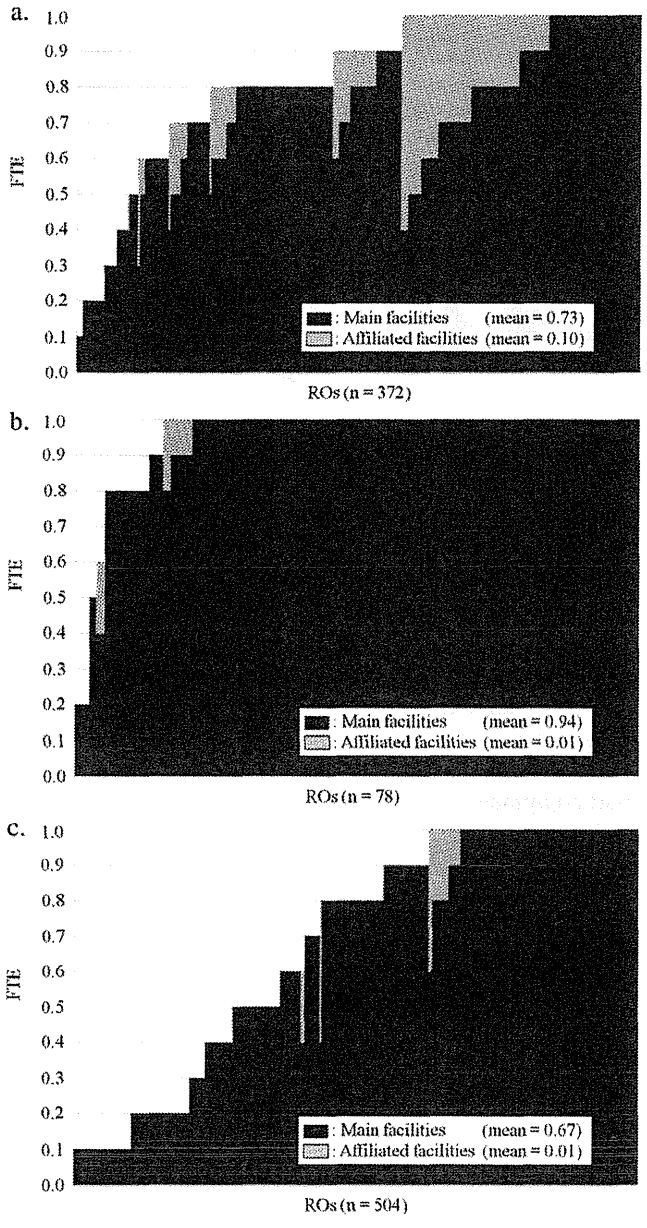

Fig. 1. Working patterns of ROs working mainly at (a) university hospitals, (b) cancer centers, and (c) other radiotherapy hospitals. Distribution of FTE ratio between main and affiliated facilities on each RO. Horizontal axis represents ROs in ascending order of own total FTE. *Abbreviations:* RO = radiation oncologist; FTE = full-time equivalent (40 hours per week for radiation oncology services only).

centers and other RT hospitals was not significant ($p = 0.9459$).

DISCUSSION

In the United States, most RT facilities are supported by full-time ROs, with an FTE of 1.0 for most ROs working at their own facilities. In Japan, on the other hand, more than a half of the facilities still rely on part-time ROs. The main reason of this discrepancy is a shortage of ROs. Between 2005 and 2007, the increase in the number of cancer

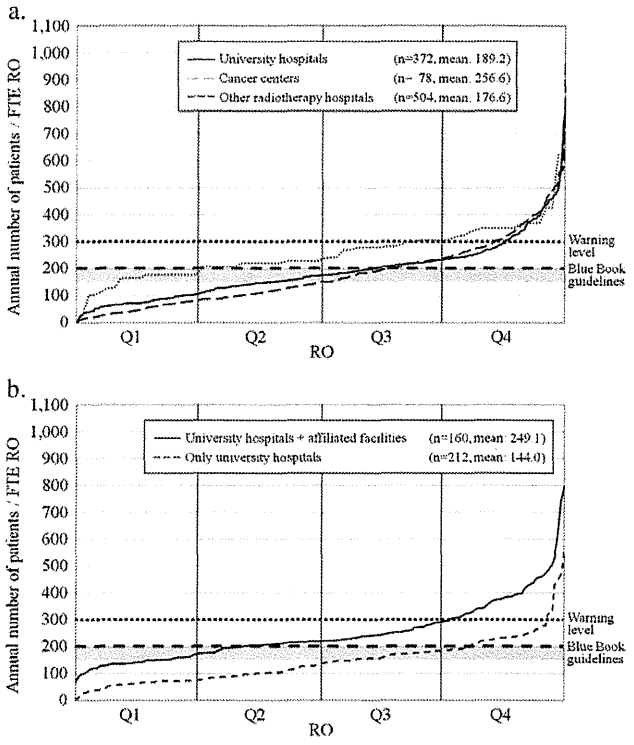

Fig. 2. Distribution of annual patient load/RO. (a) RO working mainly in university hospitals, cancer centers, and other radiotherapy hospitals. (b) RO working mainly in university hospitals. Horizontal axis represents ROs in ascending order of annual numbers of patients/RO. Q1: 0–25%, Q2: 26–50%, Q3: 51–75%, Q4: 76–100%. *Abbreviations:* RO = radiation oncologist; FTE = full-time equivalent (40 hours per week for radiation oncology services only).

patients requiring RT (7.3%) was higher than that in the number of FTE ROs (6.7%) (1). To make up for the shortage of ROs, most ROs in university hospitals must work part-time at affiliated hospitals, as is evident from the date shown in Figure 1. White parts of Figure 1 (a: 17.4%, b: 5.0% c: 32.0%) represent three types of data: (a) FTE data of ROs who were not provided in the survey questionnaire; (b) FTE data of part-time ROs whose identification data could not connect to those of full-time ROs; (c) FTE data of ROs working in nonradiation oncology services. In this survey, the data of type (a) and (b) were missing data and the data of type (c) were not collected. In other RT hospitals, the FTE of most ROs working in their own facilities is low and these ROs do not work part-time at other hospitals. There are two reasons for this. First, diagnosticians partly provide RT as ROs in their own hospitals and, second, other specialists (such as brain surgeons using gamma knife) partly function as ROs to provide RT. Because those facilities have few cancer patients, their patient load is less than that of university hospitals and cancer centers. These findings are evident from Figure 2(a). There was a major difference in the working patterns of ROs between university hospitals and cancer centers. FTE at their own facilities of most ROs working in university hospitals is less than 1.0, whereas that of most ROs working in cancer centers is 1.0,

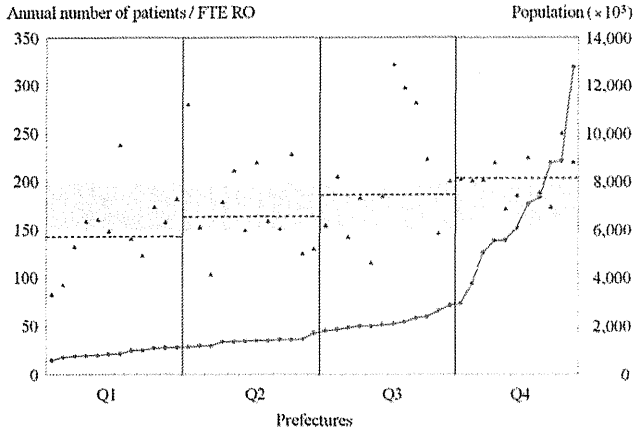

Fig. 3. Geographic distribution for 47 prefectures of annual number of patients (new plus repeat) per RO in ascending order of prefectural population. Q1: 0–25%; Q2: 26–50%; Q3: 51–75%; Q4: 76–100%. Triangles represent average annual number of patients per RO for each prefecture. Blue circles show prefectural population. Horizontal broken lines indicate the average annual number of patients per RO per quarter. The shaded area represents the Japanese Blue Book guideline (150–200 patients per RO). *Abbreviations:* RO = radiation oncologist; FTE = full-time equivalent (40 hours per week for radiation oncology services only).

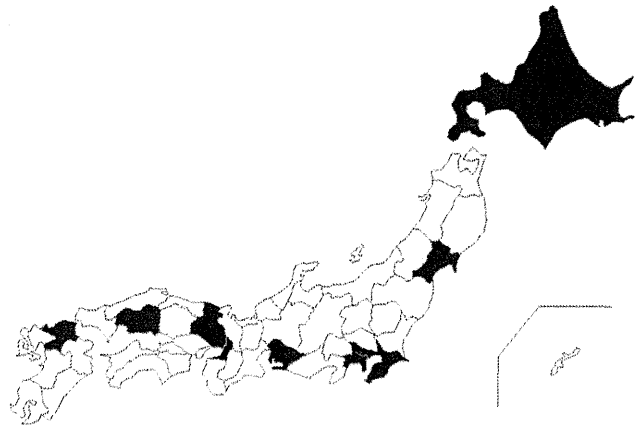

Fig. 4. The top 10 prefectures with ROs who treated more than 200 patients in descending order: Tokyo, Osaka, Kanagawa, Hokkaido, Chiba, Aichi, Fukuoka, Hyogo, Miyagi, and Hiroshima. *Abbreviation:* RO = radiation oncologist.

the same as in the United States and European countries. The shortage of ROs is not the only reason for the problems facing Japan. The pay system of ROs is another important reason. The salary of ROs in Japan is low because specialist medical fees for ROs are not covered by the Japanese healthcare insurance system. Moreover, the salary of ROs in university hospitals is lower than in other types of facilities, so that most of these ROs must work part-time at affiliated hospitals to earn a living. One advantage of this system, however, is that advanced technology is introduced sooner and faster in affiliated hospitals.

