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Abbreviations: **DPD**, dihydropyrimidine dehydrogenase; **5-FU**, 5-fluorouracil; **CDHP**, 5-chloro-2, 4-dihydroxypyridine; **OXO**, potassium oxonate; OPRT, orotate phosphoribosyltransferase; **FT**, 1-(2-tetrahydrofuryl)-5-fluorouracil (tegafur); **SCID**, severe combined immunodeficiency; **MTT**, microculture tetrazolium dye; **RP**, radical prostatectomy.

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Long-term results of first-line sequential high-dose carboplatin, etoposide and ifosfamide chemotherapy with peripheral blood stem cell support for patients with advanced testicular germ cell tumor

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Objective: Standard chemotherapy shows relatively low long-term survival in patients with poor-risk testicular germ cell tumor (GCT). First-line high-dose chemotherapy (HD-CT) may improve the result. High-dose carboplatin, etoposide, ifosfamide chemotherapy followed by autologous peripheral blood stem cell transplantation (PBSCT) was investigated as first-line chemotherapy in patients with advanced testicular GCT.

Methods: Fifty-five previously untreated testicular GCT patients with Indiana 'advanced disease' criteria received three cycles of bleomycin, etoposide and cisplatin (BEP) followed by one cycle of HD-CT plus PBSCT, if elevated serum tumor markers were observed after three cycles of the BEP regimen.

Results: Thirty patients were treated with BEP alone, because the tumor marker(s) declined to normal range. Twenty-five patients received BEP and HD-CT. One patient died of rhabdomyolysis due to HD-CT. Three and six (13% and 25%) out of 24 patients treated with BEP and HD-CT achieved marker-negative and marker-positive partial responses, respectively. The other patients achieved no change. Fifteen (63%) are alive and 14 (58%) are free of disease at a median follow-up time of 54 months. Severe toxicity included treatment-related death (4%).

Conclusions: HD-CT with peripheral stem cell support can be successfully applied in a multicenter setting. HD-CT demonstrated modest anticancer activity for Japanese patients with advanced testicular GCT and was well tolerated. This regimen might be examined for further investigation in randomized trials in first-line chemotherapy for patients with poor-risk testicular GCT.

Key words: chemotherapy, germ cell tumor, high-dose, peripheral blood stem cell transplantation (PBSCT), testis.

Introduction

Cisplatin-based combination chemotherapy has improved the prognosis of patients with metastatic germ cell tumor (GCT), and the long-term cure rate is approximately 80%.[1,2] However, patients with the 'advanced disease' criteria according to the Indiana University classification or the 'poor prognosis' criteria of the International Germ Cell Cancer Collaborative Group classification show survival rates of only 50–60% following standard-dose cisplatin-based chemotherapy.[3–5] Several attempts have been undertaken to improve the outcome of this patient group, including the use of double-dose cisplatin regimens or alternating dose-dense chemotherapy sequences.[6–8] However, doubling the dose of cisplatin did not lead to an improved outcome as compared with a standard cisplatin-dose regimen. Recently, high-dose chemotherapy (HD-CT) followed by autologous peripheral stem cell support or autologous bone marrow support has also been studied in these patients.[9–11] The rationale for the use of HD-CT in patients with GCT is based on preclinical and clinical data suggesting a dose–response relationship for certain drugs used in the treatment of GCT, particularly for etoposide and ifosfamide.[12,13] Dose finding studies in the high-dose setting, usually using a combination of carboplatin, etoposide and cyclophosphamide, ifosfamide, or thiotepa, have been conducted in heavily pretreated patients with relapsed or refractory disease.[14,15]

Although single center phase II trials have reported 2-year survival rates of 70–80% using first-line HD-CT approaches in poor prognosis patients, results of large phase II trials or phase III trials are lacking.[9–11] In addition, it is unclear at present whether the reported survival rates of 70–80% are maintained with longer follow up. The present study investigated the long-term results of first-line HD-CT with autologous stem cell support in Japanese patients with advanced testicular GCT in a multicenter setting. Patients with relapsed testicular GCT were excluded in this study.

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Methods

Patients

Fifty-five patients with advanced testicular GCT were entered onto this institutional review board-approved prospective trial between March 1996 and December 1999. All patients gave informed consent before they were enrolled onto the study.

Eligibility included 'advanced disease' criteria according to the Indiana classification.[3] According to the International Germ Cell Cancer Collaborative Group (IGCCCG) criteria, the numbers of good, intermediate and poor prognosis were 7, 26 and 22, respectively. All patients had histologically confirmed testicular GCT and no prior chemotherapy. Patients were also required to have a pretreatment leukocyte count greater than 3000/μL, pretreatment platelet count greater than 100 000/μL, glomerular filtration rate of 60 mL/min or higher, and serum creatinine level less than or equal to 1.5 times the upper level of the institutional norm.

Before chemotherapy, each patient was evaluated with a history and physical examination, chest radiography, computed tomography (CT) of the abdomen/pelvis, and screening chemistries, which included serum tumor markers (alpha-fetoprotein [AFP], human chorionic gonadotropin-β [HCG-β] and lactate dehydrogenase [LDH]). CT of chest or brain and bone scintigraphy were performed as indicated. All patients had chest CT, if chest X-ray suggested metastases.

Mobilization and harvest of peripheral blood stem cells

A dose of 5 μg/kg of recombinant human granulocyte colony-stimulating factor (G-CSF) was given to each patients s.c. once daily from the day of the nadir of neutrophil count after bleomycin, etoposide and cisplatin (BEP) combination chemotherapy. After white blood cells (WBC) recovered to 5000/μL, leukapheresis collections of peripheral blood stem cells were carried out for 2–3 consecutive days using a CS 3000 blood cell separator (Baxter Limited, Deerfields, IL, USA). A total volume of 10–15 L of blood was processed in each patient. Mononuclear cells containing hematopoietic stem/progenitor cells were cryopreserved in liquid nitrogen. The target harvest was more than 2.0×10^6 CD34-positive nucleated cells/kg patient bodyweight.

Conventional-dose chemotherapy

All patients were treated with three cycles of BEP as induction chemotherapy. The doses of anticancer agents, treatment schedule and treatment-related toxicity have been described previously (bleomycin 30 mg, i.v., days 2, 9, 16; etoposide 100 mg/m^2, i.v.; days 1–5; cisplatin 20 mg/m^2, i.v., days 1–5).[16] After three cycles of BEP therapy, patients whose tumor marker(s) were still elevated received one cycle of HD-CT with peripheral blood stem cell transplantation (PBSCT). If the serum tumor markers declined to normal range, the patients did not receive HD-CT.

