared with eight (25.0%) and four (12.5%) non-NVB preservation sides, respectively ($P = 0.002$, $P = 0.022$). Neither a positive SM nor EPE in NVB was observed in NVB sides preserved according to the algorithm.

Table 1. Clinical characteristics of all patients

Number of patients	82
Median age (range)	66 (52–77)
Median preoperative PSA (range) (ng/ml)	7.1 (1.4–29.6)
Clinical stage (%)	
T1c	52 (63.4)
T2a	19 (23.2)
T2b	7 (8.5)
T2c	4 (4.9)
Gleason sum on biopsy (%)	
≤6	36 (43.9)
7	32 (39.0)
≥8	14 (17.1)
Median number of total biopsy cores (range)	8 (6–14)
Median positive core rate (range) (%)	23.2 (7.1–100)
Positive core in apex (%)	
No	33 (40.2)
Yes	49 (59.8)
Median IIEF5 score (range)	13 (0–25)
Median penile circumferential change (range) (mm)	30 (5–50)
Indication of nerve-sparing RP (%)	
Bilateral nerve sparing	47 (57.3)
Ipsilateral nerve sparing	21 (25.6)
Bilateral non-nerve sparing	14 (17.1)

IIEF5, International Index of Erectile Function Questionnaire 5; RP, radical prostatectomy; PSA, prostate-specific antigen.

Table 2. Pathological features in sides according to the algorithm

	Favorable algorithm side group ($n = 115$)	Unfavorable algorithm side group ($n = 49$)	P value
Gleason sum on RP (%)			0.018[a]
≤6	25 (21.7)	3 (6.1)	
7	76 (66.1)	34 (69.4)	
≥8	14 (12.2)	12 (24.5)	
Organ-confined disease (%)	105 (91.3)	28 (57.1)	<0.001[b]
Extraprostatic extension (%)	6 (5.2)	19 (38.8)	<0.001[b]
Seminal vesicle involvement (%)	6 (5.2)	6 (12.2)	0.129[b]
Median maximal diameter of tumor in apex[c] (mm)	5.0 (1.0–17.0)	7.0 (2.0–20.0)	0.019[a]

[a]Mann–Whitney U-test.
[b]Chi-square test.
[c]Tumor in apex appeared in 79 sides.

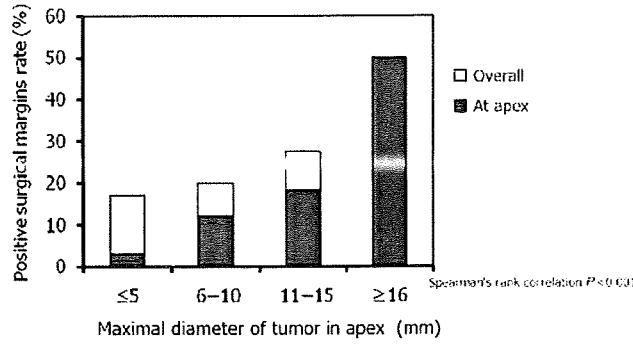

Figure 2. The relationship between surgical margin (SM) status and maximal diameter of the tumor in the apex. The incidence of positive SMs related to the maximal diameter of the tumor in the apex ($P < 0.001$). In addition, the incidence of positive SMs at the apex increased with tumor extension in the apex ($P < 0.001$).

DISCUSSION

NVB preservation is mandatory to maintain the erectile function of patients with RP (1). The rate of recovery of the postoperative erectile function, although depending on age, is higher in patients with bilateral NVB preservation than in those with unilateral preservation (14). Our previous study revealed that the efficacy of phosphodiesterase type 5 inhibitors also depended on NVB preservation (15). A recent study suggested that bilateral NVB preservation could also contribute to early recovery of urinary continence after RP (16). Therefore, it is important to increase the number of candidates for nerve-sparing RP with respect to quality of life, as long as it does not compromise SM status.

Who is an ideal candidate for nerve-sparing RP? The criteria to select candidates may require decreasing the incidence of positive SMs and increasing the proportion of NVBs preserved (9). The incidence of positive SMs has been reported to range from 2.5% to 34% in nerve-sparing RP based on various criteria (7–10,17,18). Several preoperative parameters, including the clinical T stage, PSA value, Gleason sum, number of positive biopsy cores and tumor length of a single core, are thought to be factors that affect the SM status (2,7,9–12). The incidence of positive SMs is known increase depending on the tumor extension (2,7–11). Several studies have shown that EPE commonly occurs in the posterolateral region near the NVB (2,10). This indicates that the majority of cancer with EPE is likely to have a risk of a positive SM with the nerve-sparing procedure. Walsh (17) reported that it was possible to preserve NVBs in all patients with OCD without compromising SM status. Therefore, the selection criteria for NVB preservation need to predict OCD by using preoperative factors that affect SM status. Moreover, this assessment should be performed for each prostate side separately in order to maximize the proportion of NVBs preserved (9–11).