The geographic patterns demonstrated significant differences in the patient load among prefectures, ranging from 83.2 to 321.4 patients per RO. There were more ROs in metropolitan than other areas. However, the number of ROs who had more than 200 patients (new plus repeat) was strongly associated with population (correlation coefficient: 0.94), so that the number of ROs in metropolitan area remained insufficient.

Gomi *et al.* reported that the survival rate of patients treated in academic RT facilities (university hospitals and cancer centers) was better than that of those treated in nonacademic RT facilities in Japan (10). In this study, the proportion of facilities with part-time ROs in nonacademic RT facilities group was higher than that in academic RT facilities group. Part-time ROs have less care time per patient because they had a limit to working hours. On the basis of the presented evidence, the relative practice index for patients of ROs was calculated as one way to valuate quality of cancer care in this study. Concerning ROs working primarily in university hospitals, the average relative practice index for patients in affiliated facilities was less than that in main facilities (university hospitals). Teshima *et al.* reported that academic RT facilities (university hospitals and cancer centers) had better equipments and manpower than nonacademic RT facilities (1). Therefore, ROs at large-scale university hospitals might be given sufficient support because large-scale university hospitals tend to have state-of-the-art equipment, practice leading-edge medical treatment techniques, and employ enough medical staff members. On the other hand, ROs of most affiliated facilities could provide only minimal cancer care because these facilities

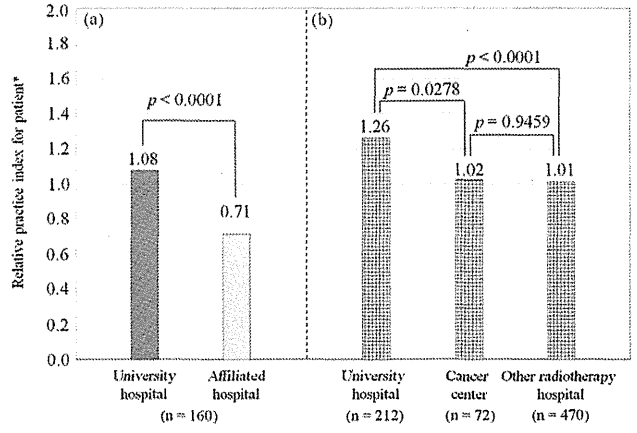

Fig. 5. Relative practice index for patients of ROs. (a) Relative practice index for patients in university hospitals and affiliated hospitals (targeted ROs were working mainly in university hospitals and part-time in affiliated hospitals). (b) Relative practice index for patients in university hospitals, cancer centers, and other radiotherapy hospitals (targeted ROs were working only in university hospitals or cancer centers only or only in other radiotherapy hospitals). *The formula used for calculating relative practice index for patients is: $\frac{\sum_{k=1}^{n} f_k}{\sum_{k=1}^{n} a_k} \times 200$ n: number of facilities that the RO works in (n = 1, 2, 3, …, k). f_k : FTE of the RO in facility k a_k : annual number of patients per RO in facility k. *Abbreviations:* RO = radiation oncologist; FTE = full-time equivalent (40 hours per week for radiation oncology services only).

tend to lack sufficient equipment and medical staff. Moreover, commuting between large-scale university hospitals and affiliated facilities resulted in a waste of time and in tiredness. Therefore, the quality of cancer care in affiliated facilities was worse than that in large-scale university hospitals. Although the annual number of patients per RO in cancer centers was higher than that in university hospitals and other RT hospitals, the average relative practice index for patients of ROs working only in cancer centers was lower than that for patients of ROs working only in university hospitals and equal to that for patients of ROs working only in other RT hospitals. It can thus be concluded that ROs in cancer centers worked efficiently.

The utilization rate of RT for new cancer patients in Japan is much lower than that in European countries and the United States. Because there are enough RT facilities distributed nationwide in Japan, an increase in the number of Ros would likely result in a spectacular improvement in the utilization rate of RT for new cancer patients. To increase the number of ROs, it is necessary to improve the work environment and conditions for radiation oncology in medical care facilities. One, feasible suggestion is for RT facilities to set up a new department of radiation oncology, so that the position of RO will be established at every such facility and the status of radiation oncology will improve as a result. In addition, the Cancer Control Act was approved in 2006 and the Basic Plan to Promote Cancer Control Program was approved by the Japanese Cabinet in 2007 to promote RT and education for ROs as well as other RT staff members. For the implementation of this law and plan, the availability of basic data of RO working conditions is essential. As a start, an education program called "Cancer Professional Training Plan" was started in April 2008 with the support of the Ministry of Education, Culture, Sports, Science and Technology.

Quality of cancer care was evaluated in this study with the aid of the relative practice index for patients. However, data concerning the processes and outcomes for cancer care using RT should be used for a more accurate evaluation of cancer care. In the United States, the National Cancer Data Base has been collecting data for cancer care. The data of National Cancer Data Base are useful for quality evaluation of cancer care (11, 12). Furthermore, PCS has been performed every 4 or 5 years since 1973 for a survey of the structure, processes, and outcomes of radiation oncology facilities (13). As PCS evolved into Quality Research in Radiation Oncology, periodic assessments of radiation oncology have been conducted for evaluation of practice quality on a national basis. In Japan, the structure, processes and outcomes for cancer care using RT have been investigated by PCS every 4 years (7, 8). The Japanese PCS has evaluated the quality of cancer care with RT and provided evidence of the disparity in quality of RT among facilities (14–18). However, these data are insufficient because PCS is a two-stage cluster sampling survey. We have recently established a database system based on available radiation oncology data and the collection of cancer care data by means of this system is now in preparation.

This study based on the JASTRO structure survey has indicated that the current national medical care system may impede fostering of true specialization of radiation oncologists in Japan because it is suffering from systemic fatigue. Although private hospitals make much money by receiving fee-for-service reimbursement, public hospitals face major deficit problems. It is therefore necessary to redistribute the burden of medical costs. On the other hand, the Japanese medical care system is beneficial for patients and national finances. Japan has had a universal health insurance system since 1961. Even though the per-capita medical costs in Japan were less than half of those in the United States and the medical costs in relation to the gross domestic product in Japan were about half of those in the United States as of 2007 (19), the outcome of cancer treatment in Japan is the same or better than in the United States. It is therefore very important to collect at regular intervals detailed information about all cancer care facilities for evaluation of quality of care and medical care systems for cancer. In Japan, the JASTRO structure survey has collected structural data of radiation oncology. Furthermore, a database system for the collection of data regarding the processes and outcomes for cancer care has recently been established in Japan as well as an information infrastructure for evaluation of the quality of care in radiation oncology.

In conclusion, our survey found that ROs working in university hospitals and their affiliated facilities treated more patients than did other ROs. In terms of patient care time only, the quality of cancer care in affiliated facilities might be worse than that in university hospitals. Under the current national insurance system, working patterns of ROs in academic facilities in Japan tend to impede the fostering of true specialization of radiation oncologists.

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Expression and Role of HMGA1 in Renal Cell Carcinoma

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Purpose: Although molecular targeted therapy has improved the clinical outcome of metastatic renal cell carcinoma, a complete response is rare and there are various side effects. Identifying novel target molecules is necessary to improve the clinical outcome of metastatic renal cell carcinoma. HMGA1 is over expressed in many types of cancer and it is associated with metastatic potential. It is expressed at low levels or not expressed in normal tissue. We examined HMGA1 expression and function in human renal cell carcinoma.

Materials and Methods: HMGA1 expression in surgical specimen from patients with renal cell carcinoma was examined by immunoblot. HMGA1 expression in 6 human renal cell carcinoma cell lines was examined by immunoblot and immunofluorescence. The molecular effects of siRNA mediated knockdown of HMGA1 were examined in ACHN and Caki-1 cells.

Results: Immunoblot using surgical specimen showed that HMGA1 was not expressed in normal kidney tissue but it was expressed in tumor tissue in 1 of 30 nonmetastatic (3%) and 6 of 18 metastatic (33%) cases ($p = 0.008$). Immunoblot and immunofluorescence revealed significant nuclear expression of HMGA1 in ACHN and Caki-1 cells derived from metastatic sites. HMGA1 knockdown remarkably suppressed colony formation and induced significant apoptosis in ACHN and Caki-1 cells. HMGA1 knockdown significantly inhibited invasion and migration in vitro, and induced anoikis associated with P-Akt down-regulation in ACHN cells.

Conclusions: HMGA1 is a potential target for novel therapeutic modalities for metastatic renal cell carcinoma.

Key Words: apoptosis; carcinoma, renal cell; HMGA1 protein; molecular targeted therapy; anoikis

Abbreviations and Acronyms

HMGA1 = high mobility group protein AT-hook 1

mRCC = metastatic RCC

P-Akt = phosphorylated Akt

poly-HEMA = poly 2-hydroxyethyl methacrylate

RCC = renal cell carcinoma

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ALTHOUGH molecular targeted therapy has improved the clinical outcome for patients with mRCC, a complete response is rare and therapy has various adverse effects.[1] Identifying novel target molecules is needed to develop new therapeutic modalities for mRCC. Targeting molecules that have a role in cell growth and survival, and are expressed exclusively in cancer cells but not in normal cells should result in significant antitumor effects with few adverse events.

