Table 1. Patient backgrounds; family history of prostate cancer, age at diagnosis and PSA value at diagnosis

	2001	2002	2003	Total	%
Family history					
No history	5959	6104	1771	13 834	79.9
Within 2nd degree of relationship	120	128	29	277	1.6
Within 3rd degree of relationship	11	8	3	22	0.1
Don't know	1412	1453	314	3179	18.4
Total	7502	7693	2117	17 312	100.0
Age at diagnosis					
<60	329	320	61	710	4.1
60–64	596	620	161	1377	8.0
65–69	1197	1265	331	2793	16.1
70–74	1935	2037	567	4539	26.2
75–79	1798	1889	562	4249	24.5
≥80	1647	1562	435	3644	21.0
Total	7502	7693	2117	17 312	100.0
PSA at diagnosis					
<4	255	269	73	597	3.4
4–<10	1680	1863	556	4099	23.7
10–<20	1470	1628	493	3591	20.7
20–<50	1459	1514	387	3360	19.4
≥50	2612	2401	606	5619	32.5
No description	26	18	2	46	0.3
Total	7502	7693	2117	17 312	100.0

Number of Registered Patients

As shown in Fig. 2, 17 872 patients were registered by October 2003. This survey investigated patients who were first diagnosed with prostate cancer at the registered institutions during this period. Respectively, 7952 and 8195 new patients were reported in 2001 and 2002 by 246 and 216 institutions. Of these new patients, 5969 and 6064 were newly administered hormone therapy, and 5646 and 5651 were registered with J-CaP. In summary, it is shown that 75% of new patients were given hormone therapy in some form and 70% registered with J-CaP.

Patient Background Factors

Of the 17 872 registered patients at the time of data compilation, data were collected from 17 312 patients. 529 cases without any record of hormone therapy commencement date were excluded, as were 31 cases whose therapy was reported as commencing in 2000. Family history, age at diagnosis and PSA value at diagnosis are given in Table 1.

Tumor Background Factors

A summary of Gleason score, histological grade, TNM classification and clinical stage (TNM) is given in Table 2.

Table 2. Tumor backgrounds; Gleason score, histological grade, TNM classification, TNM clinical stage

	2001	2002	2003	Total	%
Gleason score					
2–4	654	551	150	1355	7.8
5	696	744	251	1691	9.8
6	1029	1250	401	2680	15.5
7	1595	1958	579	4132	23.9
8–10	1801	2337	590	4728	27.3
No description	1727	853	146	2726	15.7
Total	7502	7693	2117	17 312	100.0
Histological differentiation					
Well	1489	1554	453	3496	20.2
Moderate	3360	3362	990	7712	44.5
Poor	1995	1997	513	4505	26.0
Unknown	103	119	16	238	1.4
No description	555	661	145	1361	7.9
Total	7502	7693	2117	17 312	100.0
T stage					
T0	1	3	0	4	0.0
T1	1630	1813	518	3961	22.9
T2	2566	2680	832	6078	35.1
T3	2597	2509	589	5695	32.9
T4	673	657	157	1487	8.6
Tx	27	25	12	64	0.4
No description	8	6	9	23	0.1
Total	7502	7693	2117	17 312	100.0
N factor					
N0	6000	6315	1767	14 082	81.3
N1	1004	917	210	2131	12.3
Nx	462	427	119	1008	5.8
No description	36	34	21	91	0.5
Total	7502	7693	2117	17 312	100.0
M factor					
M0	5380	5696	1634	12 710	73.4
M1	157	119	12	288	1.7
M1a	83	77	11	171	1.0
M1b	1496	1428	327	3251	18.8
M1c	100	71	19	190	1.1
Mx	250	268	93	611	3.5
No description	36	34	21	91	0.5
Total	7502	7693	2117	17 312	100.0
Clinical stage					
II	3684	3987	1188	8859	51.2
II	1273	1327	326	2926	16.9
IV	2082	1945	444	4471	25.8
No description	463	434	159	1056	6.1
Total	7502	7693	2117	17 312	100.0

Table 3. Purpose of hormone therapy

	2001	2002	2003	Total	%
Hormonal therapy					
Main	5926	5914	1585	13 425	77.5
Adjuvant	306	366	81	753	4.3
Neoadjuvant	1270	1413	451	3134	18.1
Total	7502	7693	2117	17 312	100.0
Hormonal therapy detail					
Orchiectomy only	236	214	63	513	3.0
Orchiectomy + medication	605	427	96	1128	6.5
LH-RHa only	826	1065	319	2210	12.8
LH-RHa + anti-androgen	4431	4703	1249	10 383	60.0
Anti-androgen only	392	584	251	1227	7.1
Other	1012	700	139	1851	10.7
Total	7502	7693	2117	17 312	100.0

HORMONE THERAPY

As to the reason for hormone therapy, primary application of hormone therapy was the most prevalent, comprising 77.5% of the total, followed by 18.1% neoadjuvant and 4.3% adjuvant (Table 3).

Table 3 also indicates an overview of the types of hormone therapy. The combined use of LH-RHa + anti-androgen drug is the largest, comprising 60%. Anti-androgen monotherapy was 7.1% and LH-RHa monotherapy was 12.8%.

Table 4 shows the relations between the purpose of hormone therapy and T category, clinical stage, Gleason score and age. A notable feature is that in all categories, primary use of hormone therapy was the most common.

Table 5 shows the relations between the type of hormone therapy and T category, clinical stage, Gleason score and age. In all categories and ages, combined androgen blockade (CAB) was used in the main. In Table 6, details are given of the main treatment methods when hormone therapy was administered as neoadjuvant, as well as the details of main treatment methods when used as adjuvant.

COMPLIANCE OF SURVEY DATA

Omission of data entry among registered data included 0.2% of patients for whom PSA values were not recorded. Meanwhile, omission of histological grade accounted for 7.8% and omission of clinical stage 6.1%. As for Gleason score, 23% of registered cases in 2001 had no entry, but in 2002 this had decreased to 11.9% and by 2003, to 6.5%. This is thought to be because in the First Edition of the Japanese Urological Association and Japan Society of Pathology's General Rules for Clinical and Pathological Studies on Prostate Cancer, Gleason score entry was not compulsory. Only in the Second Edition did Gleason score become required.

Table 4. Relations between the purpose of hormone therapy and T category, TNM clinical stage, Gleason score and patient age

	Main	Adjuvant	Neoadjuvant	Total	%
T stage					
T0	4 (0.1%)			4	0.0
T1	2689 (67.9%)	218 (5.5%)	1054 (26.6%)	3961	22.9
T2	4260 (70.1%)	333 (5.5%)	1485 (24.4%)	6078	35.1
T3	4965 (87.2%)	174 (3.1%)	556 (9.8%)	5695	32.9
T4	1425 (95.8%)	26 (1.7%)	36 (2.4%)	1487	8.6
Tx	60 (93.8%)	2 (3.1%)	2 (3.1%)	64	0.4
No description	22 (95.7%)		1 (4.3%)	23	0.1
Total	13 425 (77.5%)	753 (4.3%)	3134(18.1%)	17 312	100.0
Clinical stage					
II	5847 (66.0%)	537 (6.1%)	2475 (27.9%)	8859	51.2
III	2263 (77.3%)	145 (5.0%)	518 (17.7%)	2926	16.9
IV	4362 (97.6%)	44 (1.0%)	65 (1.5%)	4471	25.8
No description	953 (90.2%)	27 (2.6%)	76 (7.2%)	1056	6.1
Total	13 425 (77.5%)	753 (4.3%)	3134 (18.1%)	17 312	100.0
Gleason score					
2–4	996 (73.5%)	69 (5.1%)	290 (21.4%)	1355	7.8
5	1214 (71.8%)	91 (5.4%)	386 (22.8%)	1691	9.8
6	1902 (71.0%)	120 (4.5%)	658 (24.6%)	2680	15.5
7	3179 (76.9%)	175 (4.2%)	778 (18.8%)	4132	23.9
8–10	3966 (83.9%)	185 (3.9%)	577 (12.2%)	4728	27.3
Unknown	2168 (79.5%)	113 (4.1%)	445 (16.3%)	2726	15.7
Total	13 425 (77.5%)	753 (4.3%)	3134 (18.1%)	17 312	100.0
Age at diagnosis					
<60	364 (51.3%)	48 (6.8%)	298 (42.0%)	710	4.1
60–64	767 (55.7%)	95 (6.9%)	515 (37.4%)	1377	8.0
65–69	1613 (57.8%)	234 (8.4%)	946 (33.9%)	2793	16.1
70–74	3305 (72.8%)	226 (5.0%)	1008 (22.2%)	4539	26.2
75–79	3808 (89.6%)	116 (2.7%)	325 (7.6%)	4249	24.5
≥80	3568 (97.9%)	34 (0.9%)	42 (1.2%)	3644	21.0
Total	13 425 (77.5%)	753 (4.3%)	3134 (18.1%)	17 312	100.0

FOLLOW-UP DATA

For approximately 92% of the registered cases in 2001 and 75% of the registered cases in 2002, the input of follow-up data was confirmed at least once. The period (median) from the start of hormone therapy to the latest follow-up data entry was 406 days (between 0 and 964) for 2001-registered cases and 189 (between 0 and 615) for 2002-registered cases.

DISCUSSION

In Japan, the General Rules for Clinical and Pathological Studies on Prostate Cancer issued by the Japanese Urological Association and Japan Society of Pathology were first published in

Table 5. Relations between the type of hormone therapy and T category, TNM clinical stage, Gleason score and patient age

	Orchiectomy only	Orchiectomy + medication	LH-RHa only	LH-RHa + anti-androgen	Anti-androgen only	Other	Total	%
T stage								
T0				1 (25.0%)	1 (25.0%)	2 (50.0%)	4	0.0
T1	112 (2.8%)	169 (4.3%)	719 (18.2%)	2333 (58.9%)	427 (10.8%)	201 (5.1%)	3961	22.9
T2	158 (2.6%)	277 (4.6%)	921 (15.2%)	3737 (61.5%)	532 (8.8%)	453 (7.5%)	6078	35.1
T3	196 (3.4%)	466 (8.2%)	490 (8.6%)	3513 (61.7%)	215 (3.8%)	815 (14.3%)	5695	32.9
T4	44 (3.0%)	208 (14.0%)	69 (4.6%)	752 (50.6%)	46 (3.1%)	368 (24.7%)	1487	8.6
Tx	1 (1.6%)	5 (7.8%)	8 (12.5%)	36 (56.3%)	4 (6.3%)	10 (15.6%)	64	0.4
No description	2 (8.7%)	3 (13.0%)	3 (13.0%)	11 (47.8%)	2 (8.7%)	2 (8.7%)	23	0.1
Total	513 (3.0%)	1128 (6.5%)	2210 (12.8%)	10 383 (60.0%)	1227 (7.1%)	1851 (10.7%)	17 312	100.0
Clinical stage								
II	262 (3.0%)	360 (4.1%)	1527 (17.2%)	5366 (60.6%)	841 (9.5%)	503 (5.7%)	8859	51.2
III	111 (3.8%)	157 (5.4%)	325 (11.1%)	1959 (67.0%)	135 (4.6%)	239 (8.2%)	2926	16.9
IV	115 (2.6%)	559 (12.5%)	246 (5.5%)	2449 (54.8%)	132 (3.0%)	970 (21.7%)	4471	25.8
No description	25 (2.4%)	52 (4.9%)	112 (10.6%)	609 (57.7%)	119 (11.3%)	139 (13.2%)	1056	6.1
Total	513 (3.0%)	1128 (6.5%)	2210 (12.8%)	10 383 (60.0%)	1227 (7.1%)	1851 (10.7%)	17 312	100.0
Gleason score								
2–4	31 (2.3%)	54 (4.0%)	187 (13.8%)	820 (60.5%)	157 (11.6%)	106 (7.8%)	1355	7.8
5	65 (3.8%)	91 (5.4%)	247 (14.6%)	1032 (61.0%)	152 (9.0%)	104 (6.2%)	1691	9.8
6	80 (3.0%)	146 (5.4%)	468 (17.5%)	1579 (58.9%)	241 (9.0%)	166 (6.2%)	2680	15.5
7	151 (3.7%)	247 (6.0%)	557 (13.5%)	2515 (60.9%)	267 (6.5%)	395 (9.6%)	4132	23.9
8–10	119 (2.5%)	445 (9.4%)	373 (7.9%)	2796 (59.1%)	232 (4.9%)	763 (16.1%)	4728	27.3
Unknown	67 (2.5%)	145 (5.3%)	378 (13.9%)	1641 (60.2%)	178 (6.5%)	317 (11.6%)	2726	15.7
Total	513 (3.0%)	1128 (6.5%)	2210 (12.8%)	10 383 (60.0%)	1227 (7.1%)	1851 (10.7%)	17 312	100.0
Age at diagnosis								
<60	5 (0.7%)	32 (4.5%)	76 (10.7%)	413 (58.2%)	65 (9.2%)	119 (16.8%)	710	4.1
60–64	11 (0.8%)	88 (6.4%)	176 (12.8%)	816 (59.3%)	120 (8.7%)	166 (12.1%)	1377	8.0
65–69	57 (2.0%)	175 (6.3%)	319 (11.4%)	1674 (59.9%)	239 (8.6%)	329 (11.8%)	2793	16.1
70–74	96 (2.1%)	248 (5.5%)	564 (12.4%)	2826 (62.3%)	300 (6.6%)	505 (11.1%)	4539	26.2
75–79	153 (3.6%)	302 (7.1%)	566 (13.3%)	2556 (60.2%)	259 (6.1%)	413 (9.7%)	4249	24.5
≥80	191 (5.2%)	283 (7.8%)	509 (14.0%)	2098 (57.6%)	244 (6.7%)	319 (8.8%)	3644	21.0
Total	513 (3.0%)	1128 (6.5%)	2210 (12.8%)	10 383 (60.0%)	1227 (7.1%)	1851 (10.7%)	17 312	100.0