High-dose chemotherapy

For treatment with HD-CT and autologous PBSCT, performance status 0 or 1 was required. HD-CT consisted of 1250 mg/m^2 of carboplatin, 1500 mg/m^2 of etoposide, and 7.5 g/m^2 of ifosphamide followed by 300 mg/m^2 of Mesna (bolus i.v. every 8 h, days 1–5). HD-CT was administered in five divided doses from day −7 to day −3. PBSCT was given i.v. on day 0. All patients received 5 μg/kg of G-CSF s.c., beginning the day following PBSCT and continuing until neutrophil count recovery. If all abnormally elevated serum tumor marker values returned to normal, surgery was performed when it was necessary to resect residual tumors.

Evaluation procedures

Serum tumor markers were determined before each treatment cycle and 4 weeks after the end of therapy. Evaluation of measurable disease by radiographic means was performed after HD-CT cycle and 4–6 weeks after the end of treatment. Subsequent follow-up tests including CT scans, serum tumor marker values and routine blood tests were performed at 3-month intervals during the first 2 years and then every 6 months up to 5 years of follow up.

Response to first-line HD-CT was defined according to World Health Organization (WHO) criteria.[17] Complete response (CR) was defined as the disappearance of all evidence of disease for at least 6 weeks when documented by imaging and all tumor markers. Partial response (PR) was defined as at least 50% reduction in the product of perpendicular diameters of each indicator lesion. PR was divided into two categories, partial response with tumor marker normalization (PR^{m-}) and marker positive partial response (PR^{m+}). Progressive disease (PD) was defined as 25% increase in the product from any lesion or the appearance of any new lesion. No change (NC) was defined as that which did not meet any of the above criteria. NC was also divided to two categories, no change with tumor marker normalization (NC^{m-}) and marker positive no change (NC^{m+}). Response and duration of survival were measured from the date of initiation of HD-CT.

Statistical analysis

Disease-specific survival was determined by the Kaplan–Meier method. For statistical analysis, a χ^2 test was used.

Results

Patient characteristics

Fifty-five patients, ranging in age 16–51 years (median, 27 years), were entered into this trial. Patient characteristics are summarized in Table 1a. Approximately 75% of all patients had lung metastasis and involvement of abdominal lymph nodes. Liver metastasis was present in 20% of patients. Four percent of patients had bone metastasis and 7% had central nervous system metastasis at diagnosis. Their histological types of GCT were four pure seminomas and 51 non-seminomas.

Response and survival

Five of 55 patients treated with three cycles of BEP achieved CR. Twenty-five of 50 patients who achieved PR or NC by induction BEP had normal concentrations of serum tumor markers. These 30 patients received another cycle of BEP therapy

Thus, following three cycles of induction BEP therapy, the remaining 25 patients whose tumor marker(s) (AFP, HCG-β and/or lactate dehydrogenase [LDH]) were still elevated when treated with one cycle of HD-CT with PBSCT. The patient characteristics are summarized in Table 1b. According to the IGCCCG criteria, the numbers of good, intermediate and poor prognoses were 1, 11 and 13, respectively. Because one patient died of rhabdomyolysis due to HD-CT, 24 patients were available to evaluate. Table 2 and Figure 1 summarize the outcome data. No patient achieved CR after one cycle of HD-CT. In all,

Table 1 Patient characteristics

Characteristics	No. of patients
(a)	
Patients age (years)	55
Median	27
Range	16–51
Histology	
Seminoma	4
Non-seminoma	51
Number of metastatic sites	
1	8
2	36
3 or more	11
Sites of metastasis	
Lung	48
Retroperitoneal lymph node	45
Mediastinal lymph node	12
Supraclavicular lymph node	3
Liver	11
Brain	4
Bone	2
Serum tumor markers	
HCG-β (ng/mL)	
Median elevated value	42
Range	0.1–120 000
AFP (ng/mL)	
Median elevated value	205
Range	10–62 274
LDH (IU/L)	
Median elevated value	1 550
Range	213–7 479
(b)	
Patients age (years)	25
Median	27
Range	16–45
Histology	
Seminoma	1
Non-seminoma	24
Number of metastatic sites	
1	5
2	13
3 or more	7
Sites of metastasis	
Lung	24
Retroperitoneal lymph node	19
Mediastinal lymph node	5
Liver	8
Brain	2
Serum tumor markers	
HCG-β (ng/mL)	
Median elevated value	1.35
Range	0.2–9.59
AFP (ng/mL)	
Median elevated value	52
Range	9–2 383
LDH (IU/L)	
Median elevated value	548
Range	419–781
Follow-up (months)	
Median	54
Range	10–80

AFP, alpha-fetoprotein; HCG-β: human chorionic gonadotropin-β; LDH, lactate dehydrogenase.

Table 2 Outcome data

Outcome	No. of patients (%)
Partial response	9 (37.5)
Marker-negative	3 (12.5)
Marker-positive	6 (25.0)
No change	15 (62.5)
Marker-negative	4 (16.7)
Marker-positive	11 (45.8)
Overall response rate	37.5

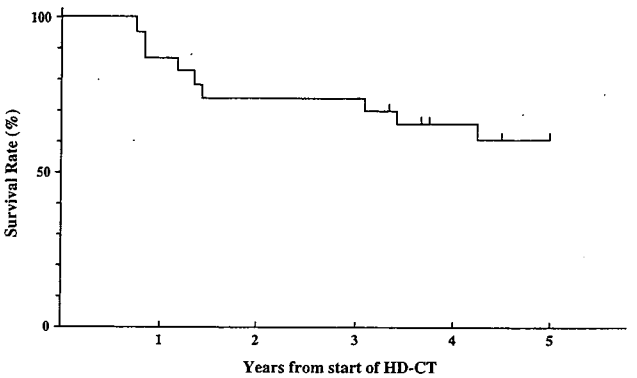

Fig. 1 Disease-specific survival of testicular germ cell tumor (GCT) patients treated with high-dose chemotherapy (HD-CT). Disease-specific survival rate was determined by the Kaplan–Meier method.

nine (38%) of 24 patients achieved PR. Marker-negative PR were achieved in only three patients. Fifteen NC and no PD were observed.

Seven patients showed high serum levels of AFP before HD-CT. The serum levels of AFP in six patients decreased after chemotherapy, but the levels in three patients were higher than normal range. In addition, serum AFP levels in one patient increased after HD-CT. Nineteen patients had high levels of serum HCG-β. The high serum HCG-β levels in five patients became less than the sensitivity of examination after HD-CT. Although the levels of serum HCG-β in 11 patients decreased after HD-CT, the serum levels were higher than normal range. The increased and same serum HCG-β levels in one and two patients were observed after HD-CT. Five patients demonstrated high serum LDH levels. The serum LDH level in three patients decreased to normal range after HD-CT, and the levels in the other two patients decreased, but to more than normal range.