However, it is notable that positive SMs occur in 2.5–27% of cases despite the presence of OCD (7–11,17). The apex, which lacks the prostate capsule, is the most common site of positive SMs (2,4,7,8). The present study revealed

Table 3. Incidence and location of EPE and positive SMs in sides according to the algorithm

NVB preservation	Number of sides	EPE (%)		Positive SM (%)		
		Overall	NVB	Overall	NVB	Apex
Favorable algorithm side group	115	6 (5.2)	3 (2.6)	10 (8.7)	2 (1.7)	4 (3.5)
NVB preservation according to algorithm	48	0	0	1 (2.1)	0	1 (2.1)
Non-NVB preservation by patient's preference	35	2 (5.7)	0	1 (2.9)	0	0
Non-NVB preservation by surgeons' assessments	32	4 (12.5)	3 (9.4)	8 (25.0)	2 (6.3)	3 (9.4)
Unfavorable algorithm side group	49	19 (38.8)	14 (28.6)	13 (26.5)	1 (2.0)	7 (14.3)

NVB, neurovascular bundle; EPE, extraprostatic extension; SM, surgical margin.

that the maximal diameter of a tumor in the apex was related to the incidence of positive SMs at the apex. The short length of the inferior pedicle allows a tumor in the apex to spread more easily into the perineural spaces (2). These findings suggested that predicting the extension of a tumor in the apex could contribute to decreasing the incidence of positive SMs in nerve-sparing RP. Moreover, it was reported that careful excision at the apex could improve the recovery of the erectile function (19). Therefore, evaluation of the tumor extension in the apex may be important for the decision-making on NVB preservation.

We developed an algorithm that aimed to decrease the incidence of positive SMs by predicting OCD and tumor extension in the apex for each prostate side. This is a simple algorithm mainly based on biopsy core features in the apex. Of all sides, 115 (70.1%) were qualified for NVB preservation according to the algorithm. The present study also confirmed that our algorithm could predict OCD and tumor extension in the apex. As a result, the incidence of positive SMs was only 2.1% when NVBs were preserved according to the algorithm. Similar to our results, several studies have shown that algorithms using risk factors for positive SMs decrease the incidence of positive SMs and increase the proportion of NVBs preserved (9,10). Shah et al. (9) developed an algorithm based on the Gleason score, percent tumor volume and perineural invasion in the biopsy specimen. Of 526 sides with clinically localized prostate cancer, 483 sides (91.8%) were qualified for NVB preservation. The incidence of positive SMs was 4.6%. Likewise, Kamat et al. (10) reported an algorithm including tumor length in the biopsy core and the location of positive cores. Their algorithm led to NVB preservation in 502 (93.3%) of 538 sides with clinically localized prostate cancer, which resulted in decreasing the incidence of positive SMs (2.5%).

In the present study, at 32 sides of the favorable algorithm side group NVBs were resected based on the surgeons' assessments contrary to the algorithm. This was associated with the positive core rate. These sides had a high incidence of positive SMs similar to that of the unfavorable algorithm side group. In addition, three sides (9.4%) had EPE in NVBs, implying a risk of positive SMs (12). If NVB were preserved in these sides, there might likely be an increase in the incidence of positive SMs. Thus, intraoperative assessment of EPE by surgeons should not be ignored in the decision-making on NVB preservation, as reported by Walsh (17). Our algorithm and the surgeons' assessments led to prevention of an increase in positive SMs in the nerve-sparing procedure. We propose that this algorithm and the surgeons' intraoperative assessments are equally important to prevent positive SMs.

We prospectively investigated the usefulness of the algorithm for the decision-making on NVB preservation before RP. However, the present study had some limitations. The total number of biopsy cores and the locations of biopsy cores varied because the majority of patients underwent prostate biopsy in other clinics. In our academic training-institution setting, multiple surgeons and pathologists were involved. The proportion of NVBs qualified for preservation was lower with our algorithm than with others. In addition, only 48 (41.7%) of all sides in the favorable algorithm side group actually had NVB preservation. However, the present algorithm was simple and safe from the aspect of cancer control. Furthermore, it became a more powerful tool when combined with surgeons' assessments. In a future study, we need to investigate other aspects of the usefulness of this algorithm such as the postoperative erectile function and prognosis.

CONCLUSIONS

We developed a simple algorithm for decision-making on NVB preservation based on the apical core characteristics, clinical T stage, preoperative PSA level and Gleason sum. NVB preservation was achieved without affecting SM status by using this algorithm. This algorithm is safe and feasible for decision-making on NVB preservation from the aspect of cancer-control in RP patients.

Conflict of interest statement

None declared.