1985 (9) and this set of rules has been widely used ever since. The document gives a guideline on diagnosis and a detailed description of rules associated with making entries on patient background, tumor background and treatment method. Most of the papers presented at such meetings, such as the academic conference of the Urological Association, follow these rules and their diffusion rate is extremely high. The J-CaP survey basically followed the rules, and the accuracy of TNM diagnoses and clinical stage diagnoses is considered to be high. The Japanese Urological Association started a prostate cancer registration system from 2001, in accordance with these rules. However, this system is a registration of all prostate cancers. Therefore, when, for example, focusing on hormone therapy, we cannot necessarily expect satisfactory outcome data.

The morbidity of prostate cancer in Japan has been remarkably lower than in Europe and North America (10). Furthermore, due to anxieties about radiotherapy and the slowness of the introduction of technical expertise in radical prostatectomy, in many cases surgical castration or estrogen administration has been conducted across the board (11). However, in recent years Japan has seen an overwhelming increase in morbidity and mortality from prostate cancer (10). Compounding this, the influx of information about prostate and surgical techniques from Europe and North America has led to a rapidly growing debate on the method of treatment. Naturally, the trend towards newer treatment is beginning with reference to European (12) and North American guidelines (13) and the trend is set to continue.

Table 6. Main treatment for adjuvant or neoadjuvant hormone therapy

	Method	2001	2002	2003	Total
Operation					
Hormonal therapy followed by surgery	Retropubic	1609	18		1627
	Laparoscopic	23			23
	Perineal	17			17
	Other	3			3
	Total	1652	18		1670
Surgery followed by hormonal therapy	Retropubic	256	3		259
	Laparoscopic	10			10
	Perineal	2			2
	Other	2			2
	Total	270	3		273
Irradiation					
Hormonal therapy followed by irradiation	External beam	647	468	69	1184
	External + brachytherapy	15	4		19
	Brachytherapy	12	6	1	18
	Other	11	6	1	118
	Total	685	484	71	1339
Irradiation followed by hormonal therapy	External beam	46	62	10	118
	External + brachytherapy	3			3
	Brachytherapy	1	3		4
	Other	1	1		2
	Total	51	66	10	127

At present, with financial assistance from the Ministry of Health, Labor and Welfare, the Japanese Urological Association is working on the drafting of a prostate cancer treatment guideline at the earliest possible date. What is of concern here is that, in addition to the circumstances previously mentioned, there have been very few clinical trials with strong evidence carried out in this country. This causes a desperate lack in clinical data specific to Japan, which is essential to establish such a guideline. Hormone therapy in Japan, which has been administered only empirically, should be re-examined correctly to determine what outcome it is actually providing for the patients. Otherwise, it is likely that Japan's treatment guideline will become a reproduction of those of Europe and North America. Ethnic and philosophical differences, religious background, differences in perceptions about sex, and economic background—these diverse factors must be taken into account in the drafting of the most appropriate guideline for a country. The general attitude toward hormone therapy in Japan is similar to other East Asian countries (14). The recent treatment and clinical trial findings on hormone therapy in Europe and North America aimed at achieving long-term stable results indicate that we should examine the outcome of hormone therapy not only in Japan but throughout the world (4–6). The CaPSURE data reported in 2003 (1) consists of the analyses of 3439 cases, showing that the proportion of primary hormone treatment on localized prostate cancer rose dramatically from 4.6% in 1989 to 14.2% in 2001 and pointed firmly to the need to review the existing guidelines.

The institutions registered with J-CaP cover 60.2% of all university hospitals. According to Japan Cancer Statistics 2003, the number of patients newly diagnosed with prostate cancer in 1998 was 15 814 (15). In view of the proportion of J-CaP registered patients obtained in the survey of new patient numbers mentioned earlier, ~50% of new prostate cancer patients were treated by hormone therapy and registered with J-CaP. J-CaP had requested reports on the number of newly diagnosed prostate cancer patients in the registered institutions. Out of 358 institutions, 246 had responded as of 2001. Based on this report, 7952 patients were newly diagnosed with prostate cancer in those 246 institutions. Of these, 5969 patients (75.1%) were treated by hormone therapy in some form. Among those patients, 5646 (71%) were registered with J-CaP. In other words, 94.6% of the patients who had initiated a hormone therapy in 2001 were registered with J-CaP. This figure is almost the same in 2002. This illustrates the breadth of significance of this study. Patient background factors and PSA values at diagnosis would not represent the general trend because of the bias that patients registered for this study are receiving hormone therapy for the first time. However, we should make a special note of the low frequency of familial prostate cancer.

For the same reason, the background to the tumor in this report would not represent the overall trend of prostate cancer in Japan. Nevertheless, considering the finding that an extremely large number of patients are receiving hormone therapy, we can safely say that they express the overall background factors of prostate cancer in Japan to a fairly high degree of accuracy.

The analysis of the purpose and types of hormone therapy shows that there is a distinctively different trend in Japan compared to Europe or North America. These are the first findings in Japan based on a large-scale organized survey. To summarize: (i) many patients are receiving hormone therapy irrespective of age, TNM, stage of illness or histological background; (ii) more than 70% of them are under primary hormone therapy; and (iii) roughly 60% undergo combined androgen blockade (CAB). Since no clear outcome investigation has yet been carried out, we should evaluate this present status of hormone therapy in Japan either as: (i) it is merely a continuation of past experience, and in the near future, it should be managed carefully by adopting European and American guidelines; or (ii) it is still difficult to judge whether the effect of hormone therapy for Japanese patients is different in the profile of effects and side-effects from that for Westerners. What is more, in T2 treatment no accurate randomized study has been conducted so far globally on whether surgical treatment and radiotherapy are truly more effective than hormone therapy. Therefore, on this point we must reserve any conclusions.

The NCI–PDQ (13) and EAU guidelines (12) attach virtually no significance to hormone therapy on T2 prostate cancer. As for T3, the emphasis is on its significance as neoadjuvant before radiotherapy and little importance is assigned to the sole application of hormone therapy. Even when there is metastasis, there is debate on whether immediate hormone therapy is appropriate and also on whether there is any point in CAB; however, no clear conclusions have been reached (16,17).

In such circumstances, there are two clinical trial results in Japan reported recently that are extremely interesting. The first (2) is the results of a randomized study on hormone therapy given to localized or locally advanced prostate cancer. This was a comparative trial of LH-RHa + chlormadinone acetate (CMA) versus LH-RHa alone on patients in whom radical prostectomy was not chosen as treatment for whatever reason. The results are interim, with an observation period less than 5 years. So far, progression-free survival is good for CAB. Even when both groups are put together, it has been determined that the same survival rate as the one expected for the population of that age group has been obtained. The other study (3) is a comparative trial of LH-RHa + bicalutamide versus LH-RHa + placebo administered for patients with locally advanced or metastatic prostate cancer. The observational period is again short, but in both PSA progression-free survival and time to PSA response, the CAB group was significantly better. Meanwhile, in a successive survey of QOL using FACT-P that was officially translated into Japanese (18), the CAB group showed a significantly better result (19). This is indicative of the perception that the effects of hormone therapy on QOL are different between Japanese and Western patients (20). Therefore, it is important to examine whether or not recent clinical trial results take into account ethnic differences in the broad sense, including the lifestyle and philosophical backgrounds of Japanese and Western people.

In future, in the treatment of prostate cancer in Japan, it is evident that the importance of hormone therapy should be investigated with specific focus on Japanese people. We await the further analysis of the outcome findings, which is the aim of the J-Cap Study.

APPENDIX

J-CAP HOME PAGE: RULES FOR USE

1. The J-CaP Home Page is to be created as an Internet server.
2. Use of the case database on the J-CaP Home Page is restricted to doctors who are joint researchers and the use of the database requires a user ID and password issued by means of prior registration.
3. Communication between the case database server and users is to be protected by encryption (SSL).
4. The names of institutions and patients (initials) displayed in the case database are to be encoded so that individual patients cannot be identified.
5. Information concerning joint researchers' institutions and patient names (initials) will only be accessible to database administrators with a special ID and password and only at the designated location (administrative secretariat).
6. The ID and password of the above-mentioned administrators will be stored as strictly confidential and no record of them will be kept.
7. The disposal of case data and information concerning joint researcher institutions and patient names (initials) after the completion of the J-CaP Study Group's research period will be determined at a later date by administrators.

References

1. Cooperberg MR, Grossfeld GD, Lubeck DP, Carroll PR. National practice patterns and time trends in androgen ablation for localized prostate cancer. *J Natl Cancer Inst* 2003;95:930–1.
2. Akaza H, Homma Y, Okada K, Yokoyama M, Usami M, Hirao Y, et al. A prospective and randomized study of primary hormonal therapy for patients with localized or locally advanced prostate cancer unsuitable for radical prostatectomy: results of the 5-year follow-up. *BJU Int* 2003;91: 33–6.
3. Akaza H, Yamaguchi A, Matsuda T, Igawa M, Kumon H, Soeda A, et al. Superior anti-tumor efficacy of bicalutamide 80 mg in combination with a luteinizing hormone-releasing hormone (LHRH) agonist versus LHRH agonist monotherapy as first-line treatment for advanced prostate cancer: interim results of a randomized study in Japanese patients. *Jpn J Clin Oncol* 2004; in press.
4. See W, Iversen P, Wirth M, McLeod D, Garside L, Morris T. Immediate treatment with bicalutamide 150mg as adjuvant therapy significantly reduces the risk of PSA progression in early prostate cancer. *Eur Urol* 2003;44:512–7.
5. Labrie F. Androgen blockade in prostate cancer in 2002: major benefits on survival in localized disease. *Mol Cell Endocrinol* 2002;198:77–87.