Seventeen of 24 patients underwent operations for residual tumors. The patients included eight PR (marker-negative PR, three; marker-positive PR, five) and nine NC (marker-negative NC, four; marker-positive NC, five). Salvage surgery was performed for 10 patients (five PR and five NC) with positive marker(s). The pathological examinations revealed nine necrosis (marker-negative, three; marker-positive, six), one mature teratoma (marker-negative, one) and seven viable malignant tumors (marker-negative, three; marker-positive, four).

Twenty-one patients received additional therapy after first-line HD-CT and/or surgical resection of residual masses. Three patients received additional HD-CT, and nine patients received salvage chemotherapy with VIP (etoposide, ifosfamide and cisplatin), taxol or camptothecin, and radiation.

Table 3 Relationship between survival and various characteristics

Characteristics	Outcome (patient no.)	
	Alive ($n = 15$)	Dead ($n = 9$)
Serum tumor marker after HD-CT:		
Positive	10	7
Negative	5	2
IGCCCG criteria:		
Good	1	0
Intermediate	5	6
Poor	9	3
Histology: choricarcinoma		
(+)	7	3
(−)	8	6
Histology: yolk sac tumor		
(+)	5	4
(−)	10	5

There was no statistical significance by χ^2 test. HD-CT, high-dose chemotherapy; IGCCCG, International Germ Cell Cancer Collaborative Group.

Table 4 Adverse events

Adverse events	Grade				
	0	1	2	3	4
Neutropenia	0	0	0	1	22
Thrombocytopenia	0	0	3	4	16
Anemia	0	0	11	6	6
Fever	19	2	2	0	0
Mucositis	7	6	9	1	0
Nausea/vomiting	1	6	8	8	0
Diarrhea	11	5	6	1	0
Liver toxicity	13	4	4	1	1
Renal toxicity	21	1	0	1	0
Skin	20	2	1	0	0

The median duration of follow up is 54 months with a range of 9–80 months. Fourteen patients are currently alive and free of GCT and one patient remains alive with disease. All six patients with marker-positive status after HD-CT, whose pathological examinations of salvage surgery revealed necrosis, are alive. Nine have died of disease. The 3-year and 5-year survival rates were approximately 75% and 63%, respectively (Fig. 1). There was no correlation between survival and various characteristics (Table 3).

Adverse effects

Table 4 describes the toxicity in this study according to the WHO scale. There was a toxic death caused by rhabdomyolysis due to HD-CT.

Neutropenia was significant in all patients, and all patients except one experienced WHO grade 4 neutropenia. Nine patients had neutropenia with fever. The median duration of neutropenia less than 500/μL was 9 days (8–15 days). All patients received G-CSF, and the median duration of use of G-CSF was 11 days (8–21 days). Similarly, grade 2–4 thrombocytopenia/anemia were reported in all patients. The median duration of thrombocytopenia less than 20 000/μL was 9 days (0–18 days). All patients received platelet transfusions during HD-CT. The median amount of platelet transfusion was 55 units (20–200 units). Twenty patients received red blood cell transfusions during the chemotherapy. The median amount of transfusion was 4 units (0–11 units). Discontinuity of chemotherapy was not necessary for this hematological toxicity.

As expected, the other most common non-hematological side-effects were mucositis and nausea/vomiting. Diarrhea was also common. The most frequent grade 3/4 complications were nausea/vomiting, which were sufficiently controlled with anti-emetic therapy. Severe neurotoxicity was rare.

No specific investigations regarding late toxicity have yet been performed. At present, no patient developed therapy-related secondary leukemia.

Discussion

This study on first-line chemotherapy with HD-CT/PBSCT consisting of carboplatin, etoposide and ifosfamide was carried out in cooperation with 30 centers within the Japan Blood Cell Transplantation Study Group. The objectives of this trial were to determine the outcome, feasibility and toxicity of HD-CT/PBSCT in a multicenter setting. The rationale for HD-CT is based on the assumption that the front-line use of HD-CT may induce cell death in a higher fraction of sensitive and intermediate-sensitive GCT before drug resistance develops. This assumption is based on the observation in several solid tumor types including lymphomas and testicular cancer, that applying chemotherapy with a higher dose-intensity may lead to improved outcome.[18,19] Several phase II studies on the use of first-line HD-CT in testicular GCT have investigated schedules consisting of two to three standard-dose cycles followed by high-dose cycles.[9–11] These phase II studies have reported 2-year survival rates of 70–80% following first-line HD-CT, indicating that a 15–20% survival advantage may be achievable with first-line HD-CT as compared with standard BEP therapy.[9–11] In the phase II study, Motzer *et al.* demonstrated that first-line high-dose chemotherapy is well tolerated, and suggested a survival advantage following this approach compared to a historical control group treated with vinblastine, actinomycin-D, cyclophosphamide, cisplatin and bleomycin.[9] In a subsequent trial by the same investigators, poor prognosis patients with insufficient marker decline following two cycles of standard-dose VIP therapy received two cycles of high-dose carboplatin, etoposide and cyclophosphamide therapy followed by autologous stem cell support. Among 58 patients treated with this approach, 50% remained disease-free as compared to 25% of control patients who only received standard-dose therapy.[10] The present study demonstrated that first-line HD-CT with PBSCT achieved a 37.5% response rate, with NC in another 62.5% of patients, and that the 2-year survival rate was 75% following this chemotherapy. This result is comparable to those phase II studies.

The only randomized study investigating HD-CT as part of the first line chemotherapy for poor-risk GCT applied HD-CT with autologous bone marrow transplantation as consolidation after three cycles at standard doses was by Chevreau *et al.*[20] In this study, patients received four cycles of cisplatin, etoposide, vinblastine and bleomycin (PVeVB) at standard doses or three cycles of PVeVB followed by one cycle of high-dose cisplatin, etoposide and cyclophosphamide. This study failed to demonstrate a survival advantage for the high-dose group. The results of this trial are difficult to interpret, because the standard regimen contained double dose cisplatin, with approximately 30% of

the patients randomized to the high-dose treatment arm not completing high-dose therapy because of toxicity or early death. Another study has conducted a matched pair analysis including 456 patients in which first-line HD-CT was compared with standard-dose chemotherapy.[21] An 11% improvement in the 2-year overall survival rate was demonstrated in the HD-CT group and a multivariate analysis revealed the use of first-line HD-CT to be an independent positive predictive factor for improved survival. One recent phase III randomized controlled trial failed to show improvement of three cycles of standard-dose VIP chemotherapy followed by one cycle of HD-CT (carboplatin, etoposide and cyclophosphamide) compared with four cycles of VIP standard-dose chemotherapy for advanced GCT.[22] These findings suggest that first-line HD-CT might be more effective against poor prognosis testicular GCT than standard-dose chemotherapy. However, these data including the present study are limited, and large randomized clinical trials are necessary. There are two ongoing randomized clinical studies comparing multicycles of HD-CT with standard-dose chemotherapy (four cycles of BEP) for patients with poor prognostic GCT. HD-CT arms are two cycles of BEP followed by HD-CT (carboplatin, etoposide and cyclophosphamide), and one cycle of VIP followed by three cycles of HD-CT (VIP), respectively. These studies will clarify the role of HD-CT for GCT.