References

1. Rabbani F, Stapleton AM, Kattan MW, Wheeler TM, Scardino PT. Factors predicting recovery of erections after radical prostatectomy. *J Urol* 2000;164:1929–34.
2. Wieder JA, Soloway MS. Incidence, etiology, location, prevention and treatment of positive surgical margins after radical prostatectomy for prostate cancer. *J Urol* 1998;160:299–315.
3. Karakiewicz PI, Eastham JA, Graefen M, Cagiannos I, Stricker PD, Klein E, et al. Prognostic impact of positive surgical margins in surgically treated prostate cancer: multi-institutional assessment of 5831 patients. *Urology* 2005;66:1245–50.
4. Hashimoto K, Masumori N, Takei F, Fukuta F, Takahashi A, Itoh N, et al. Prognostic value of surgical margin status for biochemical recurrence following radical prostatectomy. *Jpn J Clin Oncol* 2008;38:31–5.
5. Suzuki K, Sato Y, Horita H, Adachi H, Kato R, Hisasue S, et al. The correlation between penile tumescence measured by the erectometer and penile rigidity by the RigiScan. *Int J Urol* 2001;8:594–8.
6. Walsh PC. Anatomic radical prostatectomy: evolution of the surgical technique. *J Urol* 1998;160:2418–24.
7. Ward JF, Zincke H, Bergstralh EJ, Slezak JM, Myers RP, Blute ML. The impact of surgical approach (nerve bundle preservation versus wide local excision) on surgical margins and biochemical recurrence following radical prostatectomy. *J Urol* 2004;172:1328–32.
8. Palisaar RJ, Noldus J, Graefen M, Erbersdobler A, Haese A, Huland H. Influence of nerve-sparing (NS) procedure during radical prostatectomy (RP) on margin status and biochemical failure. *Eur Urol* 2005;47: 176–84.
9. Shah O, Robbins DA, Melamed J, Lepor H. The New York University nerve sparing algorithm decreases the rate of positive surgical margins following radical retropubic prostatectomy. *J Urol* 2003;169: 2147–52.
10. Kamat AM, Jacobsohn KM, Troncoso P, Shen Y, Wen S, Babaian RJ. Validation of criteria used to predict extraprostatic cancer extension: a tool for use in selecting patients for nerve sparing radical prostatectomy. *J Urol* 2005;174:1262–5.
11. Graefen M, Haese A, Pichlmeier U, Hammerer PG, Noldus J, Butz K, et al. A validated strategy for side specific prediction of organ confined prostate cancer: a tool to select for nerve-sparing radical prostatectomy. *J Urol* 2001;165:857–63.
12. Tsuzuki T, Hernandez DJ, Aydin H, Trock B, Walsh PC, Epstein JI. Prediction of extraprostatic extension in the neurovascular bundle based on prostate needle biopsy pathology, serum prostate specific antigen and digital rectal examination. *J Urol* 2005;173:450–3.
13. Swindle P, Eastham JA, Ohori M, Kattan MW, Wheeler T, Maru N, et al. Do margins matter? The prognostic significance of positive surgical margins in radical prostatectomy specimens. *J Urol* 2005;174:903–7.
14. Miyao N, Adachi H, Sato Y, Horita H, Takahashi A, Masumori N, et al. Recovery of sexual function after nerve-sparing radical prostatectomy or cystectomy. *Int J Urol* 2001;8:158–64.
15. Shimizu T, Hisasue S, Sato Y, Kato R, Kobayashi K, Tsukamoto T. Erectile dysfunction following nerve-sparing radical retropubic prostatectomy and its treatment with sildenafil. *Int J Urol* 2005;12: 552–7.
16. Kaiho Y, Nakagawa H, Ikeda Y, Namiki S, Numahata K, Satoh M, et al. Intraoperative electrophysiological confirmation of urinary continence after radical prostatectomy. *J Urol* 2005;173:1139–42.
17. Walsh PC. Radical prostatectomy, preservation of sexual function, cancer control. The controversy. *Urol Clin North Am* 1987;14: 663–73.
18. Hernandez DJ, Epstein JI, Trock BJ, Tsuzuki T, Carter HB, Walsh PC. Radical retropubic prostatectomy. How often do experienced surgeons have positive surgical margins when there is extraprostatic extension in the region of the neurovascular bundle? *J Urol* 2005;173:446–9.
19. Chuang MS, O'Connor RC, Laven BA, Orvieto MA, Brendler CB. Early release of the neurovascular bundles and optical loupe magnification lead to improved and earlier return of potency following radical retropubic prostatectomy. *J Urol* 2005;173:537–9.

PCAN Open

Prostate Cancer and Prostatic Diseases (2009) 12, 247–252

1365-7852/09 $32.00
www.nature.com/pcan

ORIGINAL ARTICLE

Isoflavone supplements stimulated the production of serum equol and decreased the serum dihydrotestosterone levels in healthy male volunteers

M Tanaka[1], K Fujimoto[1], Y Chihara[1], K Torimoto[1], T Yoneda[1], N Tanaka[1], A Hirayama[1], N Miyanaga[2], H Akaza[2] and Y Hirao[1]

[1]*Department of Urology, Nara Medical University, Kashihara, Japan and* [2]*Department of Urology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Japan*

The aim of this study was to evaluate the effect of supplementing healthy men with soy isoflavones on the serum levels of sex hormones implicated in prostate cancer development. A total of 28 Japanese healthy volunteers (18 equol producers and 10 equol non-producers) between 30 and 59 years of age were given soy isoflavones (60 mg daily) supplements for 3 months, and the changes in their sex hormone levels were investigated at the baseline and after administration. The serum and urine concentrations of daidzein, genistein, and the levels of equol in the fasting blood samples and 24-h stored urine samples were also measured. All 28 volunteers completed the 3-month supplementation with isoflavone. No changes in the serum levels of estradiol and total testosterone were detected after 3-month supplementation. The serum levels of sex hormone-binding globulin significantly increased, and the serum levels of free testosterone and dihydrotestosterone (DHT) decreased significantly after 3-month supplementation. Among the 10 equol non-producers, equol became detectable in the serum of two healthy volunteers after 3-month supplementation. This study revealed that short-term administration of soy isoflavones stimulated the production of serum equol and decreased the serum DHT level in Japanese healthy volunteers. These results suggest the possibility of converting equol non-producers to producers by prolonged and consistent soy isoflavones consumption.
Prostate Cancer and Prostatic Diseases (2009) **12**, 247–252; doi:10.1038/pcan.2009.10; published online 14 July 2009