From this perspective we focused on HMGA1, a nuclear matrix protein with 3 AT-hook domains that bind the minor groove of AT-rich DNA sequences.[2] HMGA1 modulates the activity of many transcription factors by inducing a conformational change in DNA via AT rich DNA sequence binding.[2] HMGA1 also modulates the molecular or cellular function of other molecules, including p53[3] and Rb,[4] via protein-protein interactions. HMGA1 is over expressed in many types of can-

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cer[5] but it is expressed at low levels or not expressed in normal adult tissue.[6] HMGA1 exerts oncogenic activity in vitro[7,8] and in vivo.[9–11] HMGA1 expression is associated with cancer metastatic potential and progression.[5,12–14]

We previously reported that HMGA1 transfection in prostate cancer cells enhanced tumor cell growth and matrix metalloproteinase 2 expression[15] while HMGA1 expression in the TRAMP (transgenic adenocarcinoma of the mouse prostate) model was confined to the later stage when metastatic lesions formed.[16] These findings suggest that HMGA1 is a strong candidate gene with a potential role in prostate cancer progression and metastasis.

Thus, we examined HMGA1 expression in RCC cell lines and RCC tissues, and the role of HMGA1 in RCC with an emphasis on HMGA1 as a target molecule for novel therapeutic modalities for mRCC, including antisense therapy.

MATERIALS AND METHODS

Cell Culture and Reagents

The human cancer cell lines PC-3 (prostate cancer), Caki-1, ACHN, NC65, A498, 786-O and RCC4 (RCC) were maintained in RPMI1640 containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37C in a humidified atmosphere of 5% CO_2. We used the pancaspase inhibitor zVAD-fmk (R & D Systems®) and poly-HEMA (Sigma®).

Immunofluorescence

After fixation in methanol and blocking, cultured cells were incubated with anti-HMGI(Y) antibodies (N19) (dilution 1:100) and then with fluorescein conjugated donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, California) (dilution 1:200). Samples were observed by fluorescence microscopy (Olympus®).[17]

Protein Preparation

Whole cell lysate was prepared from each cell line in RIPA Lysis Buffer (Santa Cruz Biotechnology) according to manufacturer instructions. Each whole cell lysate was prepared from surgical specimens in sodium dodecyl sulfate buffer, as previously described.[18] The protein concentration was measured using a BCA™ Protein Assay Kit.

Immunoblot

Protein samples were separated in 15% polyacrylamide gel (Bio-Rad®) and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, United Kingdom). Primary antibodies against HMGA1 (anti-HMGI [Y] antibodies, N19, Santa Cruz Biotechnology), P-Akt (Ser473) and Akt (Total Akt, Cell Signaling Technology®) were used (dilution 1:200, 1:1,000 and 1:2,000, respectively). Signals corresponding to HMGA1, P-Akt and Total Akt were detected using an enhanced chemiluminescence kit (GE Healthcare). A monoclonal antibody against β-actin (clone AC15, Sigma) (dilution 1:5,000) was used to control sample loading. The β-actin signal was detected with a chemiluminescence kit (GE Healthcare).

Patients

Surgical specimens from 48 patients diagnosed histopathologically with RCC who underwent radical nephrectomy were examined by immunoblot. Correlations between HMGA1 expression and clinicopathological factors were analyzed. The patients included 34 males and 14 females 33 to 83 years old. The histopathological diagnosis included clear cell carcinoma in 36 cases, spindle cell carcinoma in 5, chromophobe cell carcinoma in 3, papillary RCC in 2 and an unclassified subtype in 2. Histological classification and stage data according to the 2002 TNM classification system, 6th edition were T1a in 10 cases, T1b in 11, T2 in 4, T3a in 7, T3b in 13, T4 in 3, N0 in 43, N1 in 2, N2 in 3, M0 in 18, M1 in 30, stages I to IV in 19, 2, 9 and 18, and G1 to G3 in 3, 33 and 9, respectively. Normal and cancerous tissues were collected separately from each surgically removed kidney. Specimens were stored frozen at −80C until used for immunoblot.

This study was done with the approval of our institutional review board. Informed consent was obtained from each patient.

siRNA Mediated Knockdown

Synthetic double strand siRNAs were transfected at a concentration of 200 nM using Oligofectamine™ according to manufacturer instructions. The siRNAs used had the sequence (target sequence, sense strand) scramble (control) siRNA 5′-CAGTCGCGTTTGCGACTGGdTdT-3′ and HMGA1 siRNA 5′-GACCCGGAAAACCACCACAdTdT-3′ (Sigma).[19]

Assays

Colony formation. Two days after siRNA transfection cells were seeded at 150 live cells per well in 6-well plates. Cells were incubated in growth medium for 8 to 10 days. The number of viable colonies per well was counted.

Cell proliferation. Two days after siRNA transfection cells were seeded in replicates of 6 into 96-well plates at 5,000 live cells per well in growth medium. Cell proliferation was estimated using the Cell Counting Kit-8 (WST-8) (Dojindo Laboratories, Kumamoto, Japan) according to manufacturer instructions.

Apoptosis Detection

To detect apoptosis by flow cytometry cells were grown for the indicated duration after siRNA transfection and then treated with 0.1% Triton X-100 containing propidium iodide to stain nuclei or labeled using the FITC Annexin V Apoptosis Detection Kit 1 (BD Pharmingen™). DNA content and annexin V positive cells were measured using a FACSCalibur™ flow cytometer and CellQuest™ software. Apoptosis was evaluated by quantifying the percent of cells with hypodiploid DNA (sub-G1) as an indicator of DNA fragmentation or the percent positively stained with annexin V. For all assays 10,000 cells were counted.

Cell Invasion and Migration Assays

Migration and invasion assays were performed using uncoated and Matrigel™ coated Transwell® inserts according to manufacturer instructions. Two days after siRNA transfection 5×10^4 and 2×10^5 live cells were added to the inserts for migration and invasion assays, respectively. After 24-hour incubation at 37C in a humidified 5%

CO_2 atmosphere cells that had migrated through or invaded the inserts were stained using a Diff-Quik™ staining kit and quantified by determining the total cell number derived from 5 randomly chosen visual fields per membrane at 400× magnification.

Anoikis Evaluation

Anoikis was examined by plating cells on poly-HEMA coated 6-well plates to produce a cell suspension according to a previously reported method[20] with minor modifications. A solution of 30 mg/ml poly-HEMA in 95% ethanol was made and 0.96 ml of this solution was overlaid on 1 well of the 6-well plates. The plates were left to dry in a sterile culture hood at room temperature overnight. Before use the wells were rinsed twice with phosphate buffered saline (–) and once with culture medium. Two days after siRNA transfection cells were plated at 2.0×10^5 live cells in each poly-HEMA coated well (nonadherent condition) or in each uncoated well (adherent condition) and incubated for 24 hours at 37C in a humidified 5% CO_2 atmosphere. Apoptosis under each condition was evaluated as described. If the apoptotic fraction was greater under nonadherent conditions than under adherent conditions, anoikis induction was inferred.

Statistical Analysis

Multiple independent experiments were done for each data set. Results are shown as the mean ± SD. Student's t test was used to identify significant differences between the control and experimental groups. The correlation between clinicopathological factors and HMGA1 expression was evaluated by the Fisher exact test with differences considered statistically significant at $p < 0.05$.

RESULTS

HMGA1 Expression

Human RCC tumor tissue (surgical specimens). HMGA1 expression in kidney tumor and normal kidney tissues from the same patient after radical nephrectomy was examined by immunoblot. Immunoblots of surgical samples from 48 cases of RCC revealed that HMGA1 was not expressed in normal kidney tissue in any case while HMGA1 was expressed in tumor tissue in 1 of 30 nonmetastatic (M0) (3%) and 6 of 18 metastatic (M1) (33%) cases ($p = 0.008$) as well as in 3 of 36 G1–2 (8%) and 4 of 9 G3 (44%) cases ($p = 0.022$, see table and fig. 1, *A*). These findings suggest the preferential expression of HMGA1 in high grade and metastatic RCC.

Human RCC cells. High HMGA1 expression was detected by immunoblot in Caki-1 cells derived from skin metastasis and in ACHN cells derived from pleural effusion. Low expression was detected in NC65 cells derived from moderately differentiated primary renal cancer whose host had skin metastasis. Scarcely detectable expression was noted in 786-O cells derived from primary renal cancer whose host had metastasis after nephrectomy, RCC-4 and A498 cells (fig. 1, *B*). Immunofluorescence revealed nuclear localization of HMGA1 in Caki-1, ACHN and NC65 cells (fig. 1, *C*). Significant HMGA1 expression was confirmed in ACHN and Caki-1 cells and, thus, these lines were subsequently used to examine HMGA1 function using RNA interference.

HMGA1 expression in RCC

	Total No.	No. Pos HMGA1 (%)	No. Neg HMGA1 (%)	p Value (Fisher exact test)
Kidney:				0.012
Normal	48	0	48 (100)	
Tumor	48	7 (15)	41 (85)	
Histological grade:				0.022
G1–2	36	3 (8)	33 (92)	
G3	9	4 (44)	5 (56)	
Metastasis clinical stage:				0.008
M0	30	1 (3)	29 (97)	
M1	18	6 (33)	12 (67)	

HMGA1 Knockdown

By siRNA. HMGA1 knockdown in Caki-1 and ACHN cells was achieved using HMGA1 specific siRNA. Immunoblot revealed that transient transfection with HMGA1 specific siRNA efficiently and significantly decreased HMGA1 expression in each cell line 2 days after transfection while scrambled control siRNA barely affected the HMGA1 level (fig. 2, *A*). These knockdown experiments were applied to the following experiments to evaluate HMGA1 function in the 2 cell lines.