Overall toxicity was acceptable and the feasibility of this HD-CT regimen was demonstrated. As expected, all patients except one developed grade 4 neutropenia, but all of them recovered fully due to the stem cell support and G-CSF administration. Other hematological toxicity was also universal, but was quite manageable. Although platelet transfusions were required in all patients, there was no evidence of cumulative thrombocytopenia. No patient was removed from this study because of hematological adverse effects. Apart from hematological toxicity, side-effects consisted mainly of gastrointestinal events. Gastrointestinal side-effects were mostly manageable by supportive treatments such as anti-emetic therapy. Rhabdomyolysis was fatal only in one patient (4%). No septic death occurred during this study. Symptomatic acute severe ototoxicity, nephrotoxicity or peripheral neuropathy, which are common cisplatin-related toxicities, were rare.

Considering the high cure rate of GCT patients after first-line HD-CT, late toxicity is of particular interest. The previous report showed that 10% of patients suffered from late effects, mainly compensated renal failure and peripheral neuropathy.[23] In this study, no specific investigations regarding persistent late complications have yet been performed and therefore the incidence of late complications is unclear. However, no patient developed a therapy-related leukemia, which is an already-described serious late complication following high cumulative etoposide doses.

The results observed for the 24 poor prognosis GCT patients with a median follow up of nearly 4 years, demonstrate a 5-year disease-specific survival rate of 63%. Following standard-dose therapy, it has been known that relapses occurring more than 2 years after therapy are rare. This appears to be similar for patients receiving first-line HD-CT with only 12.5% of relapses occurring beyond 2 years.

The major goal of investigation for patients with poor-risk testicular GCT is identification of more effective chemotherapy. The results conducted at multiple centers in this study suggest that first-line HD-CT plus stem cell support for poor-risk testicular GCT might have a modest improved treatment outcome. In addition, this dose-intense chemotherapy is associated with relatively high but acceptable toxicity. Furthermore, there is only a minimal risk for severe late toxicity or secondary chemotherapy-induced cancer. However, it is necessary to define the optimal regimen for further studies. Moreover, the identification of prognostic factors for first-line HD-CT is needed and may be applied to select patients for a favorable treatment outcome, although HD-CT is effective for relapsed testicular GCT.[24]

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Association of *KLK5* overexpression with invasiveness of urinary bladder carcinoma cells

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Array-based comparative genomic hybridization (array-CGH) has powerful potential for high-throughput identification of genetic aberrations in cell genomes. We identified high-level amplification of kallikrein (*KLK*) genes, which are mapped to 19q13.3 and belong to the serine protease family, in the course of a program to screen a panel of urinary bladder carcinoma cell lines for genomic copy number aberrations using our in-house CGH-array. Expression levels of *KLK5*, *-6*, *-8* and *-9* were significantly increased in three cell lines with copy number gains of these *KLK* genes. Knockdown of these *KLK* transcripts by specific small interfering RNA significantly inhibited the invasion of a bladder carcinoma cell line through Matrigel *in vitro*. Reverse transcription–polymerase chain reaction analysis of 42 primary bladder tumor samples showed that increased expression of *KLK5* was frequently observed in invasive tumors (pT2–pT4) (14.3%, 6/42) compared with superficial tumors (pTa, pT1) (0%, 0/42; $P = 0.0052$), and expression levels of KLK5, -6, -8 and -9 mRNA were higher in invasive tumors than in superficial tumors ($P < 0.0001$, $P = 0.0043$, $P = 0.0790$ and $P = 0.0037$, respectively). These observations indicate that *KLK5*, *-6*, *-8* and *-9* may be the most likely targets of the 19q13.3 amplification, and may play a crucial role in promoting cancer-cell invasion in bladder tumor. (*Cancer Sci* 2007; 98: 1078–1086)

Urinary bladder carcinoma is the most common urogenital malignancy, with most being transitional cell carcinomas (TCC). Approximately 70–80% of primary bladder carcinomas are superficial (pTis, pTa, pT1). Patients with superficial bladder carcinoma are treated by transurethral resection or intravesical immunotherapy and chemotherapy. Their 5-year survival rate approaches 90%, but 50–70% of patients have subsequent recurrences, and 5–20% of post-therapeutic recurrences progress to muscle-invasive bladder carcinoma.[1] However, approximately 20–30% of primary cases involve muscle-infiltrating bladder cancers (pT2–pT4) at the time of first inspection. These patients with no evidence of metastasis are generally treated by cystectomy, although continent or incontinent urinary diversion with decreasing quality of life is still often required. The 5-year survival rate is only approximately 60%, with the majority of deaths due to recurrence or metastasis. Chemotherapy is used for most patients with metastasis, which is correlated with high morbidity and mortality. However, more than 90% of such patients die within the first 5 years.[2] Therefore, predicting the recurrence and progression of bladder carcinoma is crucial to improving prognosis and quality of life.