6. Labrie F, Candas B, Gomez JL, Cusan L. Can combined androgen blockade provide long-term control or possible cure of localized prostate cancer? *Urology* 2002;60:115–9.
7. UICC. TNM classification of the malignant tumors. 5th edition, edited by LH Sobin and Ch Wittekind 1997, Wiley-Liss, New York.
8. General rules for clinical and pathological studies on prostate cancer; 3rd edition. Kanehara Publication Inc, 2001. Japan Urological Association, Tokyo.
9. General rules for clinical and pathological studies on prostate cancer; 1st edition. Kanehara Publication Inc, 1985. Japan Urological Association, Tokyo.
10. Yoshimi I, Mizuno S. Mortality trends of prostate cancer in Japan: 1960–2000. *Jpn J Clin Oncol* 2003;33:367.
11. Kumamoto Y, Tsukamoto T, Umehara T, Harada M, Shimazaki J, Fuse H, et al. Clinical studies on endocrine therapy of prostatic carcinoma (3): Histopathological features of prostatic carcinoma and its prognosis. *Hinyokika Kiyo* 1990;36:295–305.
12. Aus G, Abbou CC, Pacik D, Schmid HP, van Poppel H, Wolff JM, Zattoni F; EAU Working Group on Oncological Urology. EAU guidelines on prostate cancer. *Eur Urol* 2001;40:97–101.
13. NCI–PDQ, Prostate cancer treatment. http://www.nci.nih.gov/cancerinfo/pdq/treatment/prostate/healthprofessional/
14. Akaza H, Naito S, Cheng C, Kaisary A, Soebadi DM, Umbas R, et al. Asian trends in prostate cancer hormone therapy. *Gan To Kagaku Ryoho* 2002;29:1951–61.
15. Cancer statistics in Japan 2003. Foundation for promotion of cancer research (FPCR). http://www.ncc.go.jp/jp/statistics/2003/
16. Klotz LH, Newman T. Does maximal androgen blockade (MAB) improve survival? A critical appraisal of the evidence. *Can J Urol* 1996;3:246–50.
17. Patterson SG, Balducci L, Pow-Sang JM. Controversies surrounding androgen deprivation for prostate cancer. *Cancer Control* 2002;9:315–25.
18. Hinotsu A, Niimi M, Akaza H, Miyanaga N, Takeshima H, Eremenco S, Cella D. Development of Japanese version of QOL questionnaire for bladder and prostate cancer patients using FACT-Bl and P: pilot study. *Gan To Kagaku Ryoho* 1999;26:657–66.
19. Naito S, Tachibana M, Deguchi T, Namiki M, Hirao Y, Arai Y, et al. Addition of bicalutamide 80 mg to LHRH-agonist monotherapy in patients with advanced prostate cancer: impact on quality of life. *J Clin Oncol* 2004;23:4703.
20. Mendoza JB. The role of hormone therapy in the treatment of locally advanced prostate cancer—Regional perspectives Asia. *Eur Urol* 2004; 45(Suppl 3):12–6.

Blood Isoflavone Levels during Intake of a Controlled Hospital Diet

Naomi TAKASHIMA[1], Naoto MIYANAGA[1], Kimitoshi KOMIYA[2], Mitsuru MORI[3] and Hideyuki AKAZA[1]

[1]*Department of Urology, University of Tsukuba, Tsukuba 305–8575, Japan*
[2]*Department of Nutritional Administration, Tsukuba University Hospital, Tsukuba 305–8576, Japan*
[3]*Department of Epidemiology, Sapporo Medical University School of Medicine, Sapporo 060–8556, Japan*

(Received September 3, 2003)

Summary Isoflavones are reported to have an estrogenic activity to prevent prostate cancer. Therefore, it is necessary to analyze the factors influencing the absorption and metabolism of isoflavones using detailed and precise dietary information. We evaluated the relationship between the amount of intake of soybean isoflavones in the diet and its serum levels in 88 hospitalized patients (22 with prostate cancer, 66 without cancer) with a mean age of 67.0±9.3 y. The intake amount of genistein and daidzein was significantly lower in the hospital diet than that in the ordinary daily diet. The serum levels of isoflavones were related to the amount of intake consumed during the day before blood collection (r=0.27 for genistein, r=0.33 for daidzein), but not to the last meal before blood collection. While little relationship was observed between the amount of intake and serum levels of isoflavones in the equol producers, a marked relationship was noted in the non-producers (r=0.52 for genistein, r=0.67 for daidzein). Blood isoflavone levels decreased when the duration of the hospitalization was longer than 1 wk. These observations indicate that the serum isoflavone levels are correlated with the amount of intake of isoflavones during the preceding 2 wk and serve as an effective biomarker in individuals during the intake of the hospital diet. Further investigations, including the mechanism of the metabolism on isoflavones, are necessary for the evaluation of the preventive effect of isoflavones.

Key Words isoflavone, equol producer, genistein, daidzein, hospital diet

Isoflavones are consumed in large quantities as soybeans by Asians. It has been reported that the intake of soy foods, which contain much isoflavone, contributes to the relatively low incidence of certain kinds of malignancies, such as breast cancer and prostate cancer, in China and Japan. The epidemiological research suggests that soybean-based foods contain substances that can inhibit carcinogenesis and the progression of hormone-related cancers (*1–3*). It was reported that isoflavones reduced the activity of 5-α reductase, which is indispensable for the manifestation of androgen, and stimulates the production of SHBG (sex hormone-binding hormone) to regulate hormonal activity in in vitro and in vivo experimental studies (*4, 5*). Isoflavones have been reported to have several other functions: 1) inhibition of tyrosine kinase activity, 2) inhibition of angiogenesis, 3) antioxidative effects and 4) an anti-promotion function of transformed cells (*6–8*). These reports suggest that isoflavones can work as anticancer agents.

We previously reported that blood isoflavone levels are lower in hospitalized patients. We also found that the patients younger than 70 showed lower levels of blood isoflavone levels than those at aged 70 and older (*9*). It is very important to ascertain these observations to better understand the influence of dietary intake to the onset of prostate cancer, which is reported to have been increasing recently in Japan. It should also be implicated that the content of the hospitalized dietary intake should be considered as the cancer-preventive aspect.

Isoflavones are contained in soybean foods mainly as glycosides (genistin, daidzin), which are converted to aglycones (genistein, daidzein) with the hydrolysis of their carbohydrate chains in the intestinal bacterial microflora for absorption (*10*). In addition, the estrogen activity of isoflavones is very weak, below 0.1% of estradiol activity (*11*). These findings indicate that analysis of the metabolism of isoflavones is important to make clear the biological effects of isoflavones. Many investigations have reported a relationship between the amount of intake of isoflavones and blood isoflavone levels using personal dietary records and food-frequency questionnaires (FFQ), but such records seem to be rough and inaccurate estimations of isoflavone intake (*12*). In addition, the individual nutrient balance has possible biases on the absorption and metabolism of isoflavones. Therefore, it is necessary to analyze the factors influencing the absorption and metabolism of isoflavones using detailed and precise dietary information.

In this study, we compared isoflavone levels between hospital and ordinary diets. We also compared isofla-

E-mail: naohei@sakura.cc.tsukuba.ac.jp

vone levels in terms of the examinees' age, and finally examined the relationship between the amount of dietary intake of isoflavones during hospitalization and serum isoflavone levels to analyze the metabolism of individual isoflavones ingesting similar amounts of isoflavones.

MATERIALS AND METHODS

The subjects were 88 hospital in-patients who were admitted to the Tsukuba University Hospital from December 2000 to June 2002, of which 22 patients suffered from prostate cancer (eligible with clinical stage and treatment), and 66 were ophthalmological cases. The ophthalmological patients included 37 patients with cataracts, 11 with retinal detachment, 5 with pterygium, 4 with glaucoma and 9 with other diagnoses. The ages of the patients were 44 to 92 y, with 67.0±9.3 y on average (urological patients, 65.1±8.2, and ophthalmological patients, 67.6±9.6). The mean value of the body mass index (BMI) was 23.0±3.1 (urological patients, 22.4±3.5, and ophthalmological patients, 23.2±3.0). Eligible patients consumed the hospital diet for 2 d before the blood test. Fasting patients were excluded, as were patients who were receiving dietary control for diabetes mellitus, or who ate anything other than the hospital diet. In the prostate cancer patients, patients were excluded if their dietary intake was reduced or if the ability of absorption was compromised. The average hospitalization period was 13.8 d. Informed consent was obtained with a form letter from all patients with their signature.

Dietary survey. The content of the diet for 2 d prior to blood collection was referred from the menu obtained from the Department of Nutritional Administration of the hospital. Dietary intake was calculated from the intake recorded in the hospital dietary chart of the individual patient. The dietary intake amount of isoflavones (genistein and daidzein) was calculated using the "isoflavone-intake table in Japan" reported by Kimira et al. (*13*). Personal information was obtained, especially on the use of antibiotics, to make clear their influence on the metabolism of isoflavones. Also, a food-frequency questionnaire was used to assess each patient's ordinary daily dietary intake of nutrients and soybean foods for 1 y before hospitalization. In addition, in prostate cancer cases, information on ordinary daily intake was obtained for 1 y prior to diagnosis. The mean value of the daily intake of energy and nutrients was calculated from these records using the "Standard Food Composition Table" published from the Science and Technology Agency of Japan in 2001.

Blood samples. Blood was collected from the individuals after fasting for 12 h following dinner, and the obtained sera were cryo-preserved at −20°C. We measured the unconjugated forms in serum after hydrolyzing with β glucuronodase and sulfatase. The concentration of isoflavones (genistein, daidzein) and equol were assayed using the high-performance liquid chromatography–multiple reaction ion monitoring mass spectrometry (HPLC-MS) method. The lower limit of detection of this method is 0.5 ng/mL (*14*).

Statistical analysis. The relationship between the amount of the dietary intake of isoflavones and blood isoflavone levels was analyzed using Pearson's correlation coefficient. The dietary intake of isoflavones was adjusted as the total energy intake. Wilcoxon's test and the Kruskal-Wallis test (non-parametric) were used to compare the duration of hospitalization and the production of equol in two groups. Paired *t*-test was used to compare the amount of dietary intake of isoflavones between the hospital diet and the ordinary daily diet, and $p<0.05$ was determined to be the statistical significance. The statistical software used was SAS JMP4.0.

RESULTS

Table 1 shows the mean values of intake of total energy and the balance of nutrition from a hospital diet for 2 d before blood collection, and the patients' ordinary daily diet. A statistically significant difference was observed in total amounts of energy, carbohydrates, salt, dietary fiber, carotene and vitamins (A, B_1 and C) between the hospital diet and ordinary diet. Because blood samples were collected from each patient on a different day, and not all the patients had the same diet for the 2 d before blood collection, some variations in the hospital dietary intake were found.

Table 2 indicates the types and amount of soybean foods, and the proportion of isoflavones in the hospital diet and the ordinary daily diet. The three main items containing isoflavones in the hospital diet were tofu (42.3%), half-fried tofu (23.5%) and boiled soybeans (10.7%), and were tofu (53.4%), natto (20.8%) and miso (13.2%) in the ordinary diet. The intake amounts of tofu, natto, miso, half-fried tofu and fried tofu were significantly higher in the ordinary diet than in the hospital diet.

In Table 3, the intake amounts of isoflavones in the hospital diet, the ordinary diet and serum isoflavone levels are shown. In this table, the data on equol were obtained from equol-producing patients only (equol-producers) (9). The intake amount of isoflavones is shown at three points: the last meal before blood collection, 1 d before collection and 2 d before collection. There were some days when no soybean food was consumed, and genistein varied from 0 to 46.8 mg/d for the range. The ratio of the amount of intake of genistein to daidzein was 3 to 2. The ordinary diet contained significantly higher genistein and daidzein than the hospital diet. In the serum isoflavone levels, genistein was the highest, and the median value was 3.7 times higher than that of daidzein. Equol production was 46.6% (41 samples in total 88 samples).

Table 4 indicates the intake amount of isoflavones in soy-foods during ordinary intake. The intake amount of daidzein showed higher levels in the patients aged 70 and older than in those younger.

Table 5 shows a comparison of the average nutritional intake for 2 d before blood collection, the amount of intake of isoflavones and the serum concentration of isoflavones between equol-producers and non-produc-

Table 1. Nutrients in hospital diet and ordinary daily diet (n=88).

Nutrient	Hospital diet			Ordinary diet			p^a
	Median	Range	Mean±SD	Median	Range	Mean±SD	
Energy (kcal/d)	1,976.8	1,267.0–2,174.0	1,905.7±202.9	1,583.0	945.0–3,212.0	1,647.9±462.6	<0.0001***
Protein (g/d)	79.2	52.1–86.6	77.1±7.4	75.3	33.7–161.8	78.1±27.8	0.737
Fat (g/d)	49.2	26.0–67.7	48.0±7.9	43.1	14.9–105.0	46.2±18.0	0.391
Carbohydrate (g/d)	295.7	113.4–484.1	284.4±41.0	219.1	110.4–423.6	224.1±64.6	<0.0001***
NaCl (g/d)	11.0	7.0–13.9	11.1±1.0	6.6	1.9–10.7	6.8±1.7	<0.0001***
Fiber (g/d)	5.0	3.0–6.7	5.1±0.9	12.6	5.4–27.3	13.6±4.9	<0.0001***
Carotene (μg/d)	5,285.1	2,735.0–7,311.0	5,322.8±972.1	3,072.0	658.0–8,108.0	3,463.2±1,535.5	<0.0001***
Vitamin A (IU/d)	3,501.7	1,970.0–6,125.5	3,488.0±779.9	2,384.3	838.2–5,131.5	2,580.6±1,006.5	<0.0001***
Vitamin B_1(mg/d)	1.35	0.6–1.8	1.3±0.24	0.93	0.49–1.97	0.96±0.34	<0.0001***
Vitamin B_2 (mg/d)	1.35	0.8–1.8	1.33±0.19	1.7	0.5–3.6	1.8±0.72	0.158
Vitamin C (mg/d)	150.7	90.5–198.2	148.5±26.7	181.0	31.0–191.4	191.4±87.2	<0.0001***

* p<0.05, ** p<0.01, *** p<0.001.
[a] Paired t-test.