Keywords: isoflavones; equol; dihydrotestosterone; cancer prevention

Introduction

Epidemiological studies have shown that the incidences of malignancies, such as prostate cancer, breast cancer and colon cancer, are commonly high in the Western Europe and United States of America, but low in the Asian populations who consume large amounts of soy bean foods.[1] Soy bean products are rich in isoflavones, such as genistein and daidzein. Isoflavones have been suggested as the principal chemical constituents responsible for the potential preventive effect of soy bean against prostate cancer.[2] Possible mechanisms have been proposed for the anti-tumor activity of soy isoflavones against prostate cancer, including estrogen-like effects,[3] prevention of oxidative DNA damage,[4] reduction in cancer cell proliferation[5] and inhibition of angiogenesis.[6]

Correspondence: Dr M Tanaka, Department of Urology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan.
E-mail: masa-t@naramed-u.ac.jp
Received 28 October 2008; revised 13 March 2009; accepted 13 March 2009; published online 14 July 2009

It has been argued for long whether the lower incidences of prostate cancer among the Asian people are because of the inhibitory effects of isoflavones (contained abundantly in soy beans) and equol (directly metabolized from daidzein by the intestinal bacterial microflora) against prostatic carcinogenesis. Equol has a weak phytoestrogen activity.[7] Especially, equol and isoflavones have a binding affinity to estrogen receptor β.[8] In addition, equol can bind to the sex hormone-binding globulin (SHBG),[9,10] and inhibit the growth of prostate cells *in vitro*.[11,12] Therefore, equol can act as an anti-androgen and inhibit the development of sex hormone-dependent tumors, such as mammary gland cancer and prostate cancer.[12] Earlier, we reported that some people can metabolize daidzein into equol, whereas others cannot, and showed that the intake quantity of isoflavones and the proportions of equol producers differed significantly among the races and age stratifications.[13]

In this intervention study, to elucidate the biological impact of isoflavones on equol-producing ability and sex hormonal variation in association with prostate carcinogenesis, we investigated whether the constant daily consumption of isoflavone supplements influenced the

serum levels of isoflavones, equol and sex hormonal biomarkers in healthy Japanese men.

Participants and methods

A total of 28 Japanese healthy volunteers (18 equol producers and 10 equol non-producers), between 30 and 59 years of age were given soy isoflavones (60 mg per day) for 3 months, and the changes in the serum levels of sex hormones, cholesterol and isoflavones were measured at the baseline, and at 1 month and 3 months later. This protocol was designed with reference to earlier studies.[14–18] This study was conducted to determine the persistence of diet-induced effects on isoflavone metabolism and disposition. Whether a volunteer is an equol producer or non-producer was determined as in our earlier study, in which we conducted a food survey on the daily intake of soybean isoflavone and measured the serum concentrations of isoflavone and equol in all participants.[13] Five tablets of Isofla A (Fuji Oil Co. Ltd, Osaka, Japan) were administered immediately before breakfast and dinner twice a day to all volunteers. In total, 10 tablets per day contained 60 mg isoflavone, 2.4 g carbohydrate, 80 mg protein, 70 mg lipid, 100 mg calcium, 60 mg magnesium, 30 IU vitamin D3, and 3 mg vitamin E. Isoflavone (60 mg) consisted of 19.1 mg daidzin, 3.5 mg genistin, 10.4 mg glycitin, 8.1 mg malonyl daidzin, 2.2 mg malonyl genistin, 3.4 mg malonyl glycitin, 7.3 mg acetyl daidzin, 1.9 mg acetyl genistin, 3.6 mg acetyl glycitin, 0.2 mg daidzein, 0.1 mg genistein and 0.3 mg glycitein.

The institutional reviewer board approved this study, and a written informed consent was obtained from all the volunteers. Each volunteer was given packages of Isofla A tablets with a log at the baseline visit. At 1 month and 3 months later, the residual tablets, used packages and log for counting the residual tablets were collected from all the volunteers to check the intake adherence. The intake of isoflavones from daily foods varied among the volunteers, but they maintained the same life style, including diet, exercise, work and sleep patterns, while being enrolled in this study.

Blood samples at 0800 hours before breakfast (after 10-h fasting) and 24-h urine samples were taken at the baseline, and at 1 month and 3 months after starting isoflavone administration. For all volunteers, blood samples were taken before breakfast, and the separated sera were stored at −20 °C or lower. Urine was taken for 24 h in dark plastic bottles containing 2 g ascorbic acid, and kept in a cool place during storage. The urine was mixed well and a sample was frozen directly after measuring the volume. These serum and urine samples were subsequently transported on dry ice to the laboratory of SRL Co. Ltd (Tokyo, Japan). The sample size of all volunteers for detecting the serum isoflavone concentration was not calculated, but it was regulated by the feasibility for each investigator.