Colony formation and cell proliferation inhibition. The effects of HMGA1 knockdown on cell growth and survival in Caki-1 and ACHN cells were examined using a colony formation assay. HMGA1 knockdown using HMGA1 specific siRNA in ACHN cells remarkably inhibited colony formation. However, transfecting control siRNA with a scrambled sequence scarcely affected the number of colonies (control vs HMGA1 siRNA mean 127.7 ± 5.4 vs 5.7 ± 2.5, $p < 0.01$, fig. 2, *B* and *C*). HMGA1 knockdown in Caki-1 cells also remarkably suppressed colony formation (control vs HMGA1 mean 86.0 ± 5.3 vs 7.3 ± 1.5, $p < 0.01$, fig. 2, *B* and *C*). Similarly HMGA1 knockdown in ACHN and Caki-1 cells remarkably suppressed cell proliferation (fig. 2, *D*).

Apoptosis induction. Since the results of our colony formation and cell proliferation assays suggested an inhibitory effect of HMGA1 knockdown on cell survival and growth, we examined how HMGA1 knockdown affected apoptosis induction. Transfection of HMGA1 specific siRNA induced significant apoptosis in ACHN and Caki-1 cells in a time dependent, siRNA sequence specific manner. Four days after transfection the mean ACHN sub-G1 fraction was 2.3% ± 0.4% vs 39.8% ± 0.7% and the mean Caki-1

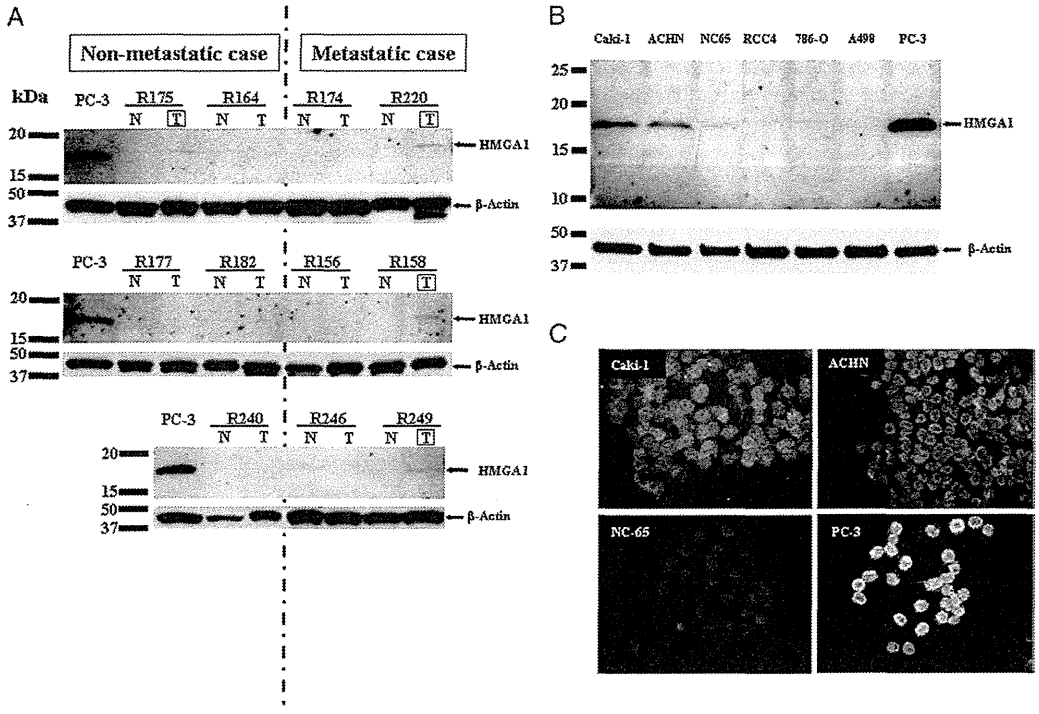

Figure 1. HMGA1 expression in RCC. PC-3 served as loading positive control. *A*, HMGA1 and β-actin expression in normal (*N*) and tumor kidney tissue from same patients was assessed by immunoblot. Boxed *T*, kidney tumor tissue expressing HMGA1. *T*, kidney tumor tissue with no HMGA1 expression. *B*, HMGA1 and β-actin expression in human RCC cell lines was assessed by immunoblot. *C*, immunofluorescence revealed HMGA1 nuclear localization in Caki-1, ACHN, NC-65 and PC-3 cells at different intensities.

sub-G1 fraction was 7.1% ± 1.0% vs 41.6% ± 1.7% for control siRNA vs HMGA1 specific siRNA, respectively (each $p < 0.01$, fig. 3, *A*). The blocking effect of the pancaspase inhibitor zVAD-fmk on apoptosis induction by HMGA1 knockdown was significant in ACHN but minimal in Caki-1 cells (fig. 3, *B*).

These findings suggest that HMGA1 has inhibitory effects on caspase independent and dependent apoptosis in RCC cells. The induction of apoptosis by transfection of HMGA1 specific siRNA in ACHN and Caki-1 cells was also confirmed by the emergence of annexin V positive cells (fig. 3, *C*).

Inhibition of ACHN cell in vitro invasion and migration. In vitro invasion assay after 24-hour incubation showed that the number of untreated ACHN cells invading the Matrigel coated membrane was high enough to be counted while that of untreated Caki-1 cells was not. Thus, the role of HMGA1 in cell invasion and migration was evaluated using ACHN cells.

In vitro invasion assay revealed that transfection of HMGA1 specific siRNA significantly decreased the number of ACHN cells invading the Matrigel coated membrane compared to the transfection of control siRNA (169.3 ± 10.1 vs 718.3 ± 163.5 cells, $p < 0.01$, fig. 4, *A*). Similarly our in vitro migration assay indicated that transfection of HMGA1 specific siRNA significantly decreased the number of ACHN cells migrating through the uncoated membrane compared to the transfection of control siRNA (299.0 ± 30.8 vs 590.0 ± 112.1 cells, $p < 0.05$, fig. 4, *B*).

Anoikis induction and phosphorylated Akt down-regulation in ACHN cells. Anoikis induction was first examined using untreated ACHN and Caki-1 cells. In each untreated cell line no significant increase was noted in apoptotic cells under nonadherent vs adherent culture conditions (fig. 5, *A*). Thus, the 2 cell lines were judged to be anoikis resistant. No significant anoikis was observed in ACHN cells transfected with control siRNA while significant anoikis was observed in ACHN cells transfected with HMGA1 specific siRNA (fig. 5, *B*). In contrast, no significant anoikis was induced in Caki-1 cells transfected with control or HMGA1 specific siRNA (fig. 5, *B*).

Since it was suggested in the literature that anoikis resistance is associated with Akt phosphorylation at Ser473, the phosphorylation of Akt in each cell line 4 days after siRNA transfection was examined by immunoblot analysis. In ACHN cells HMGA1 knockdown was associated with P-Akt down-regulation compared to transfection with control siRNA while total Akt expression was least affected by transfection of HMGA1 specific or control siRNA (fig. 5, *C*). Conversely HMGA1 knockdown in Caki-1

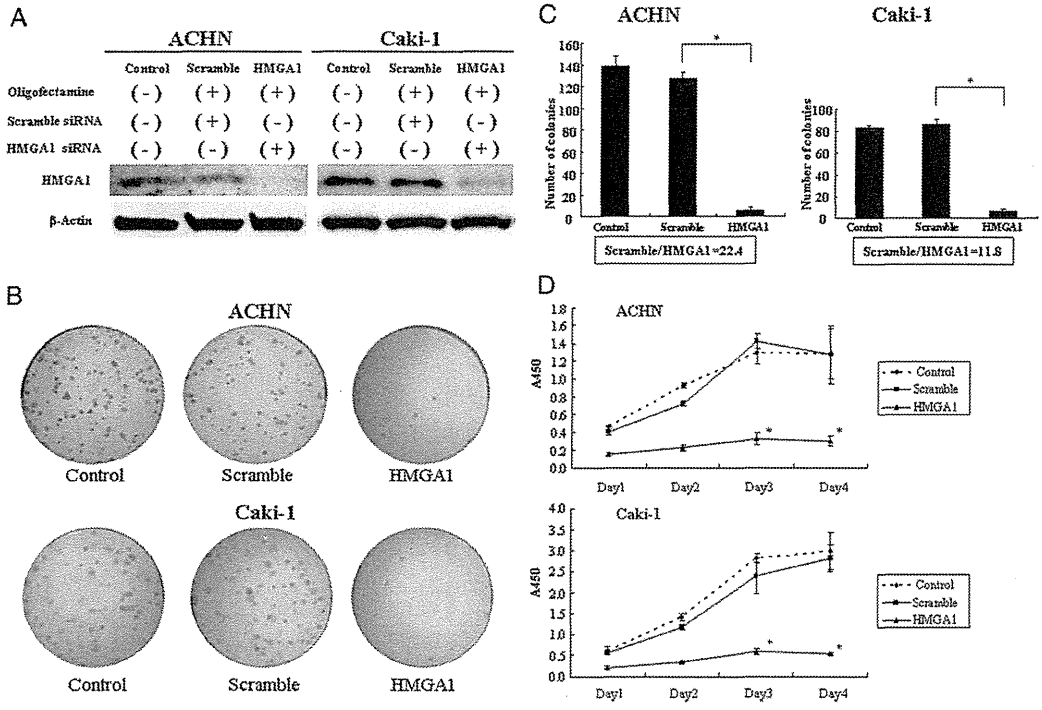

Figure 2. HMGA1 knockdown inhibited colony formation by RCC cells. *A*, expression of HMGA1 and β-actin as loading control in ACHN and Caki-1 cells was assessed by immunoblot. HMGA1 efficient decrease was confirmed in cells treated with HMGA1 specific siRNA (*HMGA1*) but not in cells treated with control siRNA (*Scramble*) vs untreated control cells. *B*, macroscopic appearance of ACHN and Caki-1 colonies treated with siRNA. Two days after siRNA transfection 150 cells were seeded per well. *C*, graphic presentation of the number of ACHN and Caki-1 colonies. Columns indicate mean of 3 preparations. Bars indicate SD. Asterisk indicates $p < 0.01$. *D*, ACHN and Caki-1 cell proliferation. Two days after transfection with siRNA on day 0 cells were seeded in 96-well plates for WST-8 assay on days 1 to 4. Points indicate mean of 6 preparations. Bars indicate SD. Asterisk indicates $p < 0.01$ vs cells treated with control siRNA.