The development and progression of bladder carcinoma is a multistep process, the result of a series of genetic alterations occurring over the lifetime of a tumor.[3] It has also been reported that the spectrum of alterations at the chromosomal level can vary depending on the grade of differentiation and the tumor stage in bladder carcinomas.[4] Genomic amplifications and homozygous deletions are believed to be useful landmarks in cancer cell genomes for identifying oncogenes and tumor-suppressor genes, respectively, critical to tumorigenesis. Therefore, the search for significant changes in copy number through the entire genome with high resolution will allow precise and rapid identification of oncogenes as well as tumor-suppressor genes in various types of cancer. For this approach, we have applied several in-house bacterial artificial chromosome (BAC)/P1-derived artificial chromosome (PAC)-based arrays for array-based comparative genomic hybridization (array-CGH) analyses.[5–8]

Here we examined seven bladder carcinoma cell lines by array-CGH and identified high-level amplification of kallikrein (*KLK*) genes. *KLK* comprise a family of 15 genes clustered together in chromosomal region 19q13.3, and encode secreted enzymes belonging to the serine protease family.[9,10] Many members of this family have been previously described as being associated with various human cancers, such as prostate, breast and ovarian cancer.[11–15] However, the significance of *KLK* genes to the pathogenesis of bladder carcinoma has never been reported. In the present study therefore we analyzed 42 primary bladder carcinoma cases, as well as seven bladder carcinoma cell lines, with regard to the frequency of the overexpression of *KLK* genes, and evaluated the association between these frequencies and the clinical characteristics of patients with bladder carcinoma. Furthermore, in order to explore the possible roles of *KLK* in cancer-cell invasiveness, we show here that the knockdown of these *KLK* mRNAs by specific small interfering RNA (siRNA) can inhibit the invasion of bladder carcinoma cell lines *in vitro* through Matrigel-containing basement membrane components. These findings suggest *KLK* to be novel molecular targets for the prevention of bladder tumor invasion.

Materials and Methods

Cell lines and primary tumor samples. Ku1, Ku7, EJ1, SNK57, NKB1, KK47 and T24 bladder carcinoma cell lines derived from TCC were used for the present study, and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin. A total of 42 frozen primary samples were obtained from TCC patients (pTis, no cases; pTa, 10 cases; pT1, 13 cases; pT2, nine cases; pT3, nine cases; and pT4, one case)

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treated at University Hospital, Kyoto Prefectural University of Medicine; University Hospital, Iwate Medical University School of Medicine and Kochi Medical School Hospital, with written consent from each patient in the formal style and after approval by the local ethics committees. All primary specimens analyzed in this study were diagnosed and classified into superficial tumors or invasive tumors by pathologists belonging to each institution. Clinical and laboratory data on all 42 of the patients with TCC were collected from the patients' records. The TNM classification of Union International Contre le Cancer was used. Three of these patients were treated with neoadjuvant methotrexate, vinblastine, doxorubicin and cisplatin chemotherapy for one to three cycles. Genomic DNA and total RNA were extracted from the cell lines and the frozen tissue using a Genomic DNA Purification kit (Gentra, Minneapolis, MN, USA) and Isogen (Nippon Gene, Toyama, Japan) according to the manufacturers' instructions. Total RNA from normal bladder was purchased from two sources: Clontech (Palo Alto, CA, USA) and Ambion (Austin, TX, USA).

Array-CGH analysis. Two in-house BAC/PAC-based arrays, the MCG Whole Genome Array-4500 containing 4523 clones covering the human genome at roughly a 0.7-Mb resolution and the MCG Cancer Array-800 containing 800 clones specifically selected to contain important tumor-associated gene loci,[5–8] were used in this study. Hybridizations were carried out as described elsewhere.[8] Hybridized slides were scanned with a GenePix 4000B and acquired images were analyzed with GenePix Pro 6.0 imaging software (Axon Instruments, Foster City, CA, USA). Fluorescence ratios were normalized so that the mean of the middle third of $\log_2$ ratios across the array was zero. Average ratios that deviated significantly (>2 SD) from zero ($\log_2$ ratio <–0.4 and >0.4) were considered abnormal.

Fluorescence *in situ* hybridization using cell lines. Metaphase chromosome slides were prepared from normal male lymphocytes and bladder carcinoma cell lines. The location of each BAC (RPCI-11 and CTC library) or PAC, used as a fluorescence *in situ* hybridization (FISH) probe, within the region of interest was compiled from information archived by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) or the University of California Santa Cruz Genome Bioinformatics (http://genome.ucsc.edu/). We confirmed or modified the mapping data according to the results of FISH using normal metaphase chromosomes. Probes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP by nicktranslation (Roche Diagnostics, Tokyo, Japan), denatured with Cot-1 DNA, and hybridized to the chromosome slides. Fluorescent detection of hybridization signals was carried out as described elsewhere.[16] The cells were counter-stained with 4′,6-diamidino-2-phenylindole.

Reverse transcription–polymerase chain reaction. Single-stranded cDNA was generated from total RNA[6] and amplified with primers specific for each gene. The primer sequences for *KLK1–KLK15* were obtained from previous reports.[17,18] The primer sequences for other genes are available on request. The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) was amplified at the same time to allow estimation of the efficiency of cDNA synthesis. Real-time reverse transcription–polymerase chain reaction (RT-PCR) was carried out using an ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), the SYBR Green PCR Master Mix (Applied Biosystems), and random-primed cDNAs.

Screening of cell lines and primary bladder tumors for amplification by quantitative real-time genomic polymerase chain reaction. We assessed gene amplification in genomic DNA isolated from the ovarian cancer cell line OVCAR-3, the gastric cancer cell lines MKN28 and MKN74, and 16 primary bladder tumors (superficial, eight samples; muscle invasive, eight samples) by SYBR Green quantitative polymerase chain reaction (PCR). All of the relevant primer sequences are available on request. PCR was carried out with the SYBR Green PCR Master Mix on an ABI Prism 7900 Sequence Detection System.

Transient siRNA oligonucleotide transfection. The 'Smart-pool' siRNA for *KLK5*, *KLK6* or *KLK8* (*KLK5*-, *KLK6*- or *KLK8*-siRNA, respectively) was purchased from Dharmacon (Lafayette, CO, USA), and was transiently transfected into Kul bladder carcinoma cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. Transfection of siRNA for the luciferase gene (CGUACGCGGAAUACUUCGA, *Luc*-siRNA) synthesized by Sigma (Tokyo, Japan) and transfection with Lipofectamine 2000 only served as controls. The efficacy of the knockdown of gene expression by the gene-specific siRNA was confirmed 48 and 96 h after transfection by RT-PCR with a specific primer set for each gene.

***In vitro* invasion assay.** To quantify the invasive activity of cancer cells *in vitro*, 24-well transwell-chamber culture systems (Becton Dickinson, Franklin Lakes, NJ, USA) were used. The upper surface of 6.4-mm-diameter filters with 8-μm pores was precoated with Matrigel. At 48 h after transfection of siRNA, 5×10^4 cells were added to the upper chamber after the lower chamber had been filled with 750 mL of DMEM with 5% FBS. Following 48 h of incubation, the non-invasive cells on the upper surface of filters were removed with sterile cotton swabs. The invasive cells on the lower surface of filters were fixed and stained with the Diff-Quik stain (Sysmex, Kobe, Japan), and stained cell nuclei were counted directly in triplicate. We assessed invasive potential using an invasion index to eliminate the influence of migration and proliferation as much as possible. Results were calculated as the percentage invasion through the Matrigel and filters relative to the migration through the control filters without Matrigel. The invasion index was also calculated as the ratio of the percentage invasion of a test cell over the percentage of a control cell.