The concentrations of genistein, daidzein and equol in the serum and urine samples were measured by reversed-phase high-performance liquid chromatography-multiple reaction ion monitoring mass spectrometry.[19] The serum concentrations of cholesterols (total, high-density lipoproteins and low-density lipoproteins), estradiol, SHBG, total and free testosterone and dihydrotestosterone (DHT) were measured at the same time points at SRL Co. Ltd.

In addition to comparing the serum concentration of isoflavones in the two participant groups, we also compared the participants on the basis of their capability of equol production. The non-producers were defined as having a serum equol concentration below the limit of detection by the present assay system, that is, 0.5 ng ml^{-1}.

Statistical analyses were carried out using non-parametric Wilcoxon's test and χ^2-test. A P-value of <0.05 was considered as representing a statistically significant difference.

Results

The demographic characteristics of the healthy volunteers are shown in Table 1. Between the equol producers and the non-producers, there was a significant difference in age. All 28 volunteers completed the 3-month isoflavone administration as scheduled. No statistically significant adverse events were reported by the study participants. Diarrhea was the most frequently reported adverse event and occurred in 3 (11%) of the 28 participants. Two participants had diarrhea of grade 1 according to the Common Terminology Criteria for Adverse Events (v3.0) several times during the consecutive 2 days, and one complained of grade 1 diarrhea once only. No participant discontinued the study regimen or withdrew from the study because of these adverse events. The mean adherence rate was more than 99% during the whole study period. During the 3 months of Isofla A administration, all participants showed no marked change in their life styles.

No significant difference was noticed in the total cholesterol between the mean serum levels at the baseline and the end of study period. Although the mean high-density lipoprotein-cholesterol level at 3 months increased significantly when compared with that of the baseline, the mean low-density lipoprotein-cholesterol significantly decreased during 3-month isoflavone administration (Table 2). No significant changes in the mean serum levels of estradiol and total testosterone after 3-month administration were noticed when compared with the baseline (Table 2). However, the mean level of SHBG significantly increased after 3 months of isoflavone administration, and the mean serum levels of free testosterone and DHT decreased

Table 1 Demography of healthy male volunteers

	Equol non-producers (n=10)	*Equol producers (n=18)*	
Age (years)	36.7 ± 5.2	43.2 ± 7.8	$P<0.05$
Height (cm)	170.8 ± 7.3	169.5 ± 6.9	ns
Weight (kg)	64.8 ± 4.1	67.8 ± 3.9	ns
BMI (kg m^{-2})	22.2 ± 0.6	23.6 ± 0.7	ns
Former or current smoker	50.0%	55.6%	ns
Family history of cancer	40.0%	44.4%	ns
Family history of benign prostatic hyperplasia	30.0%	31.1%	ns

Abbreviations: BMI, body mass index; ns, non-significant.

Table 2 Variations of the mean serum levels of cholesterols and sex hormones after isoflavone supplementation

	Before supplementation	*1 month later*	*3 months later*	
Serum cholesterol				
Total cholesterol (mg per 100 ml)	209.0 ± 35.5	206.7 ± 30.7	204.5 ± 31.7	ns
HDL-cholesterol (mg per 100 ml)	55.4 ± 11.8	57.3 ± 10.0	59.8 ± 11.4	$P<0.05$
LDL-cholesterol (mg per 100 ml)	132.2 ± 34.9	127.0 ± 30.9	118.4 ± 30.1	$P<0.05$
Serum sex hormones				
Estradiol (pg ml^{-1})	25.0 ± 5.6	25.9 ± 5.6	25.2 ± 6.5	ns
Sex hormone-binding globulin (nmol l^{-1})	52.2 ± 19.8	47.1 ± 15.1	61.2 ± 19.9	$P<0.05$
Dihydrotestosterone (ng ml^{-1})	0.96 ± 0.27	0.78 ± 0.23	0.79 ± 0.23	$P<0.05$
Free testosterone (pg ml^{-1})	74.9 ± 3.5	71.8 ± 11.9	70.9 ± 11.2	$P<0.05$
Total testosterone (pg ml^{-1})	541.0 ± 125.0	569.0 ± 108.0	576.0 ± 135.0	ns

Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; ns, non-significant.

Table 3 Variations of the mean serum and urine levels of genistein, daidzein and equol after isoflavone supplementation

	Before supplementation	*1 month later*	*3 months later*	
Serum isoflavone				
Genistein (ng ml^{-1})	81.7 ± 80.0	108.0 ± 106.4	97.6 ± 97.8	$P<0.05$
Daidzein (ng ml^{-1})	40.5 ± 48.4	132.7 ± 109.7	133.8 ± 107.0	$P<0.01$
Equol (ng ml^{-1})	21.0 ± 44.8	41.3 ± 69.7	64.7 ± 125.9	$P<0.01$
Urine isoflavone				
Genistein (nmol per day)	23.9 ± 20.5	88.7 ± 66.7	103.1 ± 73.7	$P<0.01$
Daidzein (nmol per day)	35.8 ± 36.2	41.7 ± 28.7	50.1 ± 41.6	$P<0.05$
Equol (nmol per day)	12.7 ± 19.8	36.4 ± 40.5	37.9 ± 61.4	$P<0.05$

after 3-month administration when compared with the baseline (Table 2).