cells did not lead to significant P-Akt down-regulation (fig. 5, *C*). These findings suggest that HMGA1 knockdown in cases associated with P-Akt down-regulation is involved in anoikis induction in a subset of RCC cells.

DISCUSSION

The introduction of molecular targeted drugs in clinical settings has conferred significant survival benefits for patients with mRCC but the antitumor effect of these drugs is relatively weak since they rarely achieve a complete response. Also, various side effects are commonly associated with molecular targeted drugs.[1] Identifying novel candidate target molecules with significant molecular functions is mandatory to develop new therapeutic modalities for mRCC. If the expression of these candidate molecules is observed exclusively in RCC cells but not in normal cells, the incidence of side effects should be minimal. In addition, if these candidate molecules have a significant role in cell growth or survival, therapy targeting and inhibiting these molecules should have significant antitumor effects. To this end we examined the expression and molecular function of HMGA1 in RCC cells.

HMGA1 expression is high in normal embryonic tissue but low or almost undetectable in adult tissue.[6] In contrast, HMGA1 is over expressed in cancer of various types, including cancer of the pancreas,[21] colon,[22] prostate,[13,14] ovary[23] and testis,[24] especially in association with high grade and metastasis.[2,5,21,25] HMGA1 is also a potent oncogene.[7,8,10,11] Furthermore, various important functions and roles in cancer have been reported for HMGA1. To our knowledge this is the first report to describe the expression and role of HMGA1 in RCC. We examined HMGA1 expression in RCC using surgical specimens and cell lines. We also estimated the molecular function of HMGA1 in RCC by HMGA1 knockdown using RNA interference.

HMGA1 was preferentially expressed in metastatic RCC tumor tissue and cell lines but not in normal tissue. Our functional analysis revealed that HMGA1 knockdown induced remarkable inhibition of colony formation and significant apoptosis in RCC cells. These findings make this molecule a candidate

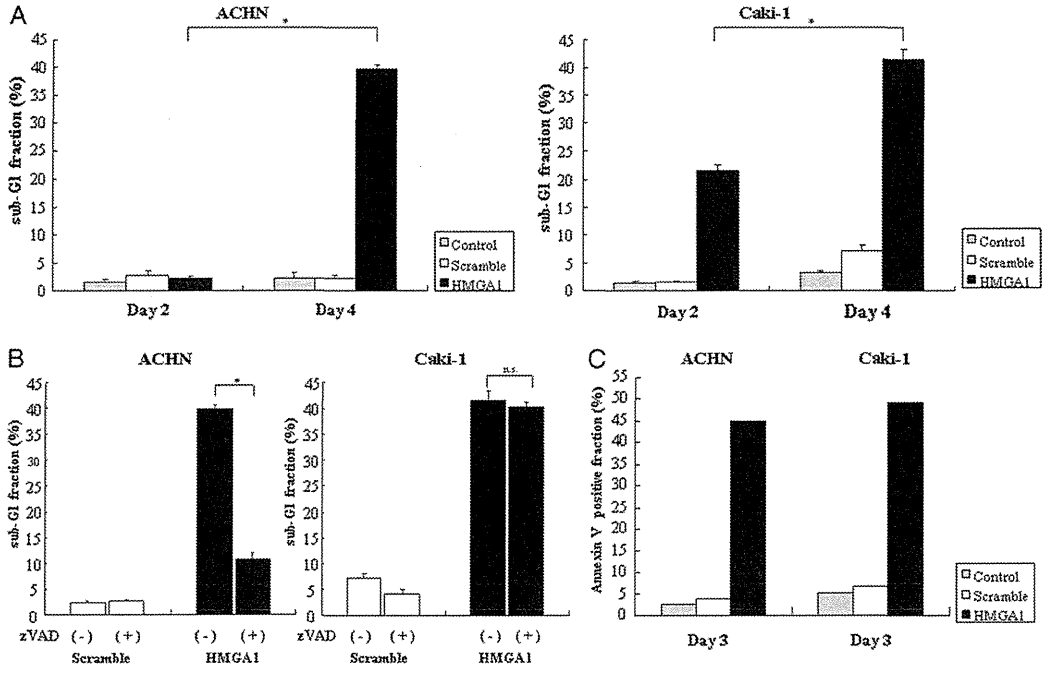

Figure 3. HMGA1 knockdown induced apoptosis in ACHN and Caki-1 RCC cells. siRNA treatment was done as described. *A* and *B*, apoptosis (sub-G1 fraction) was identified by flow cytometry. *A*, apoptosis time dependent induction. *B*, effect of pancaspase inhibitor zVAD-fmk on apoptosis. *C*, representative flow cytometry data on apoptosis. Apoptosis was evaluated by percent of annexin V positive fraction 3 days after siRNA transfection. *Scramble*, cells treated with control siRNA. Columns indicate mean of 3 preparations. Bars indicate SD. *n.s.*, not statistically significant ($p > 0.05$). Asterisk indicates $p < 0.01$.

for molecular targeted therapy for mRCC and suggest a significant antitumor effect with minimal side effects.

Our study also suggests that HMGA1 has a role in the metastatic cascade, including the acquisition of invasion potential and anoikis resistance. After cancer cells detach from a primary site upon acquiring invasion potential they must survive anoikis to develop metastatic lesions. Resistance to anoikis or detachment induced apoptosis was suggested as a phenotypic hallmark of metastatic cancer cells.[26] The PI3 kinase/Akt pathway activation is reportedly involved in resistance to anoikis.[26]

Notably Liau et al reported that HMGA1 in pancreatic cancer cells is involved in anoikis resistance and associated with Akt activation.[20] Our study suggests that HMGA1 knockdown in cases associated with P-Akt down-regulation is involved in anoikis

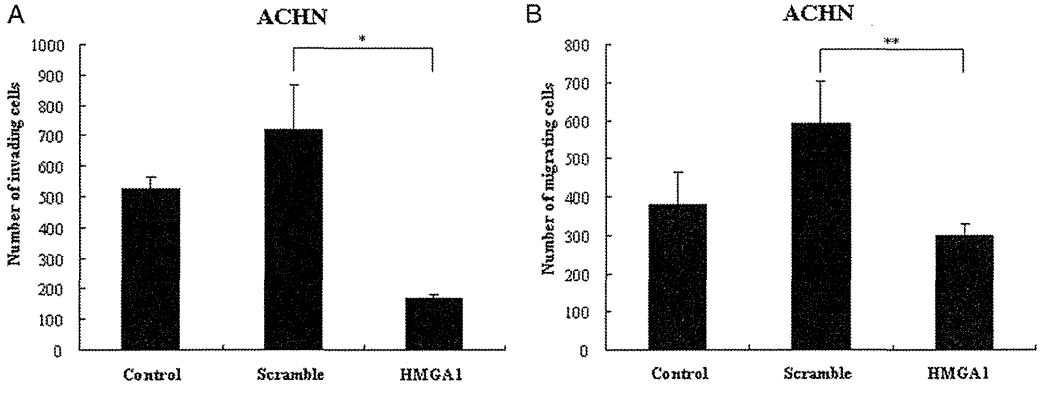

Figure 4. HMGA1 knockdown inhibited ACHN cell invasion and migration in vitro. siRNA treatment was done as described. *Scramble*, cells treated with control siRNA. Columns indicate mean of 3 preparations. Bars indicate SD. *A*, asterisk indicates $p < 0.01$. *B*, asterisks indicate $p < 0.05$.