Table 2. Soybean foods and their contribution to isoflavone intake in hospital diet and ordinary daily diet (n=88).

Soy food	Hospital diet				Ordinary diet				p^a
	Median	Range	Mean±SD	% of isoflavone intake	Median	Range	Mean±SD	% of isoflavone intake	
Tofu (g/d)	33.3	0.0–70.0	24.6±19.8	42.3%	50.0	0.0–300.0	72.9±61.2	53.4%	<0.0001***
Half-fried tofu (g/d)	0.0	0.0–53.3	7.7±12.5	23.5%	2.3	0.0–20.0	3.3±3.8	4.3%	0.002**
Boiled soybeans (g/d)	3.3	0.0–21.7	3.5±4.8	10.7%	—				
Natto (g/d)	0.0	0.0–16.7	3.0±6.5	7.5%	17.1	0.0–100.0	19.8±18.5	20.8%	<0.0001***
Miso (g/d)	12.7	0.0–27.0	12.8±5.4	6.8%	20.0	2.0–60.0	26.3±16.3	13.2%	<0.0001***
Grilled tofu (g/d)	0.0	0.0–26.7	2.5±7.1	4.4%	—				
Fried tofu (g/d)	0.0	0.0–10.0	1.1±1.7	3.3%	1.4	0.0–7.1	1.7±1.8	2.2%	0.008**
Soy sause (g/d)	24.1	9.8–41.3	23.7±6.6	1.5%	—				
Soy milk (g/d)	—				0.0	0.0–200.0	9.9±38.2	6.1%	

* p<0.05, ** p<0.01, *** p<0.001.
[a] Paired t-test.

Table 3. Intake of genistein and daidzein and their serum levels (n=88).

Isoflavone	Hospital diet			Ordinary diet			p^b
	Median	Range	Mean±SD	Median	Range	Mean±SD	
Intake							
Genistein in the last meal (mg)	0.0	0.0–27.9	4.2±8.7				
Genistein on the previous day (mg/d)	4.3	0.0–46.8	13.4±14.8				
Average of genistein for 2 d (mg/d)	14.6	1.03–36.2	14.2±8.4	17.5	1.4–84.9	22.9±15.9	<0.0001***
Daidzein in the last meal (mg)	0.0	0.0–21.9	3.0±6.2				
Daidzein on the previous day (mg/d)	3.4	0.0–36.3	9.8±11.0				
Average of daidzein for 2 d (mg/d)	10.2	0.8–29.1	10.4±6.4	12.5	0.97–58.1	15.9±10.8	<0.001***
Serum							
Genistein (ng/mL)	71.2	7.2–570.2	108.5±112.6				
Daidzein (ng/mL)	19.4	2.0–275.4	39.4±52.6				
Equol (ng/mL)[a]	8.0	0.6–904.7	39.4±141.1				

* p<0.05 ** p<0.01 *** p<0.001.
[a] Equol producers only n=41/88.
[b] Paired t-test.

Table 4. Genistein and daidzein intake in patients <70 y of age and ≧70 y during the ordinary intake.

Isoflavone		<70 y old	≧70 y old	p^a
Genistein (mg/d)	Median	16.53	23.39	0.062
	Range	1.4–84.9	8.9–59.5	
Daidzein (mg/d)	Median	11.63	15.74	0.041*
	Range	1.0–58.1	6.1–41.0	

* $p<0.05$.
[a] Wilcoxon's test.

Table 5. Serum isoflavone levels and intake in equol producers and in non-producers.

Various	Equol producers $n=41$		Equol non-producers $n=47$		p^a
	Median	Range	Median	Range	
Serum Genistein (ng/mL)	78.3	10.9–570.2	70.8	7.2–387.0	0.78
Daidzein (ng/mL)	12.8	2.0–275.4	19.9	2.8–127.6	0.85
Intake Genistein in the last meal (mg)	0.0	0–27.9	0.0	0–27.8	0.53
Genistein on the previous day (mg/d)	5.2	0–46.8	4.3	0–46.8	0.81
Average of genistein for 2 d (mg/d)	15.2	1.3–30.0	12.3	1.0–36.2	0.19
Daidzein in the last meal (mg)	0.0	0–20.7	0.0	0–21.9	0.53
Daidzein on the previous day (mg/d)	3.5	0–36.3	3.4	0–34.2	0.82
Average of daidzein for 2 d (mg/d)	10.4	0.9–23.1	9.4	0.8–29.1	0.20
Energy (kcal/d)	1,969.5	1,267.0–2,174.0	1,998.0	1,298.6–2,149.5	0.32
Protein (g/d)	79.0	53.7–86.6	79.7	52.1–86.6	0.35
Fat (g/d)	46.8	26.0–67.2	50.0	28.6–67.7	0.06
Carbohydrate (g/d)	296.4	113.4–484.1	295.7	201.9–316.7	0.73
Dietary fiber (g/d)	5.3	3.3–6.7	4.8	3.0–6.7	0.02*
Vitamin A (IU/d)	3,570.1	1,970.0–5,766.6	3,365.5	1,998.0–6,125.5	0.09
Vitamin B_1 (mg/d)	1.4	0.8–1.8	1.3	0.6–1.8	0.48
Vitamin B_2 (mg/d)	1.4	0.9–1.8	1.3	0.8–1.8	0.15

* $p<0.05$.
[a] Wilcoxson's test.

Table 6. Pearson's correlation coefficients between isoflavone levels in equol producers and in non-producers.

Isoflavone	All			Equol producers		Equol non-producers	
	Genistein	Daidzein	Equol	Genistein	Daidzein	Genistein	Daidzein
	$n=88$		$n=41$	$n=41$		$n=47$	
Intake[a] Genistein at the last meal	0.16	0.16	−0.08	−0.35	−0.13	0.52***	0.69***
Genistein on the previous day	0.27*	0.32*	−0.06	0.12	0.30	0.31*	0.64***
Average of genistein for 2 d	0.24*	0.23*	−0.04	−0.18	−0.24	0.32*	0.30*
Daidzein at the last meal	0.18	0.16	−0.06	−0.35	−0.13	0.61***	0.67***
Daidzein on the previous day	0.27*	0.33**	−0.06	0.11	0.31	0.30**	0.61***
Average of daidzein for 2 d	0.23*	0.28*	−0.09	−0.04	0.11	0.41**	0.52**

* $p<0.05$, ** $p<0.01$, *** $p<0.001$.
[a] Energy adjusted values.

ers. There was no difference between the two groups in the intake amount of isoflavones and the serum concentration of isoflavones. However, equol producers tended to consume significantly greater amounts of dietary fiber ($p=0.02$) compared with the non-producers.

Table 6 shows the relationship between the inake amount of isoflavones and serum isoflavone levels. The serum levels of isoflavones (genistein, daidzein) significantly increased when the intake amount for 1 d, and for 2 d before blood collection was increased. However, no correlation was observed between serum isoflavone levels and the intake amount of isoflavones at the last meal before blood collection. Equol non-producers showed a significant correlation with the amount of

Table 7. Isoflavone intake and serum isoflavone levels by duration of hospitalization.

Isoflavone			≦3 d	4–7 d	8–15 d	≧16 d	p^a
		n	22	21	22	23	
Intake (previous day) (mg/d)	Genistein	Median	6.9	4.3	4.2	4.0	0.908
		Range	0.0–45.2	0.0–30.5	0.0–46.4	0.0–46.8	
		Mean±SD	13.8±14.1	12.7±13.3	12.4±14.0	14.7±17.9	
	Daidzein	Median	5.0	3.4	3.1	2.9	0.754
		Range	0.0–36.3	0.0–23.1	0.0–33.9	0.0–34.2	
		Mean±SD	10.4±11.1	9.4±10.0	8.7±9.8	10.9±13.3	
Serum (ng/mL)	Genistein	Median	49.0	122.2	77.8	44.1	0.006**
		Range	10.9–570.2	24.0–323.6	7.2–548.3	16.1–387.0	
		Mean±SD	106.6±143.6	136.5±78.6	116.5±124.2	76.9±90.7	
	Daidzein	Median	13.4	32.0	17.8	10.2	0.141
		Range	3.2–275.4	3.1–152.0	3.0–248.1	2.0–120.3	
		Mean±SD	52.0±73.9	46.4±40.3	37.4±55.7	22.7±28.1	
	Equol-producers	*n*	14/22(64%)	8/21(38%)	9/22(41%)	10/23(44%)	
	Equol	Median	6.8	6.1	6.2	12.3	0.687
		Range	2.2–85.3	1.4–904.7	0.6–137.0	0.7–53.6	
		Mean±SD	17.7±26.0	120.1±317.1	22.5±43.6	20.2±18.4	

** $p<0.01$.
[a] Kruskal-Wallis test.

Table 8. Pearson's correlation of coefficients between of genistein and daidzein intake, and serum isoflavone levels by duration of hospitalization.

Isoflavone		Serum											
		Genistein				Daidzein				Equol			
		≦3 d	4–7 d	8–15 d	≧16 d	≦3 d	4–7 d	8–15 d	≧16 d	≦3 d	4–7 d	8–15 d	≧16 d
	n	22	21	22	23	22	21	22	23	14	8	9	10
Intake[a]	Genistein in the last meal	0.04	0.58**	0.17	−0.05	0.06	0.30	0.21	−0.03	0.37	−0.59	−0.14	0.12
	Genistein on the previous day	0.37	0.52*	0.68**	−0.29	0.42	0.42	0.76***	−0.29	0.41	−0.27	0.74*	−0.46
	Average of genistein for 2 d	0.12	0.09	0.18	0.25	0.02	0.20	0.48*	0.30	0.11	−0.28	−0.54	0.36
	Daidzein at the last meal	0.06	0.56**	0.17	−0.06	0.07	0.28	0.22	−0.04	0.36	−0.60	−0.14	0.13
	Daidzein on the previous day	0.40	0.48*	0.70***	−0.26	0.41	0.39	0.77***	−0.30	0.43	−0.26	0.77*	−0.48
	Average of daidzein for 2 d	0.16	0.40	0.62**	−0.11	0.17	0.17	0.63**	−0.12	0.44	−0.28	0.87**	−0.37

* $p<0.05$. ** $p<0.01$. *** $p<0.001$.
[a] Energy adjusted values.

intake of isoflavones at the last meal before blood collection to serum isoflavone levels. In contrast, no such relationship was observed in equol-producers. There was no relationship between the intake amount of isoflavones and blood equol levels.

Table 7 shows the comparison of the intake amount of isoflavones and blood isoflavone levels for the four divided periods during hospitalization. There was no significant difference in the intake amount of isoflavones compared with the duration of hospitalization. There was no significant correlation between the number of hospitalized days during a week and serum isoflavone levels. However, after 1 wk of hospitalization, blood isoflavone levels were slightly decreased. Blood levels of genistein were significantly decreased after a 16-d stay in the hospital.

Table 8 shows the relationship between the intake amount of isoflavones and blood isoflavone levels depending on the number of hospitalization days. In the group hospitalized for 3 d or less, no correlation was observed between the intake amount of isoflavones and blood isoflavone levels. A relationship between the intake amount of genistein and blood genistein levels was noted in the group for 4–7 d hospitalization and in the group for 8–15 d hospitalization. A relationship between the intake amount of daidzein and blood daidzein levels was observed only in the group for 8–15 d hospitalization. Although a significant relationship was not observed between the intake amount of isoflavones and blood equol levels in total, there was observed a significant relationship in the 8–15 d hospitalization group.

DISCUSSION

In this paper we estimated the intake amount of isoflavones (genistein, daidzein) ingested during a comparatively quantitative hospital diet and an ordinary diet, compared the levels between patients' ages, and analyzed the relationship between their intake amounts and blood isoflavone levels. The median daily dietary intake of isoflavones by Japanese was reported to 27.9 mg for genistein and 14.7 mg for daidzein (*15*). Another study reported the intake amount of genistein to be 31.4 mg and daidzein 18.3 mg in 122 males and 125 females, based on an FFQ (*16*). The total amount of isoflavones ingested daily was reported to be 39.5 mg in Japan (*13*). In the present study, the intake amount of genistein and daidzein in hospitalized patients (Tsukuba University Hospital) was 14.2 mg/d and 10.4 mg/d, respectively for 2 d before blood collection. These data were lower levels than what was previously reported, and were also lower than that reported in the ordinary diet.