The mean serum concentration of isoflavones and equol at the baseline, and at 1 month and 3 months after isoflavone administration are shown in Table 3. Genistein increased more significantly in the urine than in the serum. Daidzein and equol showed marked increases in the serum as well as in the urine after 3-month administration. Two equol non-producers became equol producers after 3 months of isoflavone administration. Of these two volunteers, one had serum equol levels of <0.5 ng ml^{-1}, <0.5 ng ml^{-1} and 1.1 ng ml^{-1}, whereas the other had <0.5 ng ml^{-1}, 1.1 ng ml^{-1} and 24.1 ng ml^{-1} at the baseline, 1 month, and 3 months after isoflavone administration, respectively.

The mean serum levels of cholesterol, SHBG, estradiol, testosterone and its metabolites were analyzed in the equol producers and non-producers (Table 4). The mean high-density lipoprotein-cholesterol level significantly increased and the mean low-density lipoprotein-cholesterol level showed a significant decrease after 3-month isoflavone administration in the equol producers. Neither the equol producers nor the non-producers showed any changes in the mean serum level of estradiol during the study period. Equol producers showed a significant increase in the mean SHBG level and a significant decrease in the mean DHT level 3 months later. The free testosterone level showed a significant decrease in the equol producers, but not in the non-producers, whereas the total testosterone level showed no significant increase in both groups.

Discussion

Prostate cancer has recently become an increasingly important public health issue in Japan. Despite the extensive studies on the pathogenesis and clinical behavior of prostate cancer, the etiological origins or the host-environmental risk factors, which promote its progression, have not yet been well elucidated.[20] In fact, the African-American men have the highest morbidity rates of prostate cancer in the world, whereas the Asian men natives to their countries, such as the Japanese, Korean and Chinese, have the lowest rates.[21]

However, the incidence of prostate cancer in Japan has recently increased as the diets and life styles became westernized. Although latent or clinically insignificant prostate cancer is detected at a high rate—similar to that in the American men—in autopsy studies on the Asian men, the morbidity rate of clinically significant prostate cancer is 80-fold higher in USA.[22] This suggests that the same dietary factors may also promote the progression of latent or microscopic prostate cancer to clinically significant and metastatic prostate cancer.[23] Some epidemiological studies indicated that the level of dietary soy consumption may be linked to a decreased risk of prostate cancer.[24] The standard intake of isoflavones by the American men was commonly a couple of milligrams per day. The ninefold decrease in prostate cancer mortality among the Japanese men as compared with that in USA may be attributed, in part, to the high soy protein content in the Japanese diet.[25]

Isofla A tablets are commercially sold in Japan as a supplement, and the most dominant flavonoid was an

Table 4 Variations of the mean serum levels of cholesterols and sex hormones in equol producers and non-producers after isoflavone supplementation

	Before supplementation	*1 month later*	*3 months later*	
Total cholesterol (mg per 100 ml)				
Equol producers	209.2 ± 40.1	205.6 ± 30.2	204.1 ± 34.3	ns
Equol non-producers	208.5 ± 27.3	208.7 ± 33.0	205.2 ± 28.3	ns
HDL-cholesterol (mg per 100 ml)				
Equol producers	53.1 ± 10.3	56.1 ± 10.7	57.7 ± 11.6	$P<0.05$
Equol non-producers	59.5 ± 13.8	59.5 ± 8.8	63.5 ± 10.4	ns
LDL-cholesterol (mg per 100 ml)				
Equol producers	134.9 ± 38.4	126.3 ± 31.5	117.1 ± 30.8	$P<0.05$
Equol non-producers	127.4 ± 28.9	128.7 ± 33.0	120.8 ± 30.3	ns
Estradiol (pg ml^{-1})				
Equol producers	24.7 ± 4.7	25.7 ± 5.1	24.6 ± 5.4	ns
Equol non-producers	25.5 ± 7.2	26.3 ± 6.7	27.0 ± 7.6	ns
Sex hormone-binding globulin (nmol l^{-1})				
Equol producers	50.1 ± 17.2	45.9 ± 14.9	60.7 ± 22.1	$P<0.05$
Equol non-producers	56.1 ± 24.3	49.3 ± 15.8	62.0 ± 16.3	ns
Dihydrotestosterone (ng ml^{-1})				
Equol producers	0.93 ± 0.25	0.77 ± 0.24	0.75 ± 0.18	$P<0.05$
Equol non-producers	1.03 ± 0.32	0.82 ± 0.22	0.86 ± 0.23	ns
Free testosterone (pg ml^{-1})				
Equol producers	74.6 ± 3.8	74.0 ± 4.0	70.1 ± 4.6	$P<0.05$
Equol non-producers	75.4 ± 3.0	67.8 ± 19.2	73.6 ± 2.6	ns
Total testosterone (pg ml^{-1})				
Equol producers	529.0 ± 119.0	564.0 ± 107.0	541.0 ± 114.0	ns
Equol non-producers	561.0 ± 140.0	578.0 ± 114.0	638.0 ± 153.0	ns

Abbreviations: HDL, high-density lipoproteins; LDL, low-density lipoproteins; ns, non-significant.

isoflavone glycoside daidzin. The second dominant flavonoid glycitin is an isoflavone glycoside present in foods containing soybean. The metabolite of glycitin synthesized by intestinal microflora is glycitein, which scavenges the intracellular reactive oxygen species. The other ingredients are malonyl or acetyl glycosides and isoflavone aglycones. Diverse bacterial strains of human origin have specific β-glucosidase and β-glycuronidase activity in the metabolism of dietary isoflavones from glycosides to aglycones. Aglycone and sulfate of genistein and daidzein have estrogen-like activity and so does equol.