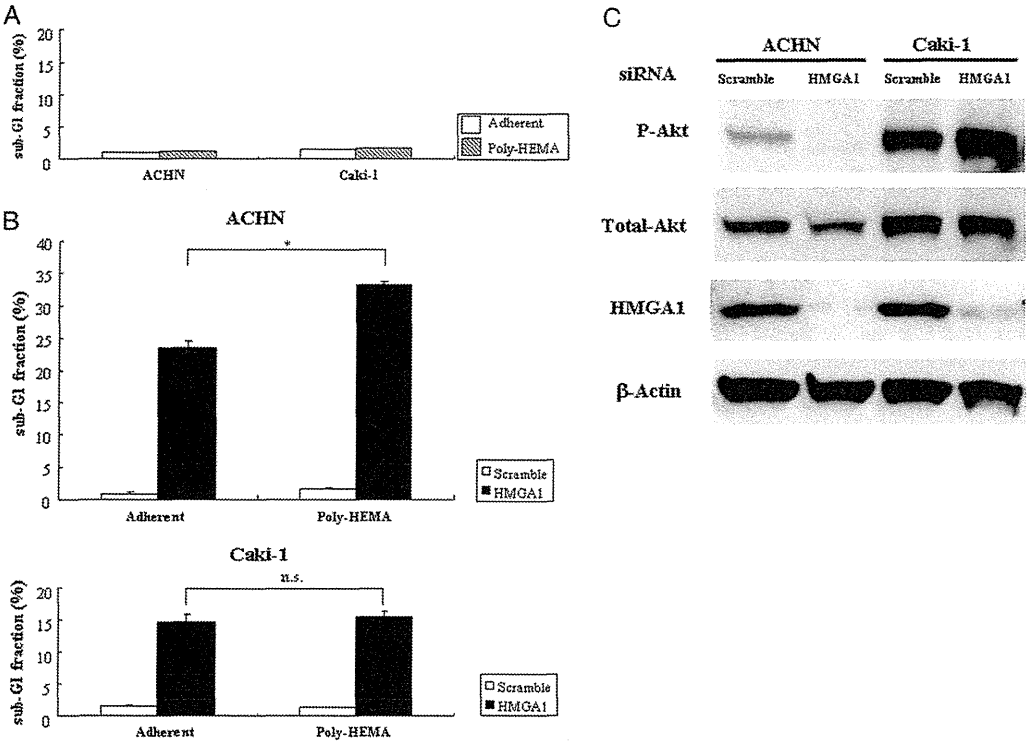

Figure 5. HMGA1 knockdown affected RCC cell anoikis. siRNA treatment was done as described. *A*, ACHN and Caki-1 were resistant to anoikis. No increase in apoptosis under nonadherent conditions with poly-HEMA was noted vs adherent conditions in each cell line. *B*, HMGA1 knockdown induced significant anoikis in ACHN but not in Caki-1 cells. *Scramble*, cells treated with control siRNA. Columns indicate mean of 3 preparations. Bars indicate SD. *n.s.*, not statistically significant ($p > 0.05$). Asterisk indicates $p < 0.01$. *C*, immunoblot produced using antibodies against P-Akt, total Akt, HMGA1 and β-actin showed that HMGA1 knockdown led to down-regulation of P-Akt associated with anoikis induction in ACHN but not Caki-1 cells.

induction in RCC cells. This indicates that therapy targeting HMGA1 could inhibit the formation of metastatic lesions that develop from existing metastatic lesions, which would significantly affect the survival of patients with mRCC.

Several drugs that affect HMGA1 DNA binding activity have been examined to estimate their efficacy and toxicity in animal models and clinical trials, including distamycin, FR900482, FK317, netropsin and NOX-A50.[27,28] Alternatively other groups have targeted important molecules downstream of HMGA1, including COX-2, STAT3 and MMP-2.[29] HMGA1 has multiple important biological roles in cancer cells, including oncogenic activity in vitro[7,8] and in vivo,[10,11] cell growth,[8,15] metastatic potential,[5,7,14,16] chromosomal rearrangement,[17] and p53[3] and Rb[4] inhibition, in addition to the regulation of gene expression.[2] Thus, antisense therapy for HMGA1 that suppresses the expression of only 1 gene would produce multiple effects simultaneously, which might contribute greatly to mRCC control. Chi et al developed an antisense construct for clusterin and applied it clinically to treat castration resistant prostate cancer.[30] Further in vivo studies and the development of an antisense construct are warranted to confirm whether HMGA1 is a good candidate molecule for targeted therapy of mRCC.

CONCLUSIONS

HMGA1 was preferentially expressed in mRCC but not expressed in normal kidney tissue. Functional analysis revealed that HMGA1 knockdown markedly inhibited colony formation, significantly induced apoptosis, inhibited invasion potential and induced anoikis. These findings suggest that HMGA1 is a potential target molecule for novel molecular targeted therapy of mRCC.

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Original Article

Routine Clinical Use of the One-Step Nucleic Acid Amplification Assay for Detection of Sentinel Lymph Node Metastases in Breast Cancer Patients

Results of a Multicenter Study in Japan

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BACKGROUND: The objective of this study was to confirm, by means of a multicenter study conducted in Japan, the reliability and usefulness of the one-step nucleic acid amplification (OSNA) assay in routine clinical use for sentinel lymph node biopsy (SLNB) of breast cancer patients. **METHODS:** Patients with Tis-T2N0M0 breast cancer who underwent SLNB before systemic chemotherapy comprised the study cohort. A whole sentinel lymph node (SLN) was examined intraoperatively with the OSNA assay except for a 1-mm-thick, central slice of the lymph node, which underwent pathologic examination after the operation. For patients who underwent axillary dissection, non-SLNs were examined with routine pathologic examination. **RESULTS:** In total, 417 SLNBs from 413 patients were analyzed. SLN metastases were detected with greater sensitivity by the OSNA assay than by pathologic examination (22.5% vs 15.8%; $P < .001$), as expected from the difference in size of the specimens examined. Patients who had SLN metastases assessed with the OSNA assay proved to harbor non-SLN metastases with an overall risk ratio of 33.7%. The risk of non-SLN metastasis was significantly lower for patients who had positive SLNs assessed as OSNA+ than for those who had SLNs assessed as OSNA++ (17.6% vs 44%; $P = .012$). **CONCLUSIONS:** The OSNA assay can be used for routine clinical SLNB, and its assessment for volume of metastasis may be a powerful predictive factor for non-SLN metastasis. Further studies with more patients are needed to confirm the usefulness of this assay for selection in the clinical setting of patients who do not need axillary dissection. ***Cancer* 2012;118:3477-83.** © *2012 American Cancer Society.*

KEYWORDS: breast, sentinel, cytokeratin, messenger RNA, one-step nucleic acid amplification assay, metastasis.

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INTRODUCTION

Sentinel lymph node biopsy (SLNB) has been a standard procedure for patients with early stage breast cancer.[1,2] However, to date, the method for examining sentinel lymph nodes (SLN) has not been standardized. Hematoxylin and eosin (H&E) staining for multistep sections with or without immunohistochemistry for cytokeratin (CK) generally is recommended,[3] although it is not known how many specimens should be examined. To overcome this problem, automated molecular detection systems for lymph node metastases, such as the one-step nucleic acid amplification (OSNA) assay (Sysmex, Kobe, Japan) and the Geneseach breast lymph node (BLN) assay (Veridex, Raritan, NJ) have been developed and are receiving much attention recently.[4,5] Several studies have shown that these new tests can detect lymph node metastases with the same statistically determined accuracy as the conventional pathologic examination,[6-15] which indicates that a molecular test may constitute an alternative to pathology. However, in those previous studies, only half the volume of a lymph node was examined with the molecular test, because the remaining half was used for pathologic examination as the standard procedure. Essentially, some results obtained with the 2 methods are discrepant, especially when a lymph node harbors micrometastases. The molecular test originally was supposed to examine a whole lymph node with high sensitivity for detecting cancer deposits and also with much less labor than what is required for a thorough pathologic examination of a great number of sections. Nevertheless, currently, pathology remains the gold standard, and using the molecular tests may generate some anxiety about, for example, technical failure and mechanical trouble. How to use the molecular tests for SLNB in the daily clinical setting is therefore still controversial.

The OSNA assay, a molecular diagnostic system for lymph node metastasis that detects cytokeratin 19 (CK19) messenger RNA (mRNA) of cancer cells, was approved by the Japanese Ministry of Health, Labor and Welfare in June 2008 and has been covered by the Japanese National Health Insurance system since November 2008. In view of these developments, we conducted a multicenter study of the clinical use of the OSNA assay for SLNB, in which most of an SLN was examined with the OSNA assay, and only a central, 1-mm-thick slice of the SLN was preserved as a permanent pathologic section. The reliability and usefulness of the OSNA assay in clinical use and the relation between the OSNA assessment and the risk of non-SLN metastasis are described in this report.

MATERIALS AND METHODS

Study Design

The objective of this study was to determine the usefulness of the OSNA assay for clinical use in SLNB of breast cancer. The primary endpoint was to examine the superiority of the OSNA assay for detecting metastases in SLN compared with pathologic examination with H&E staining for a single SLN section. The secondary endpoint was to investigate the relation between non-SLN metastasis and the OSNA assessment for CK19 mRNA copy numbers in SLN. SLNs were detected using both radiocolloids and blue dye, radiocolloids only, or blue dye only. Removed SLNs were prepared according to the protocol detailed below and were assessed immediately with the OSNA assay. Patients had axillary lymph node dissection (ALND) recommended when necessary according to the OSNA assessment and/or other clinicopathologic factors. The level of axillary dissection was determined by the surgeon according to the patient's condition and institutional guidelines. Non-SLNs were examined with a routine pathologic examination using H&E staining. Each patient received appropriate postoperative adjuvant therapy and/or radiotherapy based on the clinicopathologic findings and in accordance with guidelines if necessary, and each patient was followed at the treating center.

The study group comprised 11 hospitals, which are the central institutions for breast cancer therapy and research in each area of Japan. The study protocol was approved by the institutional review board of each center.