Cell growth assay. Cells transiently transfected with *KLK*-siRNA and control cells (3×10^3) were seeded in 96-well plates and allowed to grow for 4 consecutive days. Viable cells were assessed with the microtiter plate colorimetric water-soluble trazolium salt (WST) assay (Cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan). The proliferation rate was taken as the ratio of the optical density at 450–650 nM on the second day to the density on the fourth day.

Statistical analysis. The Fisher's exact test or the χ^2-test was used to examine categorical data. The Mann–Whitney *U*-test was used to compare the level of gene expression. All tests of significance were two-sided and considered significant at the level $P < 0.05$.

Results

Array-CGH analysis of bladder carcinoma cell lines. Copy number gains and losses were seen to some degree in all of the seven bladder carcinoma cell lines examined by both array-CGH analyses. Among the genetic aberrations detected, high-level amplifications and homozygous deletions are believed to be useful for identifying oncogenes and tumor-suppressor genes, respectively, critical to tumorigenesis. Therefore, we paid attention to significant patterns of chromosomal abnormalities. Supplementary Tables S1 and S2 summarize the clones showing high-level amplifications ($\log_2$ ratio >2.0) and homozygous deletions ($\log_2$ ratio <–2.0), respectively. Eight loci with high-level amplifications (5p13.3, FRA5A; 11p13, FRA11E; 11q13.2-13.3, FRA11H/FRA11A; 19q13.3, FRA19A) or homozygous deletions (3p14.2, FRA3B; 9p21.3, FRA9A/FRA9C) detected in this study are located at fragile sites.

Homozygous deletions were detected in five of the bladder carcinoma cell lines (Ku7, EJ1, SNK57, KK47, T24) in 12 clones, and five loci were represented (Suppl. Table S2). Among them, BAC RP11-259 N12 at 1p21.1 and RP11-43B19 at 6q26

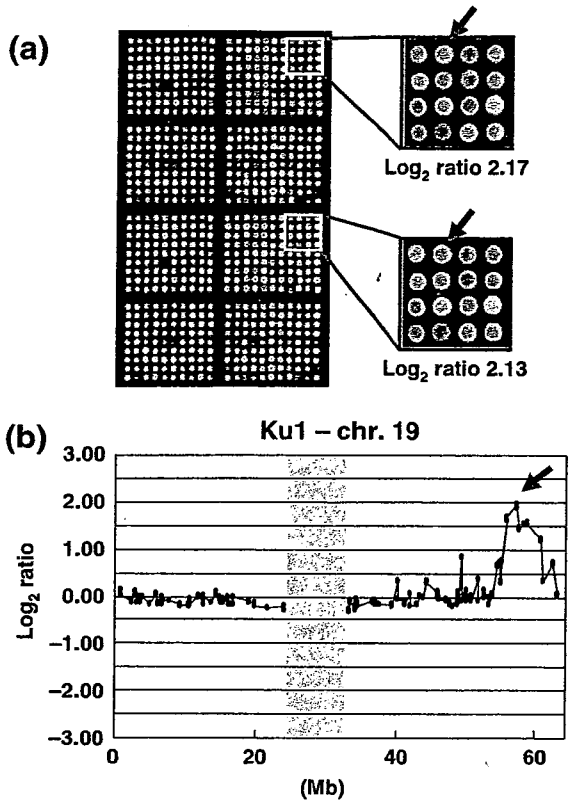

Fig. 1. Array-based comparative genomic hybridization (CGH-array) analysis of bladder carcinoma cell lines. (a) Representative duplicate CGH-array image of the Ku1 cell line. A significant increase in the copy number ratio (log_2 ratio) of *KLK* at 19q13.3 was detected as a clear green signal. (b) Copy-number profile of chromosome 19 in the Ku1 cell line. The arrow indicates a candidate spot showing the pattern of high-level amplification (log_2 ratio >2) at 19q13.3. A vertical shade marks the centromeric region.

are located within regions of large-scale copy number variation in the human genome (Database of Genomic Variants, http://projects.tcag.ca/variation/), and BAC RP11-91E8 at 3p21.31 harbors no putative tumor-suppressor gene. A homozygous deletion of the region containing *CDKN2A/p16* at 9p21.3 is frequently observed in bladder carcinoma, and can be detected by means of array-CGH analysis.[3,19] *FHIT* at 3p14.3 is a well-known tumor suppressor gene in patients with bladder carcinoma.[20]

High-level amplifications were detected in the Ku1, SNK57 and NKB1 cell lines in 10 clones, and five loci were represented (Suppl. Table S1). Several cancer-related genes, *CCND1*, *CD44*, *CDH6*, *EHF*, *KLK1-KLK15* and *PDZD2*,[16,21–24] are located at these loci. These loci were reported as regions with copy number gains in a BAC array-based CGH analysis of 22 bladder carcinoma cell lines, although only one of the 22 cell lines overlapped with our series of cell lines.[19] Within the 19q13 region, interestingly, copy number gains at 19q13.1 and 19q13.2 were reported in primary bladder tumors,[3,4] but there is no report about high-level amplifications at 19q13.3, which prompted us to further analyze this region to identify target genes for this alteration.

Definition of the 19q13.3 amplicon by FISH. High-level amplification at 19q13.3 was identified in our array-CGH analysis (Fig. 1). *KLK1–KLK15* are located at 19q13.3, and have been reported as promising biomarkers of several cancers, including prostate, ovarian, testicular and breast cancers. However, hK3 translated from KLK3 mRNA has had the greatest impact on the screening, diagnosis, staging and monitoring of prostate cancer.[25,26] The *KLK6* gene is known to be overexpressed in ovarian cancers with gene amplification,[27] although no other *KLK* genes are known to be targets for amplification. We therefore focused on the high-level amplification at this locus, and first generated a defined amplicon map in three bladder carcinoma cell lines, Ku1, SNK57 and NKB1, which showed copy number gains at this locus in our array-CGH analysis. A FISH analysis was carried out using 15 BAC spanning the amplified region as probes. Relative positions of these BAC on a map of the 19q13.3 region are indicated in Fig. 2a. Copy numbers, as well as the molecular organization of the amplicon, were assessed by analyzing hybridization patterns of metaphase and interphase chromosomes. In the Ku1 cell line, 10 BAC (RP11-795B6, RP11-10I11, RP11-891J20, RP11-99I13, RP11-1108B3, RP11-105H4, RP11-749C22, RP11-690A4, 79A3 and RP11-344A8) produced the highest number of signals (14 copies) on marker chromosomes (Fig. 2b,c). Fewer signals were detected with the remaining five BAC (45F3, 1051H12, 22I5, 184K19 and 79I16), suggesting that they were located outside the amplicon. The other two cell lines examined by FISH yielded more than five signals; the number of signals in each line did not differ among the 15 BAC, except for one increase observed between BAC RP11-1051H12 and RP11-105H4 in the NKB1 cell line. Therefore, the smallest region of overlap (SRO) was defined between BAC RP11-795B6 and RP11-105H4. The size of the SRO was 870 kb, according to information in the University of California, Santa Cruz (UCSC) Genome Bioinformatics database (http://genome.ucsc.edu/), and was adopted as a critical region harboring targets.