Yamamoto et al. reported that the main item for the intake of isoflavones in soybean foods in Japan was natto, which accounted for 33% to 34% of the total intake, and miso, tofu and natto accounted for up to 90% (*16*). Another report revealed that only four items (tofu for miso soup, other tofu types, miso and natto) contributed to more than 80% of the total intake of isoflavones (*17*). Our present report showed that tofu, natto and miso accounted for 87.4% of the total intake of isoflavones in the ordinary diet. However, in the hospital diet, tofu and miso accounted for 49.1% of the total amount of isoflavone intake, and natto accounted for only 7.5%. It should be taken into consideration that natto has an odor, and that 50% of the breakfasts served in Tsukuba University Hospital are now western-style ones.

Arai et al. reported a relationship between the intake amount, blood levels and urinary excretion levels of isoflavones, including equol (*15*). Adlercreutz et al. observed a positive relationship between the daily intake of soybean foods and urinary isoflavone excretion levels, including equol in 19 patients in Kyoto (*18*). Xu et al. reported that dietary intake amounts and urinary excretion levels showed a positive relationship with an intervention trial using soymilk given to young women (*19*). Our observations indicated that there is a positive relationship between the amount of isoflavones ingested 1 and 2 d before blood tests and blood isoflavone levels (genistein and daidzein). Regarding the hospital diet, there was an interesting finding. At least 1 wk after hospitalization was needed for blood isoflavone levels to decrease. For the first 3 d of hospitalization, the relationship was not observed because of the influence of daily dietary intake on blood isoflavone levels. In the group hospitalized for over 1 wk, blood isoflavone levels were decreased, and a relationship between the amount of intake of isoflavones and blood isoflavone levels was observed. Watanabe et al. reported that, with an intervention trial that was designed to reduce the subjects' blood isoflavone level to zero, 72 h is necessary for the recovery to normal blood isoflavone levels (*20*). The influence of a daily dietary intake that is rich in soybean foods disappeared after 1 wk of the hospital diet. We also observed that in the group hospitalized for 16 d or longer, blood isoflavone levels were significantly decreased, and a relationship between dietary isoflavone intake and blood isoflavone levels was not demonstrated. Fukui et al. reported that blood isoflavone levels were increased remarkably by day 4 during a high isoflavone-loading test. However, blood isoflavone levels came down after day 11 in spite of continuing the high amount of loading (*21*). They speculated that a change in the internal absorption influenced blood isoflavone levels. Our observations on longer hospitalized groups were probably also influenced by this mechanism, but the disease-status should also be considered because 16 of 23 patients in this group were sufferring from prostate cancer. In our study, it was revealed that the hospital diet, which should be ideal to support patient health, was not the ideal one for the prevention of cancer from the point of dietary intake of isoflavones. It is assumed that a hospital diet should be planned to provide functional foods for the prevention of cancer.

We observed a significant difference in the amount of the intake of isoflavones during the ordinary intake between the ages under 70 and above 70. Similar observations were obtained on the blood levels of isoflavones in our previous study (*9*). These data indicate that the intake of isoflavones is changing in Japan. Younger generations in Japan have been pointed out to consume less soybean foods, and to remove most of the fat from meat (*22*). It has been reported that the intake content in younger ages may influence the onset of breast cancer (*23*). In Japan, it has been also reported that cases of prostate cancer have been increasing in number (*24*). Therefore, it is very important to check the ordinary intake during younger ages for the prevention of prostate cancer.

Watanabe et al. reported that genistein and daidzein showed maximum blood levels 6 h after intake, and that the half-life of each was 8.95 h and 6.31 h, respectively, using an intervention trial with kinako-loading in seven males (*20*). Based on this observation, it was supposed that the amount of the dietary intake of isoflavone at the last meal before blood collection and blood isoflavone levels should be related significantly, but no relationship was observed. However, the relationship was observed in equol non-producers. King and Bursill reported that, in an intervention trial, urinary excretion of equol appeared 6 h after isoflavone intake and continued for over 11 to 35 h (*25*). This observation indicates that the production of equol by the intestinal bacterial microflora can influence blood daidzein levels for over 10 h after the intake of daidzein, and also indicates that the metabolism to dihydrogenistein by the intestinal bacterial microflora shows a similar tendency for daidzein and genistein, which are increased in the blood with a similar time dependency (*26*). Therefore, further investigation into the mechanisms and pharma-

cokinetics of isoflavones, including equol production, is necessary. It also seems very important to examine cases more precisely to compare isoflavone levels and to estimate the relationship on the disease-status as a case-controlled study.

REFERRENCES

1) Lee HP, Gourley L, Duffy SW, Esteve J, Lee J, Day NE. 1991. Dietary effects on breast-cancer risk in Singapore. *Lancet* **337**: 1197–1200.
2) Severson RK, Nomura AM, Grove JS, Stemmermann GN. 1989. A prospective study of demographics, diet, and prostate cancer among men of Japanese ancestry in Hawaii. *Cancer Res* **49**: 1857–1860.
3) Goodman MT, Hankin JH, Wilkens LR, Lyu LC, McDuffie K, Liu LQ, Kolonel LN. 1997. Diet, body size, physical activity, and the risk of endometrial cancer. *Cancer Res* **57**: 5077–5085.
4) Evans BA, Griffiths K, Morton MS. 1995. Inhibition of 5 alpha-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. *J Endocrinol* **147**: 295–302.
5) Munro IC, Harwood M, Hlywka JJ, Stephen AM, Doull J, Flamm WG, Adlercreutz H. 2003. Soy isoflavones: a safety review. *Nutr Rev* **61**: 1–33.
6) Makela S, Poutanen M, Kostian ML, Lehtimaki N, Strauss L, Santti R, Vihko R. 1998. Inhibition of 17beta-hydroxysteroid oxidoreductase by flavonoids in breast and prostate cancer cells. *Proc Soc Exp Biol Med* **217**: 310–316.
7) Peterson G, Barnes S. 1993. Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* **22**: 335–345.
8) Molteni A, Brizio-Molteni L, Persky V. 1995. In vitro hormonal effects of soybean isoflavones. *J Nutr* **125**: 751S–756S.
9) Akaza H, Miyanaga N, Takashima N, Naito S, Hirao Y, Tsukamoto T, Mori M. 2002. Is daidzein non-metabolizer a high risk for prostate cancer? A case-controlled study of serum soybean isoflavone concentration. *Jpn J Clin Oncol* **32**: 296–300.
10) Slavin JL, Karr SC, Hutchins AM, Lampe JW. 1998. Influence of soybean processing, habitual diet, and soy dose on urinary isoflavonoid excretion. *Am J Clin Nutr* **68**: 1492S–1495S.
11) Breinholt V, Larsen JC. 1998. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. *Chem Res Toxicol* **11**: 622–629.
12) Thompson FE, Byers T. 1994. Dietary assessment resource manual. *J Nutr* **124**: 2245S–2317S.
13) Kimira M, Arai Y, Shimoi K, Watanabe S. 1998. Japanese intake of flavonoids and isoflavonoids from foods. *J Epidemiol* **8**: 168–175.
14) Coward L, Kirk M, Albin N, Barnes S. 1996. Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin Chim Acta* **247**: 121–142.
15) Arai Y, Uehara M, Sato Y, Kimira M, Eboshida A, Adlercreutz H, Watanabe S. 2000. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J Epidemiol* **10**: 127–135.
16) Yamamoto S, Sobue T, Sasaki S, Kobayashi M, Arai Y, Uehara M, Adlercreutz H, Watanabe S, Takahashi T, Iitoi Y, Iwase Y, Akabane M, Tsugane S. 2001. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. *J Nutr* **131**: 2741–2747.
17) Wakai K, Egami I, Kato K, Kawamura T, Tamakoshi A, Lin Y, Nakayama T, Wada M, Ohno Y. 1999. Dietary intake and sources of isoflavones among Japanese. *Nutr Cancer* **33**: 139–145.
18) Adlercreutz H, Honjo H, Higashi A, Fotsis T, Hamalainen E, Hasegawa T, Okada H. 1991. Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am J Clin Nutr* **54**: 1093–1100.
19) Xu X, Wang HJ, Murphy PA, Cook L, Hendrich S. 1994. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr* **124**: 825–832.
20) Watanabe S, Yamaguchi M, Sobue T, Takahashi T, Miura T, Arai Y, Mazur W, Wahala K, Adlercreutz H. 1998. Pharmacokinetics of soybean isoflavones in plasma, urine and feces of men after ingestion of 60 g baked soybean powder (kinako). *J Nutr* **128**: 1710–1715.
21) Fukui Y, Nasada M, Toda T, Okuhira T, Hayashi A. 1999. Influence of soy isoflavones in Japanese men. *Kenkou Eiyo Syokuhin Kenkyu* **2**: 63–71 (in Japanese).
22) Kenko Eiyo Joho Kenkyukai. 2003. Kokumin Eiyo no Genjyo 2000. Daiichi Shuppan, Tokyo (in Japanese).
23) Colditz GA, Frazier AL. 1995. Models of breast cancer show that risk is set by events of early life: prevention efforts must shift focus. *Cancer Epidemiol Biomarkers Prev* **4**: 567–571.
24) Tominaga H, Oshima A, Kuroiwa T. 1999. Gann•Toukei Hakusyo—1999. Shinohara Shuppan Sinsha, Tokyo (in Japanese).
25) King RA, Bursill DB. 1998. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am J Clin Nutr* **67**: 867–872.
26) Kinjo J. 2000. Phytoestrogens. *Nippon Rinsho* **58**: 2434–2438 (in Japanese).

Synergistic antitumor effect of combined use of adenoviral-mediated *p53* gene transfer and antisense oligodeoxynucleotide targeting *clusterin* gene in an androgen-independent human prostate cancer model

Kazuki Yamanaka,[1] Martin E. Gleave,[1,2] Isao Hara,[3] Mototsugu Muramaki,[1] and Hideaki Miyake[4]

[1]Prostate Centre, Vancouver General Hospital; [2]Division of Urology, University of British Columbia, Vancouver, British Columbia, Canada; [3]Department of Urology, Kobe University School of Medicine, Kobe, Japan; and [4]Department of Urology, Hyogo Medical Center for Adults, Akashi, Japan

Abstract

Our recent studies showed that antisense oligodeoxynucleotide targeting antiapoptotic gene, *clusterin*, enhanced apoptosis induced by conventional therapeutic modalities using several prostate cancer models. In this study, to establish a more effective therapeutic strategy against prostate cancer, we investigated the effect of combined treatment with antisense clusterin oligodeoxynucleotide and adenoviral-mediated *p53* gene transfer (Ad5CMV-*p53*) in an androgen-independent human prostate PC3 tumor model. Treatment of PC3 cells with 500 nmol/L antisense clusterin oligodeoxynucleotide decreased clusterin mRNA by >80% compared with that with 500 nmol/L mismatch control oligodeoxynucleotide. Clusterin mRNA expression in PC3 cells was highly upregulated by Ad5CMV-*p53* treatment; however, antisense clusterin oligodeoxynucleotide treatment further suppressed clusterin expression in PC3 cells after Ad5CMV-*p53* treatment. Antisense clusterin oligodeoxynucleotide treatment significantly enhanced the sensitivity of Ad5CMV-*p53* in a dose-dependent manner, reducing the IC_{50} of Ad5CMV-*p53* by 75%. Apoptotic cell death was detected after combined treatment but not after treatment with either agent alone. *In vivo* administration of antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* resulted in a significant inhibition of s.c. PC3 tumor growth as well as lymph node metastases from orthotopic PC3 tumors compared with administration of either agent alone. Furthermore, combined treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-*p53*, and mitoxantrone completely eradicated s.c. PC3 tumors and lymph node metastases from orthotopic PC3 tumors in 60% and 100% of mice, respectively. These findings suggest that combined treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* could be a novel strategy to inhibit progression of hormone-refractory prostate cancer and that further addition of chemotherapeutic agents may help to enhance the efficacy of this combined regimen. [Mol Cancer Ther 2005;4(2):187–95]

Introduction

Prostate cancer is the most commonly diagnosed malignant disease and the second leading cause of cancer-related death in men in Western industrialized countries. Androgen ablation, however, remains the only effective therapy for patients with advanced disease. Approximately 80% of patients achieve symptomatic and/or objective response after androgen withdrawal, but progression to androgen independence ultimately occurs in almost all cases (1). Although a number of nonhormonal agents have been evaluated in patients with hormone-refractory disease, these agents have limited antitumor activity with an objective response rate of <20% and failed to show a survival benefit (2). Accordingly, novel therapeutic strategies targeting molecular mechanisms mediating resistance to conventional treatments must be developed to achieve a significant impact on the survival of patients with advanced prostate cancer.