Patients and Sentinel Lymph Node Biopsy

The enrolment for this study comprised patients with tumor in situ (Tis) through T2, clinically lymph node-negative primary breast cancer who underwent SLNB between August 2009 and December 2010 at 1 of the participating hospitals. Patients who had a preoperative diagnosis of ductal carcinoma in situ (DCIS) were enrolled in the study when a surgeon judged SLNB was needed. Patients who underwent SLNB before receiving preoperative systemic chemotherapy (PSCT) also were eligible for the analysis of sensitivity of the OSNA assay, although those who received chemotherapy or hormone therapy before SLNB were excluded from the study. Men also were excluded. Patients received the necessary information about the study, and only those who gave their consent and underwent SLNB successfully were enrolled.

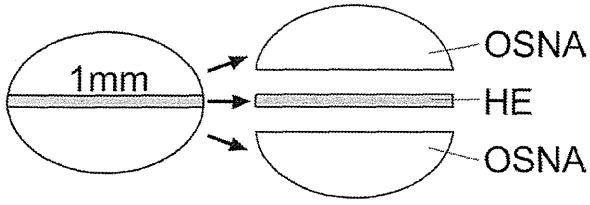

Figure 1. A 1-mm-thick slice was cut out from the longitudinal central part of the sentinel lymph node for staining with hematoxylin and eosin (HE), and the remaining parts were examined by using the one-step nucleic acid amplification (OSNA) assay.

Preparation of Sentinel Lymph Nodes and the One-Step Nucleic Acid Assay

Preparation of an SLN is shown in Figure 1. Fat tissue surrounding the SLN was trimmed off. A 1-mm-thick slice was then cut out from the longitudinal central part of the SLN, fixed as a permanent section for staining with H&E, and examined postoperatively by a pathologist at one of the hospitals. The remaining part of the lymph node was immediately examined with the OSNA assay by laboratory technicians at the hospitals in the manner described previously.[7]

An SLN was assessed with the OSNA assay according to the cutoff level of calculated CK19 mRNA copy numbers per microliter determined by Tsujimoto et al, and the results were reported according to the manufacturer's instructions: that is, as negative ($<2.5 \times 10^2$ copies/μL), + positive ($\geq 2.5 \times 10^2$ and $<5.0 \times 10^3$ copies/μL), ++ positive ($\geq 5.0 \times 10^3$ copies/μL), or positive +i (inhibited in the regular sample and $\geq 2.5 \times 10^2$ copies/μL in the diluted sample).[4]

Statistical Analysis

Sensitivity of the OSNA assay and of pathologic examination for the detection of metastasis was compared and analyzed with the McNemar test. The risk of non-SLN metastases for OSNA-positive patients was calculated with the chi-square test.

RESULTS

In total, 439 patients, including 9 women with bilateral breast cancer, were enrolled in this study. Five of the 9 women with bilateral disease underwent unilateral SLNB, and the remaining 4 women underwent bilateral SLNB, and the biopsy specimens were examined with the OSNA assay. Twenty-one of the originally enrolled patients were excluded from the analysis because of significant violations against the study protocol, including 8 patients who

Table 1. Patient Characteristics

Characteristic	No. of Patients (%)
Average age [range], y	56.1 [25-90]
Menopausal status	
Premenopausal	169 (40.9)
Postmenopausal	243 (58.8)
Unknown	1 (0.2)
Clinical tumor classification	
Tis	50 (12)
T1	254 (60.9)
T2	111 (26.6)
T3	2 (0.5)
Timing of SLNB	
Preoperative	47 (11.3)
Intraoperative	370 (88.7)
Method of SLNB	
Dye only	107 (25.7)
RI only	51 (12.2)
Dye and RI	259 (62.1)
Operation	
Total mastectomy	156 (37.4)
Partial mastectomy	248 (59.5)
Others	2 (0.5)
Surgery after PSCT	11 (2.6)
Axillary dissection	
Not done	305 (73.1)
Level I only	49 (11.8)
Levels I and II	52 (12.5)
Unknown[a]	11 (2.6)
Pathologic type	
Ductal carcinoma in situ	53 (12.7)
Invasive ductal carcinoma	305 (73.1)
Invasive lobular carcinoma	24 (5.8)
Others	25 (6)
Unknown	10 (2.4)
Tumor grade	
1	183 (43.9)
2	110 (26.4)
3	70 (16.8)
Unknown	54 (12.9)
Hormone receptor status	
Positive	335 (80.3)
Negative	66 (15.8)
Unknown	16 (3.8)
Her2 status	
Positive	51 (12.2)
Negative	334 (80.1)
Unknown	32 (7.7)
Lymphatic invasion	
Positive	30 (7.2)
Negative	376 (90.2)
Unknown	11 (2.6)

Abbreviations: Her2, human epidermal growth factor receptor 2; SLNB, sentinel lymph node biopsy; PSCT, preoperative systemic chemotherapy; Tis, tumor in situ; R1

[a] Patients received PSCT after SLNB.

received PSCT before SLNB, 10 patients who were not examined with the OSNA assay, 2 patients whose central sections of the SLN did not undergo pathologic examination as a permanent specimen for H&E staining, and 1 patient who was a man. Two patients who had benign intraductal papilloma confirmed after surgery, 1 who had with a clinical T4 tumor, and 2 who had clinically evident axillary lymph node metastases also were excluded because they did not meet the general criteria for SLNB candidates. Conversely, 2 patients who had T3 tumors that finally were diagnosed as DCIS and T1, invasive cancer were included. The final total enrolment was 413 patients who had 417 SLNBs eligible for analysis.

In total, 775 SLNs were obtained from 417 SLNBs, and the average number of SLNs was 1.86 (1-7 SLNs) per patient. Of those, 762 SLNs (98.3%) were examined successfully with the OSNA assay. In 5 biopsies that had multiple SLNs, >4 excess lymph nodes were assessed by means of pathology (total, 13 SLNs). One hundred and one patients underwent ALND, including 49 patients who underwent level I dissection and 52 patients who underwent level I and II dissections. Of those, 86 patients had positive OSNA assessments, and 15 patients had negative OSNA assessment. Seven OSNA-negative patients underwent delayed ALND based on pathology results after primary surgery. The final axillary status of 11 patients who received PSCT after SLNB was unknown. Patient characteristics are summarized in Table 1.

Of 417 SLNBs, including 11 from patients who received PSCT after SLNB, the OSNA assay identified SLN metastases in 94 biopsies (22.5%), and pathologic examination of a single section identified SLN metastases in 66 biopsies (15.8%) (Table 2). Thus, the OSNA assay detected significantly more metastases than pathologic examination of a single H&E-stained section ($P < .001$), as expected, because most of each SLN was examined by means of the OSNA.

There were 44 results that were discordant: that is, there were 36 OSNA-positive/pathology-negative (O+/P−) sections and 8 O−/P+ sections (Table 3). In 7 of the O−/P+ patients, only micrometastases were identified in the SLN, and macrometastasis was identified in 1 SLN with a tumor in which further immunohistochemical analysis revealed a low level of CK19 protein expression. Isolated tumor cells were identified in SLNs from 2 of the 36 O+/P− patients, and non-SLN metastases were

Table 2. Comparison of the One-Step Nucleic Acid Assay With Pathology[a]

	Pathology		
OSNA Assay	Positive	Negative	Total
Positive	58	36	94
Negative	8	315	323
Total	66	351	417

Abbreviations: OSNA, one-step nucleic acid amplification.
[a] $P < .001$ (McNemar test).

Table 3. Summary of Discordant Cases Between the One-Step Nucleic Acid Assay and Pathology

SLN Metastasis		Non-SLN Metastasis	Pathologic Diagnosis of the Main Tumor[a]
OSNA Assay	Pathology		
Negative, n = 8	Positive (macrometastasis), n = 1	Positive, n = 1	IDC, n = 1[a]
	Positive (micrometastasis), n = 7	Positive, n = 1	IDC, n = 1
		Negative, n = 5	IDC, n = 3
			MUC, n = 2
		Not assessed, n = 1	IDC, n = 1
Positive, n = 36	Negative, n = 34	Positive, n = 6	IDC, n = 4
			ILC, n = 1
			Unknown, n = 1
		Negative, n = 27	IDC, n = 17
			ILC, n = 3
			DCIS, n = 6
			Others, n = 1
		Not assessed, n = 1	Unknown, n = 1
	ITC, n = 2[c]	Positive, n = 1	IDC, n = 1
		Not assessed, n = 1	Unknown, n = 1

Abbreviations: IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; ILC, invasive lobular carcinoma; ITC, isolated tumor cells; MUC, mucinous carcinoma.
[a] Cytokeratin 19 was not detected with immunohistochemistry in the main tumor.

Table 4. The Risk of Nonsentinel Lymph Node Metastasis in One-Step Nucleic Acid Assay-Positive Patients Who Undergo Axillary Dissection

	Axillary Dissection			Non-SLN Metastases			
OSNA Assay Results[a]	No. Level I	No. Levels I+II	*P*	No. Positive	No. Negative	% Positive	*P*
Positive	40	46		29	57	33.7	
+	18	16	.421	6	28	17.6	.012
++	22	28		22	28	44.0	
+i	0	2	—	1	1	50.0	—

Abbreviations: OSNA, one-step nucleic acid amplification; SLN, sentinel lymph node.
[a] Positive OSNA results were scored as + ($\geq 2.5 \times 10^2$ copies/μL and $<5.0 \times 10^3$ copies/μL); ++ ($\geq 5.0 \times 10^3$ copies/μL), or i+ (inhibited in the regular sample and $\geq 2.5 \times 10^2$ copies/μL in the diluted sample).

identified in 7 patients. Therefore, in total, 9 of the O+/P− patients (25%) harbored cancer cells in either SLNs or non-SLNs.