Expression of genes located on the 19q13.3 amplicon in bladder carcinoma cell lines. Next, to determine whether *KLK1–KLK15* and other genes located at 19q13.3 were overexpressed in association with their amplification, we assessed the expression status of these genes in seven bladder carcinomas cell lines, one normal bladder as a normal control, and pancreas and prostate as positive controls using RT-PCR (Fig. 3). *KLK1*, *-5*, *-6*, *-7*, *-8*, *-9*, *-10*, *-11* and *-14*, *SIGLEC9* and *-10*, *GPR32*, *ATPBD3*, *ETFB*, *CLDND2*, *NKG7* and *ZNF175* were expressed in three cell lines with amplification at 19q13.3, Ku1, SNK57 and NKB1; however, the expression of other genes mapped to this amplicon was not detected in any of the cell lines, except for *CD33*, which was expressed only in the T24 cell line. Among them, the increased expression levels of *KLK5*, *-6*, *-8* and *-9* were well associated with copy number gains in the Ku1, SNK57 and NKB1 cells. In the Ku1 cell line, which had the highest copy number gain in our array-CGH analysis (Fig. 1b), the expression levels of *KLK5*, *-6*, *-8* and *-9* were much higher than in other cell lines. In addition, compared with the ovarian cancer cell line OVCAR-3, the gastric cancer cell lines MKN28 and MKN74, which had already been reported to overexpress *KLK5*, *-6*, *-8* and *-9*,[15,17] had much lower levels of these *KLK* than the Ku1 cell line (Fig. 3b). Because no change in copy number at 19q13 was observed in OVCAR-3, MKN28 or MKN74, the high-level amplification at 19q13.3 might strongly contribute to the signigficant upregulation of these *KLK* in Ku1 cells (Fig. 3a). We did not detect transcripts of *ACPT* or *LIM2* in any of the samples (data not shown).

Inhibition of the *in vitro* invasiveness of Ku1 bladder carcinoma cells by KLK5, -6, -8 and -9 siRNA. Previously, experimental evidence indicated that *KLK* might promote cancer-cell growth, angiogenesis, invasion and metastasis.[28] Furthermore, some reports showed that hK5, hK6 and hK8 can degrade fibronectin, laminin and type IV collagen *in vitro*.[12,13,29] These reports prompted us to investigate the role of *KLK5*, *-6*, *-8* and *-9* in cancer-cell invasion because our findings indicated that the overexpression of these four *KLK* was correlated with copy number gains in three cell lines with amplification at 19q13.3. Hence we examined the effects of knocking down the KLK5, -6, -8 and -9 transcripts on *in vitro* cell growth and invasion in Ku1 cells which had the highest copy number gain and expression of these four *KLK*.

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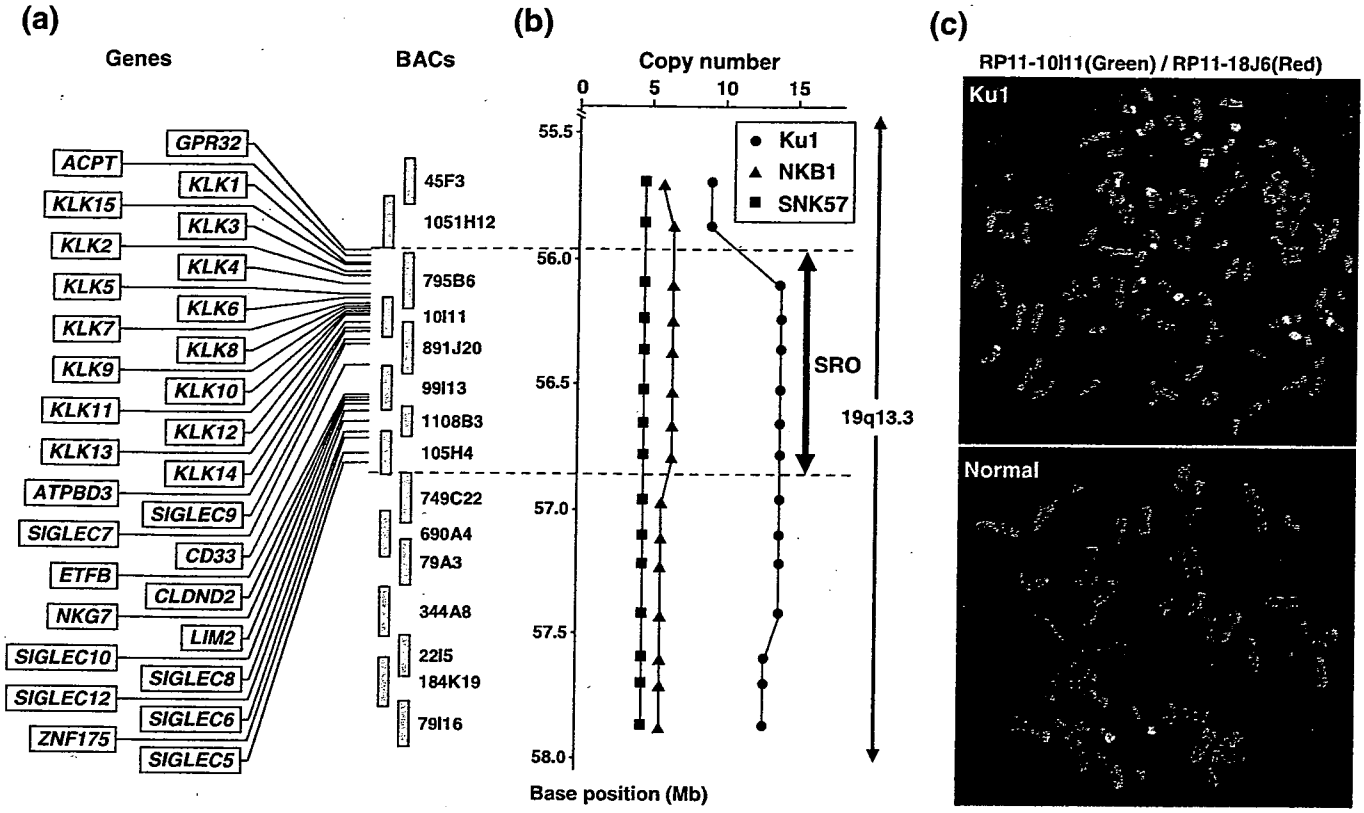