Genistein reportedly inhibits the growth of androgen-dependent and androgen-independent prostate cancer cell lines[26] and inhibits the development of prostate-related cancers in Wistar rats.[27] The mechanism of action by which genistein exerts this anti-tumor angiogenesis is not well understood. Enough scientific data have accumulated to allow clinical intervention of soy protein in human trials as an adjuvant supplementation for the treatment of prostate cancer, but the optimal and effective doses of soy protein are not yet known. In this study, we also assessed the effects of the daily consumption of isoflavone supplements on the serum high-density lipoprotein-cholesterol and low-density lipoprotein-cholesterol levels, and measured the isoflavone concentrations in the blood and urine of healthy men. Importantly, our results, as well as those of an earlier study,[14] supported the hypothesis that the continuous intake of isoflavones is likely to play a promising role in chemoprevention against cardiovascular disease.

In a recent study, it was found that isoflavones could increase the production of SHBG in the liver and bind to biologically active testosterone. Consequently, lowering the free testosterone levels and its bioavailability to the target prostate cells should theoretically halt cancer cell proliferation, inhibit tumor progression and reduce the tumor volume in accordance with the changes in the prostate-specific antigen.[16] In our study, the SHBG level significantly increased, whereas the free testosterone level and DHT level decreased after 3-month isoflavone administration as compared with the baseline. This result is in consensus with several earlier studies and a recent review on the effects of isoflavone.[11,12,28]

On the other hand, there are some limitations of the product used in our study. The interactions of isoflavones and other nutrients included in Isofla A tablets were not considered. The other ingredients, such as vitamin D or E, may have some effect on SHBG, DHT or testosterone levels, although our earlier study showed that the intake amounts of vitamin D and E were not risk factors for the development of prostate cancer in Japanese men.[29] Furthermore, supplementation is usually a high-dose and unbalanced administration unlike the physiological condition, and we donot know whether the supplemental isoflavones behave exactly the same as isoflavones from the actual food, particularly because the intestinal microflora variability or genetic differences in isoflavone metabolism may vary among the individuals or the races. It is still a question how the food substances work in the actual food in comparison with their action in the supplemental form.

In our earlier case–control study, the ability of producing equol or equol itself closely correlated with the lower incidence of prostate cancer.[30] At 3 months after soy isoflavone supplementation, serum equol was detected in two healthy volunteers among the equol non-producers group. This suggests the possibility of converting equol non-producers to producers by prolonged and consistent isoflavones consumption. Moreover, prolonged and consistent isoflavones consumption could potentially delay the onset of prostate cancer by interfering with carcinogenesis. A public health initiative now may exist to identify non-toxic therapies for cancer. Several clinical trials evaluated the role of various nutritional supplementations in the treatment of localized prostate cancer. On the basis of updated scientific evidences, numerous nutritional strategies could be used for clinical nutrition interventional trials on the use of individual supplements or dietary modification versus the incorporation of multiple nutritional strategies. As prostate cancer grows relatively slowly in comparison with other malignancies, the potential impact of nutritional intervention may spare patients from undergoing a variety of toxic treatments for prostate cancer, and therefore improve their quality of life. The results of our study warrant further preclinical and clinical trials focusing on the role of isoflavone in chemoprevention against prostate cancer.

Conclusion

This study revealed that short-term administration of soy isoflavones to Japanese healthy volunteers increased the SHBG level, and decreased the free testosterone and DHT levels. Moreover, equol was detected in the serum of two healthy volunteers among the equol non-producers group. These results suggest the possibility of converting equol non-producers to producers by prolonged and consistent soy isoflavones consumption, and that a diet based on soy isoflavones will be useful in preventing prostate cancer development.

Conflict of interest

Isoflavone tablets used in this study were kindly provided by Protein Technologies International (Soybean health Foods Laboratory), Fuji Oil Co. Ltd (Osaka, Japan). This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas, Cancer A-03 and A-04, from the Ministry of Education, Science, Sports and Culture, Japan.

Acknowledgements

We would like to express our deepest gratitude to the company manager.