Of the 86 OSNA-positive biopsies from patients who underwent axillary dissection, 34 were assessed as +, 50 were assessed as ++ and 2 were assessed as +i. In total, 18 of 34 patients with OSNA + results and 22 of 50 patients with OSNA ++ results underwent Level I ALND alone. There was no relation between the level of ALND and OSNA assessment ($P = .421$). Six patients (17.6%) who had OSNA + results and 22 patients (44%) who had OSNA ++ results had non-SLN metastases (Table 4). The risk of non-SLN metastasis was significantly lower for patients who had positive SLNs assessed as OSNA + versus those who had SLNs assessed as OSNA ++ ($P = .012$).

DISCUSSION

It has been demonstrated that the OSNA assay has the same capability for detecting lymph node metastasis as conventional pathologic examination.[6-11] However, only a few studies have presented data regarding clinical use of the assay.[10] In our study, most of each SLN was examined intraoperatively by using the OSNA assay, and the decision whether to perform axillary dissection was based in principle on the assay results. Only a single 1-mm-thick, central slice of the lymph node was used for pathologic examination. Therefore, we expected that the OSNA assay would have higher sensitivity for SLN metastasis than pathologic examination, and the results were as expected. There were some discordant cases in our study, which also was expected, because this is inevitable when 2 modalities are used to examine different parts of the lymph nodes. Of the 44 discordant results, 8 were OSNA-negative, in which postoperative pathologic examination identified metastasis. In the 7 patients who had micrometastasis identified, discordance may have occurred because of the uneven allocation of minuscule metastases in an SLN. However, in 1 patient with macrometastasis, low expression of the CK19 protein in the main tumor was confirmed as the result of further immunohistochemical examination performed by a pathologist at the concerned hospital. The incidence of low expression of the CK19 protein in breast cancer was reported previously as 1.6%.[16] However, the expression of protein and mRNA can be expected to be different, especially between the main tumor and metastatic sites. In fact, the reported incidence of discordance between OSNA and pathology caused by low expression of CK19 mRNA is very low, from 0.2% to 0.5% of examined lymph nodes in previous studies[7,9] and 0.1% of examined lymph nodes and 0.2% of all patients in our study. Lack of CK19 expression is associated significantly with the triple-negative (estrogen receptor negative, progesterone receptor negative, and human epidermal growth factor receptor 2 [Her2] negative) phenotype.[17] Some adjuvant chemotherapy is likely to be used for such patients based on other factors, although SLN is assessed as negative by the OSNA because of low expression of CK19. Therefore, this false-negative aspect may have only a minimal effect on patients' clinical prognosis, because pathologic examination of 1 preserved slice of the lymph node can negate such an effect.

Conversely, there were 36 O+/P− discordant cases, including 2 with isolated tumor cells in the SLNs that were assessed by pathology. Of the 34 patients who underwent axillary dissection, non-SLN metastases were identified in 7 patients. The OSNA assay had made an accurate assessment of these patients. It is interesting to note that there were 6 patients with DCIS among these O+/P− cases. Microinvasion was suspected in a core-needle

biopsy specimen from 1 patient. Two patients had widespread DCIS that measured >6 cm, and another had multiple lesions. The remaining 2 patients had high-grade DCIS. Ansari et al reported in their review that the estimated incidence of SLN metastases in patients who had a definitive diagnosis of DCIS alone was 3.7%.[18] Thus, the OSNA assay can detect metastases with high sensitivity even in tumors diagnosed pathologically as DCIS, and such findings may result in an upgrade of the clinical stage of such tumors.

The clinical significance of micrometastases in SLNs is controversial. de Boer et al reviewed 58 studies concerning this issue and concluded that the presence of metastases measuring ≤2 mm in greatest dimension in axillary lymph nodes detected on single-section examination was associated with poorer disease-free and overall survival.[19] Reed et al reported the results from a prospective study indicating a significant association between SLN micrometastasis and distant recurrence.[20] Conversely, Hansen et al reported that micrometastatic tumor deposits in SLNs, pN0(i+) or pN1mi, detected by H&E staining or immunohistochemistry do not have clinical significance for disease-free or overall survival.[21] In the study, >90% of patients with micrometastases received adjuvant systemic therapy, although only 66% of those without metastases received such therapy. Weaver et al reported that occult metastases were detected by means of further examination using immunohistochemistry in 15.9% of patients with pathologically negative SLNs who were enrolled in The National Surgical Adjuvant Breast and Bowel Project trial B-32.[22] That report revealed significant differences in overall survival, disease-free survival, and distant-disease-free survival between patients with and without occult metastases. Nevertheless, the authors concluded that the data did not indicate a clinical benefit of additional evaluation, including immunohistochemical analysis, of initially negative SLNs, because the magnitude of the difference in outcome was so small. However, tumor size, endocrine therapy, and radiation therapy were independent prognostic factors of death or distant disease in the patients studied, which may have reduced the difference in prognostic outcomes. Results from the Micrometastases and Isolated Tumor Cells (MIRROR) study also indicated that both isolated tumor cells and micrometastases in axillary lymph nodes were associated significantly with a worse prognosis for patients who have favorable, early stage breast cancer who did not receive adjuvant systemic therapy.[23] That report indicated that adjuvant systemic therapy could improve the 5-year disease-free survival of such patients with micrometastases with a gain in 5-year disease-free survival of nearly 10%. Thus, a precise initial evaluation of SLN metastasis is important for the accurate assessment of clinical stage and the appropriate selection of adjuvant treatment for each patient. The OSNA assay, which can evaluate the volume of metastases in SLNs semiquantitatively, is a useful tool for an accurate assessment of clinical stage of breast cancer patients.

The original objective of SLNB was to avoid axillary dissection and reduce postoperative adverse morbidity for patients without axillary lymph node metastasis. Giuliano et al indicated that axillary dissection may not be needed even for patients with 1 or 2 positive SLNs who have undergone breast-conserving surgery with postoperative whole-breast radiation and systemic adjuvant therapy, as indicated by the results from the American College of Surgeons Oncology Group Z0011 study.[24] However, it remains unknown whether axillary dissection also may be omitted for patients who have ≥3 positive SLNs and for those who have positive SLNs and undergo total mastectomy. Therefore, accurate clinical staging and selection of patients who do not need axillary dissection remain the goals of SLNB. Previous reports indicated that approximately 60% of patients with positive SLN did not have any non-SLN metastasis[25,26] and that such patients basically did not need axillary dissection. In our study, 66.3% of patients who had SLN metastases identified by the OSNA assay did not have non-SLN metastases. Conversely, 17.6% of patients with OSNA+ results and 44% of patients with OSNA++ results had non-SLN metastasis, which are ratios similar to those previously reported (range, 13%-22% for patients with SLN micrometastasis; 45%-79% for patients with SLN macrometastasis[27]), and such patients may have suffered axillary recurrence because they underwent total mastectomy and did not undergo axillary dissection. Thus, how to select patients with a high or low risk of non-SLN metastasis remains an important issue for the use of SLNB. The tumor volume in SLN is considered a significant factor for the prediction of non-SLN metastasis.[25,27,28] It is easy to assess tumor volume in SLNs semiquantitatively with the OSNA assay, and this ease of operation constitutes a major advantage over conventional pathologic examination. Data from larger numbers of patients are expected to determine the appropriate cutoff level of the OSNA assay for the selection of patients who do not need additional axillary dissection.

In conclusion, the OSNA assay is considered reliable in the clinical setting for the routine intraoperative examination of SLN and is useful because it can be performed easily by a nonpathologist. However, further studies to obtain long-term follow-up data for greater numbers of patients are needed to confirm the clinical significance, especially the prognostic impact, of results of the OSNA assay of SLNB for breast cancer.

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CONFLICT OF INTEREST DISCLOSURES

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Patterns of Practice in Intensity-modulated Radiation Therapy and Image-guided Radiation Therapy for Prostate Cancer in Japan

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Background: The purpose of this study was to compare the prevalence of treatment techniques including intensity-modulated radiation therapy and image-guided radiation therapy in external-beam radiation therapy for prostate cancer in Japan.
Methods: A national survey on the current status of external-beam radiation therapy for prostate cancer was performed in 2010. We sent questionnaires to 139 major radiotherapy facilities in Japan, of which 115 (82.7%) were returned.
Results: Intensity-modulated radiation therapy was conducted at 67 facilities (58.3%), while image-guided radiation therapy was conducted at 70 facilities (60.9%). Simulations and treatments were performed in the supine position at most facilities. In two-thirds of the facilities, a filling bladder was requested. Approximately 80% of the facilities inserted a tube or encouraged defecation when the rectum was dilated. Some kind of fixation method was used at 102 facilities (88.7%). Magnetic resonance imaging was routinely performed for treatment planning at 32 facilities (27.8%). The median total dose was 76 Gy with intensity-modulated radiation therapy and 70 Gy with three-dimensional radiation therapy. The doses were prescribed at the isocenter at the facilities that conducted three-dimensional radiation therapy. In contrast, the dose prescription varied at the facilities that conducted intensity-modulated radiation therapy. Of the 70 facilities that could perform image-guided radiation therapy, 33 (47.1%) conducted bone matching, 28 (40.0%) conducted prostate matching and 9 (12.9%) used metal markers. Prostate or metal marker matching tended to produce a smaller margin than bone matching.
Conclusions: The results of the survey identified current patterns in the treatment planning and delivery processes of external-beam radiation therapy for prostate cancer in Japan.

Key words: radiation therapy – urologic-radoncol – radiation oncology