Gene transfer techniques may provide new therapeutic approaches to a variety of malignancies (3), and among them, replication-deficient adenoviral vectors have been shown to be attractive because they are capable of efficient infection of target cells and are safe for clinical use (4). A number of studies have shown that adenoviral-mediated gene therapy of wild-type *p53* in tumor cells, including prostate cancer, suppressed tumor growth both *in vitro* and *in vivo* through induction of the apoptotic pathway. Based on the promising results of these preclinical studies, several clinical trials of *p53* gene therapy have been carried out (5–10). Recent studies, however, showed that expression of antiapoptotic genes markedly inhibits the therapeutic efficacy of adenoviral-mediated *p53* gene transfer (11, 12);

Received 7/22/04; revised 10/23/04; accepted 12/8/04.

Grant support: Ministry of Education, Science, and Culture of Japan grant-in-aid.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Hideaki Miyake, Department of Urology, Hyogo Medical Center for Adults, 13-70 Kitaohji-cho, Akashi 673-8558, Japan. Phone: 81-78-929-1151; Fax: 81-78-929-2380. E-mail: hideakimiyake@hotmail.com

therefore, these findings suggest that it would be necessary to add another type of adequate therapy if adenovirus *p53* vectors are used for cases expressing high levels of antiapoptotic genes.

Clusterin, also known as testosterone-repressed prostate message-2, sulfated glycoprotein-2, or apolipoprotein J, was first isolated from ram rete testes fluid (13) and has various biological functions, including tissue remodeling, reproduction, lipid transport, and apoptotic cell death (14). Clusterin was initially regarded as a marker for cell death, because its expression is highly up-regulated in various normal and malignant tissues undergoing apoptosis (15–18). Recent studies, however, reported conflicting findings on the association between enhanced clusterin expression and apoptotic activity (19–21). Similarly, clusterin expression is increased in regressing normal and malignant prostate tissues after androgen ablation (15), and its up-regulation has been shown to be associated with anti-apoptotic activity and disease progression in prostate cancer (22–24). We have also reported that clusterin expression in malignant prostatic tissue was significantly greater in patients who underwent preoperative neoadjuvant hormonal therapy (25), and that clusterin expression in prostate cancer cells plays a protective role against apoptosis induced by androgen ablation, cytotoxic chemotherapy, and radiation; therefore, antisense oligodeoxynucleotide therapy targeting the *clusterin* gene enhances the efficacies of several kinds of therapy, resulting in a delay in the progression to androgen independence (26–28). However, because numerous genes mediate tumor progression, inhibition of a single target gene is likely insufficient to completely suppress tumor progression. Although there has been no report demonstrating complete responses of established tumors *in vivo* using antisense oligodeoxynucleotide technique alone, combined use of antisense oligodeoxynucleotide with another oligodeoxynucleotide targeting a different gene or other type of agents, such as a chemotherapeutic agent, has been shown to induce more potent antineoplastic effects in some tumor model systems (29, 30).

The objectives of this study were to test whether induction of apoptotic cell death by adenoviral-mediated *p53* gene transfer (Ad5CMV-*p53*) is enhanced by antisense clusterin oligodeoxynucleotide treatment, and to determine whether combined use of Ad5CMV-*p53* and antisense clusterin oligodeoxynucleotide inhibits tumor growth and metastasis in the androgen-independent human prostate PC3 tumor model.

Materials and Methods

Tumor Cell Line

PC3, derived from human prostate cancer, was purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% heat-inactivated FCS.

Chemotherapeutic Agent

Mitoxantrone was purchased from Wyeth-Ayerst, Inc. (Montreal, Quebec, Canada). Stock solutions of mitoxantrone (1 mg/mL) were prepared with DMSO and diluted with PBS to the required concentrations before each *in vitro* experiment.

Adenovirus Vectors

Two previously described replication-deficient recombinant adenovirus vectors were used in this study (12). Ad5CMV-*p53* expresses wild-type human *p53* under the control of the human cytomegalovirus promoter, and Ad5CMV-*Luc* encoding *Luciferase* gene was used as a control vector. Adenoviruses were propagated in 293 cells, a human embryonic kidney cell line, which contains estrone and is thus highly permissive of the replication of the estrone replication-deficient adenovirus. It was stored as previously reported, and the viral infection titers were determined by plaque assays (12).

Antisense Clusterin Oligodeoxynucleotides

Phosphorothioate oligodeoxynucleotides used in this study were generously supplied by Dr. Brett P. Monia (Isis Pharmaceuticals, Carlsbad, CA). The sequences of antisense clusterin oligodeoxynucleotides corresponding to the human clusterin translation initiation site were 5′-CAG-CAGCAGAGTCTTCAT-3′. Two-base clusterin mismatch oligodeoxynucleotide (5′-CAGCAGCAGAGTATTTAT-CAT-3′) was used as control. Oligodeoxynucleotides were diluted in 10 mmol/L Tris (pH 7.4) and 1 mmol/L EDTA and kept at −20°C.

Treatment of Cells with Oligodeoxynucleotides

PC3 cells were treated with various concentrations of oligodeoxynucleotides after a preincubation for 20 minutes with 4 μg/mL lipofectin (Life Technologies) in serum-free OPTI-MEM (Life Technologies). Four hours later, the medium containing oligodeoxynucleotides and lipofectin was replaced with the standard culture medium described above.

Northern Blot Analysis

Total RNA was isolated from cultured PC3 cells using the acid-guanidium thiocyanate-phenol-chloroform method. Electrophoresis, hybridization, and washing conditions were carried out as previously reported (28). Human clusterin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were generated by reverse transcription-PCR from total RNA of human kidney using primers 5′-AAGGAAATTCA-AAATGCTGTCAA-3′ (sense) and 5′-ACAGACAAGATCTCCCGGCACTT-3′ (antisense) for clusterin and 5′-TGCTTTTAACTCTGG-TAAAGT-3′ (sense) and 5′-ATATTTGGCAGGTTTTTC-TAGA-3′ (antisense) for GAPDH. The density of bands for clusterin was normalized against that of GAPDH by densitometric analysis.

Real-time Reverse Transcription-PCR

Total RNA was isolated as described above, and 1 μg of each total RNA was reverse transcribed using an Oligo dT and Superscript preamplification system (Life Technologies). To examine the expression levels of clusterin, real-time quantitative PCR was done using a Sequence Detector (ABI PRISM 7700, PE Applied Biosystems, Foster City, CA).

Selected sequences of primers, and probes are as follows: 5′-GAGCAGCTGAACGAGCAGTTT-3′ (sense), 5′-CTTCGCCTTGCGTGAGGT-3′ (antisense) and 5′-ACTGGGTGTCC-CGGCT-3′ (probe) for clusterin and 5′-GAAGGTGAAGGTCGGAGTC-3′ (sense), GAPDH 5′-GAAGATGGTGATGGGATTTC-3′ (antisense), 5′-CA-AGCTTCCCGTTCTCAGCC-3′ (prove) for GAPDH. The Taqman probes consisted of an oligodeoxynucleotide with a 5′ FAM reporter dye and 3′ TAMRA quencher dye. Each cDNA was analyzed by quantitative PCR in a 50-μL volume using Master Mix (PE Applied Biosystems). The condition of thermal cycling was 50 cycles of amplification consisting of 15 seconds at 95°C and 1 minute at 60°C. Real-time quantitation was done based on Taqman assay according to the manufacturer's instruction. After the generation of a real-time amplification plot based on the normalized fluorescence signal, the threshold cycle was determined. Threshold cycle was then used for kinetic analysis and was proportional to the initial number of target copies in the sample. The starting quantity of a sample was calculated after comparison of the threshold cycles of a serial dilution of a positive control. Transcripts of the GAPDH were also quantified as an internal reference, and the quantification value of clusterin mRNA was described as each value relative to that of GAPDH mRNA.

In vitro Cell Growth Assay

The *in vitro* growth-inhibitory effects of antisense clusterin oligodeoxynucleotide and/or Ad5CMV-*p53* on PC3 cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., St. Louis, MO) as described previously (28). Briefly, 1×10^4 cells were seeded in each well of 96-well microtiter plates and allowed to attach overnight. Cells were then treated once daily with various concentrations of oligodeoxynucleotide for 2 days. Following oligodeoxynucleotide treatment, cells were treated with various concentrations of Ad5CMV-*p53*. After 48 hours of incubation, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in PBS was added to each well followed by incubation for 4 hours at 37°C. The formazan crystals were dissolved in DMSO. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware, Lincoln Park, NJ) at 540 nm. Absorbance values were normalized to the values obtained for the vehicle-treated cells to determine the percent of survival. Each assay was done in triplicate.

Measurement of Apoptosis

The nucleosomal DNA degradation was quantified by the Cell Death Detection ELISA kit using antihistone antibody (Roche, Mannheim, Germany) as described previously (31). Briefly, 1×10^5 PC3 cells were seeded in 5-cm culture dishes and allowed to adhere overnight. After treatment with oligodeoxynucleotide and/or Ad5CMV-*p53* under the same schedule as described above, cells were harvested and then assays were done in triplicate according to the manufacture's instruction.

Assessment of *In vivo* Tumor Growth

Athymic nude mice (BALB/c *nu/nu* females, 6–8 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan), and housed in a controlled environment at 22°C on a 12-hour light, 12-hour dark cycle. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Each experimental group consisted of 10 mice. PC3 cells were trypsinized, washed twice with PBS, and 1×10^6 cells were injected s.c. with 0.1 mL of Matrigel (Becton Dickinson Labware) in the flank, or directly given 1×10^6 cells into the prostate, as previously described (32). The schedules administrating oligodeoxynucleotide, viral vectors, and chemotherapeutic agents were determined based on our previously reported studies (8, 28, 33).

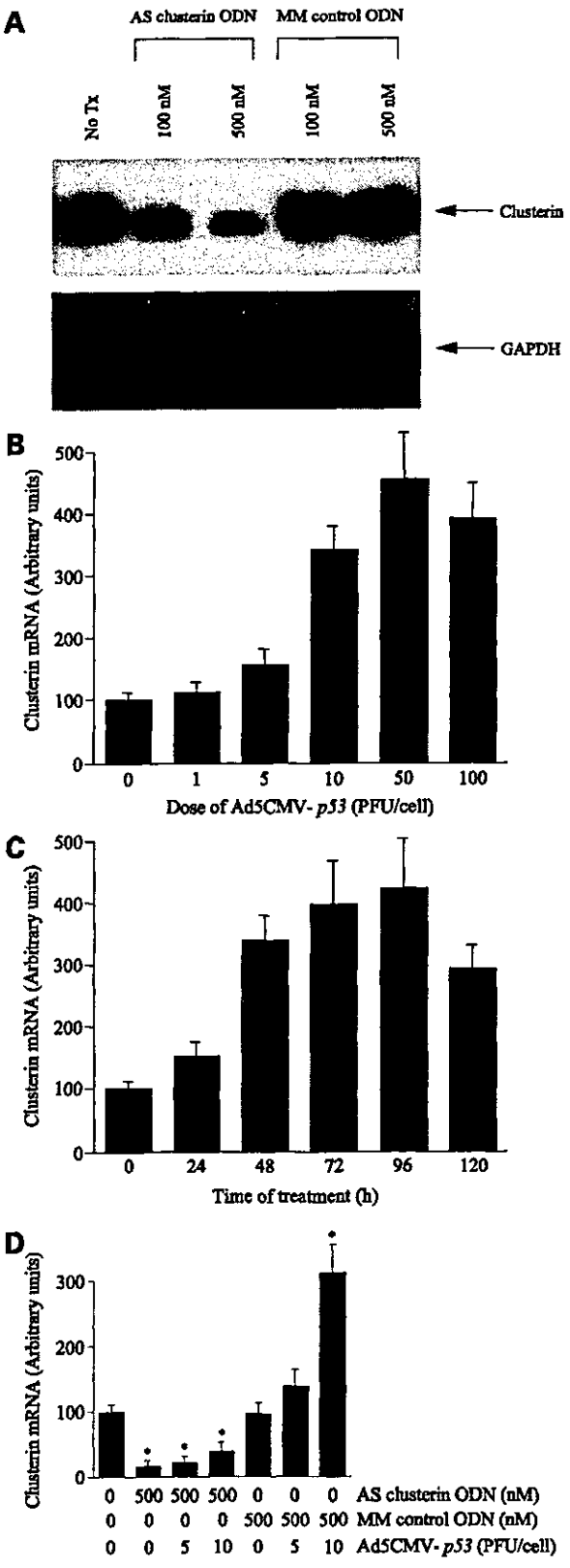
For the first experiment, 10 days after injection, mice were randomly selected for treatment with antisense clusterin oligodeoxynucleotide plus Ad5CMV-*Luc*, mismatch control oligodeoxynucleotide plus Ad5CMV-*Luc*, antisense clusterin oligodeoxynucleotide plus Ad5CMV-*p53*, or mismatch control oligodeoxynucleotide plus Ad5CMV-*p53*. After randomization, 10 mg/kg antisense clusterin or mismatch control oligodeoxynucleotide were injected i.p. once daily into each mouse for 28 days, and 200 μL of adenovirus vector [1×10^7 plaque-forming units (pfu)/mL] were injected intratumorally or i.p. into mice bearing s.c. or orthotopic tumors, respectively, twice a week for 2 weeks.