References

1 Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hämäläinen E, Hasegawa T *et al*. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am J Clin Nutr* 1991; **54**: 1093–1100.
2 Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL, Clinton SK. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. *J Nutr* 1999; **129**: 1628–1635.
3 Knight DC, Eden JA. A review of the clinical effects of phytoestrogens. *Obstet Gynecol* 1996; **87**: 897–904.
4 Wei H, Cai Q, Rahn RO. Inhibition of UV light-and Fenton reaction –induced oxidative DNA damage by the soybean isoflavone genistein. *Carcinogenesis* 1996; **17**: 73–77.
5 Shao ZM, Alpaugh ML, Fontana JA, Barsky SH. Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21WAF1/CIP1 induction, G2/M arrest and apoptosis. *J Cell Biochem* 1998; **69**: 44–54.
6 Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R *et al*. Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. *Proc Natl Acad Sci USA* 1993; **90**: 2690–2694.
7 Price KR, Fenwick GR. Naturally occurring oestrogens in foods-a review. *Food Addit Contam* 1985; **2**: 73–106.
8 Petersen DN, Tkalcevic GT, Koza-Taylor PH, Turi TG, Brown TA. Identification of estrogen receptor β2, a functional variant of estrogen receptor β expressed in normal rat tissues. *Endocrinology* 1998; **139**: 1082–1092.
9 Déchaud H, Ravard C, Claustrat F, de la Perrière AB, Pugeat M. Xenoestrogen interaction with human sex hormone-binding globulin (hSHBG). *Steroids* 1999; **64**: 328–334.
10 Martin ME, Haourigui M, Pelissero C, Benassayag C, Nunez EA. Interactions between phytoestrogens and human sex steroid binding protein. *Life Sci* 1996; **58**: 429–436.
11 Hedlund TE, Johannes WU, Miller GJ. Soy isoflavonoid equol modulates the growth of benign and malignant prostatic epithelial cells *in vitro*. *Prostate* 2003; **54**: 68–78.
12 Lund TD, Munson DJ, Haldy ME, Setchell KD, Lephart ED, Handa RJ. Equol is a novel anti-androgen that inhibits prostate growth and hormone feedback. *Biol Reprod* 2004; **70**: 1188–1195.
13 Fujimoto K, Tanaka M, Hirao Y, Nagata Y, Mori M, Miyanaga N *et al*. Age-stratified serum levels of isoflavones and proportion of equol producers in Japanese and Korean healthy men. *Prostate Cancer Prostatic Dis* 2008; 11: 252–257.
14 Urban D, Irwin W, Kirk M, Markiewicz MA, Myers R, Smith M *et al*. The effect of isolated soy protein on plasma biomarkers in elderly men with elevated serum prostate specific antigen. *J Urol* 2001; **165**: 294–300.
15 Hussain M, Banerjee M, Sarkar FH, Djuric Z, Pollak MN, Doerge D *et al*. Soy isoflavones in the treatment of prostate cancer. *Nutr Cancer* 2003; **47**: 111–117.
16 Kumar NB, Cantor A, Allen K, Riccardi D, Besterman-Dahan K, Seigne J *et al*. The specific role of isoflavones in reducing prostate cancer risk. *Prostate* 2004; **59**: 141–147.
17 Maskarinec G, Morimoto Y, Hebshi S, Sharma S, Franke AA, Stanczyk FZ. Serum prostate-specific antigen but not testosterone levels decrease in a randomized soy intervention among men. *Eur J Clin Nutr* 2006; **60**: 1423–1429.
18 Kumar NB, Krischer JP, Allen K, Riccardi D, Besterman-Dahan K, Salup R *et al*. Safety of purified isoflavones in men with clinically localized prostate cancer. *Nutr Cancer* 2007; **59**: 169–175.
19 Coward L, Kirk M, Albin N. Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin Chim Acta* 1996; **247**: 121–142.
20 Carter HB, Coffey DS. The prostate: an increasing medical problem. *Prostate* 1990; **16**: 39–48.
21 Hsing AW, Tsao L, Devesa SS. International trends and patterns of prostate cancer incidence and mortality. *Int J Cancer* 2000; **85**: 60–67.
22 Yatani R, Chigusa I, Akazaki K, Stemmermann GN, Welsh RA, Correa P. Geographic pathology of latent prostatic carcinoma. *Int J Cancer* 1982; **29**: 611–616.
23 Wynder EL, Rose DP, Cohen LA. Nutrition and prostate cancer: a proposal for dietary intervention. *Nutr Cancer* 1994; **22**: 1–10.
24 Oishi K, Okada K, Yoshida O, Yamabe H, Ohno Y, Hayes RB *et al*. A case–control study of prostatic cancer with reference to dietary habits. *Prostate* 1988; 12: 179–190.
25 Messina M, Barnes S. The role of soy products in reducing risk of cancer. *J Natl Cancer Inst* 1991; **83**: 541–546.

26 Peterson G, Barnes S. Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* 1993; **22**: 335–345.
27 Pollard M, Wolter W, Sun L. Prevention of induced prostate-related cancer by soy protein isolate/isoflavone-supplemented diet in Lobund-Wistar rats. *In Vivo* 2000; **14**: 389–392.
28 Perabo FG, Von Löw EC, Ellinger J, von Rücker A, Müller SC, Bastian PJ. Soy isoflavone genistein in prevention and treatment of prostate cancer. *Prostate Cancer Prostatic Dis* 2008; **11**: 6–12.
29 Nagata Y, Sonoda T, Mori M, Miyanaga N, Okumura K, Goto K *et al*. Dietary isoflavones may protect against prostate cancer in Japanese men. *J Nutr* 2007; **137**: 1974–1979.
30 Akaza H, Miyanaga N, Takashima N, Naito S, Hirao Y, Tsukamoto T *et al*. Comparisons of percent equol producers between prostate cancer patients and controls: case-controlled studies of isoflavones in Japanese, Korean and American residents. *Jpn J Clin Oncol* 2004; **34**: 86–89.