For the second experiment, 10 days after injection, mice were randomly selected for treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-*Luc* plus mitoxantrone, mismatch control oligodeoxynucleotide, Ad5CMV-*Luc* plus mitoxantrone, antisense clusterin oligodeoxynucleotide, Ad5CMV-*p53* plus mitoxantrone, or mismatch control oligodeoxynucleotide, Ad5CMV-*p53* plus mitoxantrone. After randomization, 10 mg/kg antisense clusterin or mismatch control oligodeoxynucleotide was injected i.p. once daily into each mouse for 28 days, 200 μL of adenovirus vector (1×10^7 pfu/mL) were injected intratumorally or i.p. into mice bearing s.c. or orthotopic tumors, respectively, twice a week for 2 weeks, and 50 μg of mitoxantrone were injected i.v. twice a week for 2 weeks.

Tumor volume was measured every 5 days and calculated by the formula length × width × depth × 0.5236 (28). Eight weeks after the injection of tumor cells in the prostate, the mice were sacrificed and the presence of metastasis was macroscopically examined in all abdominal and thoracic internal organs. The organs with metastases were removed, and the number of surface metastatic nodules was counted.

Statistical Analysis

A repeated-measure ANOVA model was used to analyze the both *in vitro* and *in vivo* cytotoxic effects of oligodeoxynucleotide, Ad5CMV-*p53* and mitoxantrone. Changes in clusterin mRNA levels were evaluated by one-factor ANOVA. Synergy of oligodeoxynucleotide and Ad5CMV-*p53* was analyzed by calculating the fractional product variable according to the fractional product method. Other



data were analyzed by Student's *t* test. The levels of statistical significance were set at $P < 0.05$, and all statistical calculations were done using Statview 4.5 software (Abacus Concepts, Inc., Berkeley, CA).

Results

Changes in Clusterin Expression in PC3 Cells after Treatment with Ad5CMV-*p53* and/or Antisense Clusterin Oligodeoxynucleotides

We initially showed the marked inhibition of clusterin mRNA expression in PC3 cells by treatment with antisense clusterin oligodeoxynucleotide using Northern blot analysis; that is, treatment of PC3 cells with 500 nmol/L antisense clusterin oligodeoxynucleotide decreased clusterin mRNA by >80% compared with that with mismatch control oligodeoxynucleotide (Fig. 1A).

Real-time reverse transcription-PCR assay was used to determine the effects of Ad5CMV-*p53* treatment on clusterin mRNA expression in PC3 cells. As shown in Fig. 1B, clusterin mRNA induction increased in a dose-dependent manner by Ad5CMV-*p53* treatment at concentrations up to 50 pfu per cell ($P < 0.05$). Time course experiment showed that Ad5CMV-*p53*-induced clusterin mRNA up-regulation peaked by 96 hours after treatment and began decreasing by 120 hours after treatment ($P < 0.05$; Fig. 1C).

We then examined whether antisense clusterin oligodeoxynucleotide treatment further suppresses clusterin expression in PC3 cells treated by Ad5CMV-*p53*, which causes up-regulation of the *clusterin* gene as described above. As shown in Fig. 1D, combined treatment with 500 nmol/L antisense clusterin oligodeoxynucleotide and 5 or 10 pfu per cell Ad5CMV-*p53* decreased clusterin mRNA levels by 84% or 87%, respectively, compared with that by 500 nmol/L mismatch control oligodeoxynucleotide treatment and 5 or 10 pfu per cell Ad5CMV-*Luc*.

Figure 1. Effects of antisense (*AS*) clusterin oligodeoxynucleotide (*ODN*) and/or Ad5CMV-*p53* treatment on clusterin expression in PC3 cells. **A**, PC3 cells were treated daily with antisense clusterin oligodeoxynucleotide or a two-base clusterin MM ODN for 2 d. Total RNA was extracted from cultured cells and analyzed for clusterin and GAPDH levels by Northern blotting. *No Tx*, untreated cells. **B**, PC3 cells were treated with various doses of Ad5CMV-*p53* for 48 h; total RNA was then extracted and analyzed for clusterin levels by real-time reverse transcription-PCR. As described in Materials and Methods, clusterin mRNA value was corrected by corresponding GAPDH mRNA value. *Columns*, mean of three independent experiments; *bars*, SD. The changes in clusterin mRNA levels were significant ($P < 0.05$ by one-factor ANOVA). **C**, PC3 cells were treated with Ad5CMV-*p53* at 10 pfu per cell for indicated intervals; total RNA was then extracted and clusterin levels were analyzed by real-time reverse transcription-PCR. As described in Materials and Methods, clusterin mRNA value was corrected by corresponding GAPDH mRNA value. *Columns*, mean of three independent experiments; *bars*, SD. The changes in clusterin mRNA levels were significant ($P < 0.05$ by one-factor ANOVA). **D**, PC3 cells were treated daily with 500 nmol/L AS clusterin ODN or a two-base clusterin mismatch (*MM*) control ODN for 2 d. Following a 48-h exposure to Ad5CMV-*p53* at 5 or 10 pfu per cell, total RNA was extracted and clusterin levels were analyzed by real-time reverse transcription-PCR. As described in Materials and Methods, clusterin mRNA value was corrected by corresponding GAPDH mRNA value. *Columns*, mean of three independent experiments; *bars*, SD. *, $P < 0.01$, differs from control (Student's *t* test).

Synergistic Induction of Apoptotic Cell Death by Ad5CMV-*p53* and Antisense Clusterin Oligodeoxynucleotides in PC3 Cells *In vitro*

To examine whether treatment with antisense clusterin oligodeoxynucleotide enhances the Ad5CMV-*p53*-induced cytotoxicity, PC3 cells were treated with various concentrations of antisense clusterin oligodeoxynucleotide once daily for 2 days and then incubated with various concentrations of Ad5CMV-*p53* for 2 days. As shown in Fig. 2A, antisense clusterin oligodeoxynucleotide treatment significantly enhanced sensitivity to Ad5CMV-*p53* in a dose-dependent manner ($P < 0.01$), reducing the IC_{50} of Ad5CMV-*p53* from 25 to 5 pfu per cell, whereas mismatch control oligodeoxynucleotide had no effect. Dose-dependent synergy between antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* was also observed by increasing the antisense oligodeoxynucleotide concentration when Ad5CMV-*p53* dose was fixed at 5 pfu per cell ($P < 0.01$; Fig. 2B).

DNA fragmentation was measured using an ELISA kit to evaluate the effects of combined antisense clusterin oligodeoxynucleotide (500 nmol/L) and Ad5CMV-*p53* (5 pfu per cell) treatment on apoptosis induction. After the same treatment schedule described above, marked DNA ladder formation was observed only after combined treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* (Fig. 2C).

Inhibition of PC3 Tumor Growth and Metastasis *In vivo* by Combined Treatment with Ad5CMV-*p53* and Antisense Clusterin Oligodeoxynucleotides

The efficacy of a regimen combining Ad5CMV-*p53* and antisense clusterin oligodeoxynucleotide for inhibiting the growth of s.c. PC3 tumors was evaluated. Athymic nude mice bearing PC3 tumors ~1 cm in diameter were randomly selected for treatment as described above. Mean tumor volume was similar at the beginning of treatment in each of these groups. Whereas changes in tumor volume in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-*Luc* were similar to those with mismatch control oligodeoxynucleotide plus Ad5CMV-*Luc*, PC3 tumor growth was inhibited slightly but definitively by treatment with mismatch control oligodeoxynucleotide plus Ad5CMV-*p53*, and combined antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* therapy showed marked growth inhibitory effects. Fifty days after tumor injection, the tumor volume in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-*p53* was 65%, 66%, or 50% smaller than that in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-*Luc*, mismatch control oligodeoxynucleotide plus Ad5CMV-*Luc*, or mismatch control oligodeoxynucleotide plus Ad5CMV-*p53*, respectively (Fig. 3); however, there were no significant differences in the body weight of mice among these four groups (data not shown).

We then examined the effects of the combined regimen using a recently reported orthotopic injection (i.e., intraprostatic injection) model (32) according the same treatment schedule described above. As shown in Table 1, the

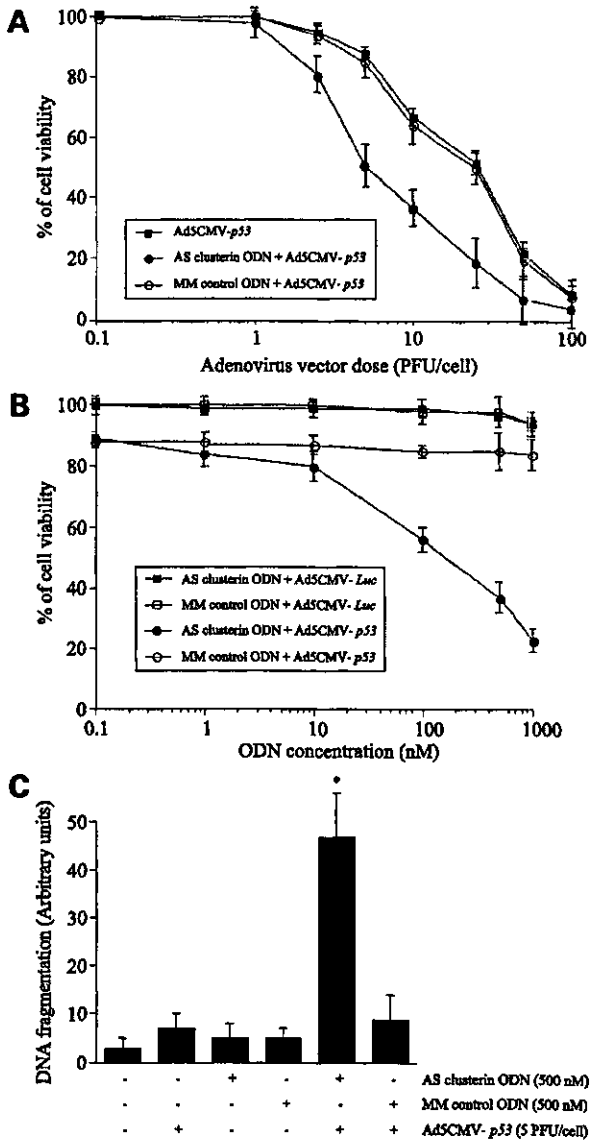


Figure 2. Effects of combined treatment with antisense (*AS*) clusterin oligodeoxynucleotide (*ODN*) and Ad5CMV-*p53* on PC3 cell growth and apoptosis. **A**, PC3 cells were treated daily with 500 nmol/L AS clusterin ODN or mismatch (*MM*) control ODN for 2 d. Following ODN treatment, the medium was replaced with medium containing various doses of Ad5CMV-*p53*. After 48 h of incubation, number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Point*, mean of triplicate analyses; *bars*, SD. The cytotoxic effect of Ad5CMV-*p53* on PC3 cells was significantly enhanced by AS clusterin ODN treatment ($P < 0.01$, by repeated measure ANOVA). **B**, PC3 cells were treated daily with various concentrations of AS clusterin ODN or MM control ODN for 2 d, then incubated for 48 h with medium alone or medium containing Ad5CMV-*p53* or Ad5CMV-*Luc* at 5 pfu per cell, and number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Point*, mean of three independent experiments; *bars*, SD. Treatment of PC3 cells with AS clusterin ODN significantly enhanced the sensitivity to Ad5CMV-*p53* ($P < 0.05$, by repeated measure ANOVA). **C**, after the same treatment schedule as described above, DNA fragmentation was quantitatively measured by Cell Death Detection ELISA kit using antihistone antibody. *Columns*, mean of three independent experiments; *bars*, SD. *, $P < 0.01$ differs from control (Student's *t* test).

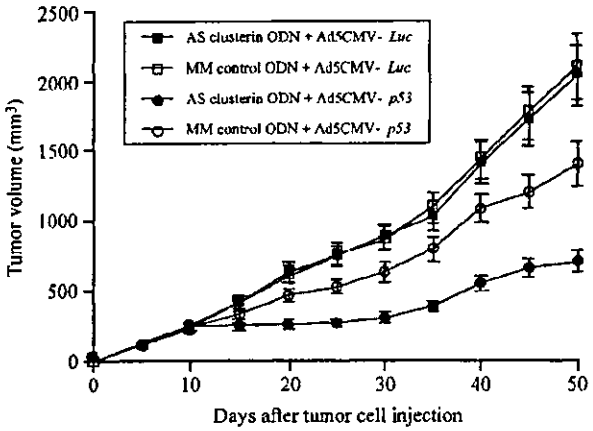


Figure 3. Effects of combined treatment with antisense (*AS*) clusterin oligodeoxynucleotide (*ODN*) plus Ad5CMV-*p53* on PC3 tumor growth. Mice bearing PC3 tumor were randomly selected for treatment with AS clusterin ODN plus Ad5CMV-*Luc*, mismatch (*MM*) control ODN plus Ad5CMV-*Luc*, AS clusterin ODN plus Ad5CMV-*p53*, or MM control ODN plus Ad5CMV-*p53*. Ten days after tumor cell injection, 10 mg/kg AS clusterin ODN or MM control ODN were injected daily i.p. for 28 d, and 200 μL of Ad5CMV-p53 or Ad5CMV-Luc (1×10^7 pfu/mL) were injected i.t. twice a week for 2 wks. Tumor volume was measured every 5 d and calculated by the formula, length × width × depth × 0.5236. *Point*, mean tumor volume in each experimental group containing 10 mice; *bars*, SD. Mean tumor volume in mice treated with AS clusterin ODN plus Ad5CMV-*p53* was significantly different from that in mice treated with other regimens ($P < 0.01$ by repeated-measure ANOVA).

combined antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* therapy substantially suppressed the primary tumor growth as well as the incidence of metastasis after orthotopic injection of PC3 cells compared with the other three treatment regimens. That is, both the weight of primary PC3 tumor and the incidences of retroperitoneal lymph node metastasis in mice treated with combined antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53* were significantly lower than those in mice treated with antisense clusterin oligodeoxynucleotide plus Ad5CMV-*Luc*, mismatch control oligodeoxynucleotide plus Ad5CMV-*Luc*, or mismatch control oligodeoxynucleotide plus Ad5CMV-*p53*. In addition, when sacrificed, there were no significant differences in the body weight of mice among these four groups (data not shown).

Complete Eradication of PC3 Tumor *In vivo* by Combined Treatment with Ad5CMV-*p53*, Antisense Clusterin Oligodeoxynucleotide, and Mitoxantrone

To achieve more potent antitumor effect against PC3 tumor growth, we evaluated the efficacy of combined regimen consisting of Ad5CMV-*p53*, antisense clusterin oligodeoxynucleotide, and mitoxantrone. Athymic nude mice bearing s.c. PC3 tumors ~1 cm in diameter were randomly selected for treatment as described above. Mean tumor volume was similar at the beginning of treatment in each of these groups. As shown in Fig. 4, combined treatment with Ad5CMV-*p53*, antisense clusterin oligodeoxynucleotide, and mitoxantrone significantly inhibited PC3 tumor growth compared with the other three regimen, and PC3 tumors in 6 of 10 mice were completely eradicated by this combined therapy. Furthermore, combined treatment with Ad5CMV-*p53*, antisense clusterin oligodeoxynucleotide, and mitoxantrone inhibited the development of lymph node metastasis in all mice after PC3 cells were orthotopically injected (Table 2). However, there were no significant differences in the body weight of mice among these four groups in s.c as well as orthotopic tumor cell injection models (data not shown).

Discussion

Despite the original hypothesis that clusterin is a marker for programmed cell death (15–18), several studies showed conflicting findings suggesting the dissociation of up-regulation of *clusterin* gene from apoptosis (19–21, 23–25). We also previously showed that overexpression of clusterin helps mediate malignant progression against apoptosis induced by therapeutic stimuli using several kinds of preclinical animal models, including prostate cancer (26–28). Collectively, these findings indicate that

Table 1. Changes in metastasis of PC3 cells injected into the prostate of nude mice after treatment with antisense clusterin oligodeoxynucleotide and Ad5CMV-*p53*

Regimen*	Incidence of lymph node metastasis (%)†	Incidence of hemorrhagic ascites (%)‡	Weight of the primary tumor (mg)
AS clusterin ODN + Ad5CMV-*Luc*	10/10 (100)	4/10 (40)	24.2 ± 8.5§
MM control ODN + Ad5CMV-*Luc*	10/10 (100)	4/10 (40)	26.1 ± 10.1
AS clusterin ODN + Ad5CMV-*p53*	3/10 (30)‖	0/10 (0)‖	13.6 ± 5.1¶
MM control ODN + Ad5CMV-*p53*	7/10 (10)	2/10 (20)	19.6 ± 7.2

*Ten days after the implantation of tumor cells, 10 mg/kg antisense clusterin oligodeoxynucleotide (AS clusterin ODN) or mismatch control oligodeoxynucleotide (MM control ODN) was injected i.p. for 28 days, and 200 μL of adenoviral vector (1×10^7 pfu/mL) were injected i.p. twice a week for 2 weeks.
†No. mice with tumor/no. injected mice.
‡No. mice with hemorrhagic ascites/no. injected mice.
§Mean ± SD.
‖The incidence of metastasis or hemorrhagic ascites was significantly different from that in mice treated with other regimens ($P < 0.05$, χ^2 test).
¶The mean weight of the primary tumor was significantly different from that in mice treated with other regimens ($P < 0.05$, Student's t test).

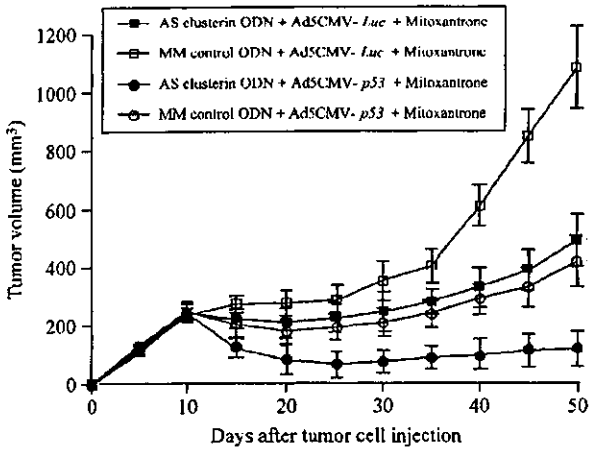


Figure 4. Effects of combined treatment with antisense (*AS*) clusterin oligodeoxynucleotide (*ODN*), Ad5CMV-*p53*, and mitoxantrone on PC3 tumor growth. Mice bearing PC3 tumor were randomly selected for treatment with AS clusterin ODN, Ad5CMV-*Luc* plus mitoxantrone, mismatch (*MM*) control ODN, Ad5CMV-*Luc* plus mitoxantrone, AS clusterin ODN, Ad5CMV-*p53* plus mitoxantrone, or MM control ODN, Ad5CMV-*p53* plus mitoxantrone. Ten days after tumor cell injection, 10 mg/kg AS clusterin ODN or MM control ODN were injected daily i.p. for 28 d, 200 μL of Ad5CMV-p53 or Ad5CMV-Luc (1×10^7 pfu/mL) were injected i.t. twice a week for 2 wks, and 50 μg of mitoxantrone were injected i.v. twice a week for 2 wks. Tumor volume was measured every 5 d and calculated by the formula, length × width × depth × 0.5236. *Point*, mean tumor volume in each experimental group containing 10 mice; *bars*, SD. Mean tumor volume in mice treated with AS clusterin ODN, Ad5CMV-*p53*, plus mitoxantrone was significantly smaller than that in mice treated with other regimens ($P < 0.01$ by repeated measure ANOVA).

clusterin expression plays a protective role against apoptosis induced by various kinds of stimuli, and thereby may confer an aggressive phenotype during cancer progression.

Advances in the field of nucleic acid chemistry offers one attractive strategy to design antisense oligodeoxynucleotide-based therapeutic agents, that specifically hybridize with complementary mRNA regions of a target gene and thereby inhibit gene expression by forming RNA/DNA duplexes (36). Rapid intracellular degradation of oligodeoxynucleotide is a potential disadvantage of antisense oligodeoxynucleotide therapy, but this problem can be overcome by substituting a nonbridging phosphoryl oxygen of DNA with a sulfur to create a phosphorothioate backbone, which stabilizes the oligodeoxynucleotide against nuclease digestion (37, 38). Recently, several antisense oligodeoxynucleotides targeted against specific genes involved in neoplastic progression have been evaluated as potential therapeutic agents (29–33, 35, 38–42). We also previously showed that antisense clusterin oligodeoxynucleotide used in this study enhanced the therapeutic effects of androgen ablation and cytotoxic chemotherapy in several kinds of prostate cancer xenograft models (26–28). These findings clarify the efficacy of antisense oligodeoxynucleotide as a novel class of antineoplastic agents when designed against appropriate molecular targets. However, because numerous genes are involved in tumor progression, inhibition of a single target gene may likely be insufficient to completely inhibit tumor growth.

Recently, the usefulness of *p53* tumor suppressor gene therapy has been reported as a possible new therapeutic approach against various kinds of malignant diseases, including prostate cancer (5–10). Among several kinds of vectors, a replication-deficient adenoviral vector encoding human wild-type *p53* under the control of a human cytomegalovirus promoter (i.e., Ad5CMV-*p53*) has been most frequently used because of various useful properties including a high-infectious ability, limited period of the transgene expression, and very low probability of integration of the transgene into the host genome (4). However, the molecular mechanism interfering with the effects of the *p53* gene therapy has been clarified; that is, if antiapoptotic genes, such as *bcl-2*, were overexpressed in target malignant lesions, the efficacy of adenoviral-mediated *p53* gene transfer would be significantly limited through the inhibition of apoptotic cell death (31, 32). In this study, therefore,

Table 2. Changes in metastasis of PC3 cells injected into the prostate of nude mice after treatment with antisense clusterin oligodeoxynucleotide, Ad5CMV-*p53*, and mitoxantrone

Regimen*	Incidence of lymph node metastasis (%)†	Incidence of hemorrhagic ascites (%)‡	Weight of the primary tumor (mg)
AS clusterin ODN + Ad5CMV-*Luc* + mitoxantrone	8/10 (80)	2/10 (20)	20.2 ± 6.9§
AS control ODN + Ad5CMV-*Luc* + mitoxantrone	9/10 (90)	2/10 (20)	24.1 ± 9.1
AS clusterin ODN + Ad5CMV-*p53* + mitoxantrone	0/10 (0)‖	0/10 (0)	7.6 ± 4.8¶
MM control ODN + Ad5CMV-*p53* + mitoxantrone	6/10 (60)	1/10 (10)	17.6 ± 6.1

*Ten days after the implantation of tumor cells, 10 mg/kg antisense clusterin oligodeoxynucleotide (AS clusterin ODN) or mismatch control oligodeoxynucleotide (MM control ODN) were injected i.p. for 28 days, 200 μL of adenoviral vector ($1 \times {}^{7}10$ pfu/mL) and 50 μg of mitoxantrone were injected i.p. and i.v., respectively, twice a week for 2 weeks.
†No. mice with tumor/no. injected mice.
‡No. mice with hemorrhagic ascites/no. injected mice.
§Mean ± SD.
‖The incidence of metastasis or hemorrhagic ascites was significantly different from that in mice treated with other regimens ($P < 0.05$, χ^2 test).
¶The mean weight of the primary tumor was significantly different from that in mice treated with other regimens ($P < 0.05$, Student's t test).