Fig. 2. Definition of the 19q13.3 amplicon by fluorescence *in situ* hybridization (FISH). (a) Map of 19q13 covering the region amplified in bladder carcinoma cell lines. Genes located within the smallest region of overlap are positioned according to the information archived by UCSC and NCBI. Bacterial artificial chromosome (BAC) probes used for FISH are indicated as vertical bars. (b) Summarized results of a copy number analysis using FISH in three different cell lines. The horizontal axis shows the number of FISH signals detected with BAC probes. The closed thick arrow indicates the smallest region of overlap. (c) Representative FISH images from probe RP11-10I11, which contains *KLK* (green signals) and with RP11-18J6 as a control (red signals) hybridized to metaphase chromosomes from the Ku1 cell line and normal male lymphocytes. RP11-10I11 shows 14 signals in the Ku1 cell line.

The effects of the knockdown were confirmed at days 2 and 4 after siRNA transfection. The transcript levels of these four *KLK* were decreased significantly by the transient transfection of each specific siRNA, whereas these transcripts were detected in cells transfected with non-specific- or *Luc*-siRNA (Fig. 4a). The knockdown of these *KLK* mRNAs did not affect the *in vitro* growth rate of the transfectants (Fig. 4b), whereas the invasive potential of these specific siRNA transfectants was significantly reduced in the Matrigel invasion assay (Fig. 4c,d). Notably, *KLK6*-siRNA was found to be a potent inhibitor of invasion through Matrigel compared with *KLK5*-, *KLK8*- and *KLK-9*-siRNA. In morphological appearance, these transfectants showed no significant differences from the parental Ku1 cells (Fig. 4c).

Expression of KLK5, -6, -8 and -9 in primary bladder carcinoma cases. Finally, we assessed the frequency and level of expression of these four *KLK* mRNAs in 42 primary bladder cancers by RT-PCR. High-level expression of *KLK5*, *-6*, *-8* and *-9*, an increase of more than two-fold in comparison with normal bladder tissue, was observed in 14.3% (6/42), 2.4% (1/42), 7.1% (3/42) and 19.0% (8/42) of primary tumors, respectively (Fig. 5a). The frequency of *KLK5* mRNA expression was significantly higher in invasive tumors (pT2–pT4: 31.6%, 6/19) than in superficial tumors (pTa, pT1: 0%, 0/23; $P = 0.0052$, Fisher's exact test). Furthermore, this frequency also differed significantly between grade 3 tumors and grade 1–2 tumors ($P = 0.0458$, Fisher's exact test). The expression levels of *KLK5*, *-6*, *-8* and *-9* were higher in invasive tumors than in superficial tumors ($P < 0.0001$, $P = 0.0043$, $P = 0.0790$, and $P = 0.0037$, respectively, Mann–Whitney *U*-test; Fig. 5b). There was no relationship between the increased expression of these four *KLK* and sex or past history of bladder carcinoma (Table 1). We could not analyze the prognostic significance because not all of the survival data was available. To confirm the association between overexpression and amplification of four KLK, we used a real-time quantitative PCR with 16 primary bladder tumors (superficial, eight samples; muscle invasive, eight samples) in which the expression levels of these KLK had been analyzed. However, copy number alterations of these *KLK* genes were not detected in these primary tumors (Suppl. Fig. 1).

Discussion

Genome-wide screening of DNA copy number aberrations, especially high-level amplifications and homozygous deletions, that could be landmarks of oncogenes and tumor-suppressor genes, respectively, in cancer cell genomes is a tremendously efficacious way to identify novel genes contributing to the development and progression of cancers. In the present study, we applied our in-house MCG Whole Genome Array-4500, covering the entire human genome with a total of 4523 BAC, and a MCG Cancer Array-800, containing 800 clones specifically selected to contain important tumor-associated genes and loci, to scan the genomic aberrations in a panel of bladder carcinoma cell lines.[5] Based on the results of our array-CGH analysis in bladder carcinoma cell lines, we focused on chromosomal region 19q13.3, which harbored a high-level amplification in one of the cell lines, because (i) 19q13 is

Table 1. Correlation between *KLK* expression and clinicopathological characteristics of bladder carcinoma patients

	KLK5 expression			*KLK6* expression			*KLK8* expression			*KLK9* expression		
	Positive	Negative	*P*-value	Positive	Negative	*P*-value	Positive	Negative	*P*-value	Positive	Negative	*P*-value
Total number (%)	6 (14.3%)	36 (85.7%)		1 (2.4%)	41 (97.6%)		3 (7.1%)	39 (92.9%)		8 (19.0%)	34 (81.0%)	
Age median (years) (range)	74.5 (56–80)	70.0 (30–86)		78.0	70.0 (30–86)		61.0 (56–78)	71.0 (30–86)		72.0 (56–78)	69.5 (30–86)	
Sex												
Male	6	26	0.3076	1	31	>0.9999	3	29	>0.9999	7	25	0.6545
Female	0	10		0	10		0	10		1	9	
T classification	0	10	0.0023	0	10	0.4400	0	10	0.3274	0	10	0.2788
pTa												
pT1	0	13		0	13		1	12		2	11	
pT2	1	8		0	9		0	9		3	6	
pT3	4	5		1	8		2	7		3	6	
pT4	1	0		0	1		0	1		0	1	
pTa–pT1	0	23	0.0052	0	23	0.4524	1	22	0.5813	2	21	0.1122
pT2–pT4	6	13		1	18		2	17		6	13	
Grade												
G1	0	2	0.0798	0	2	0.2779	0	2	0.9142	0	2	0.7343
G2	2	26		0	28		2	26		6	22	
G3	4	8		1	11		1	11		2	10	
G1–G2	2	28	0.0458	0	30	0.2857	2	28	>0.9999	6	24	>0.9999
G3	4	8		1	11		1	11		2	10	
Initial/recurrence												
Initial	5	31	>0.9999	1	35	>0.9999	3	33	>0.9999	6	30	0.3193
Recurrence	1	5		0	6		0	6		2	4	

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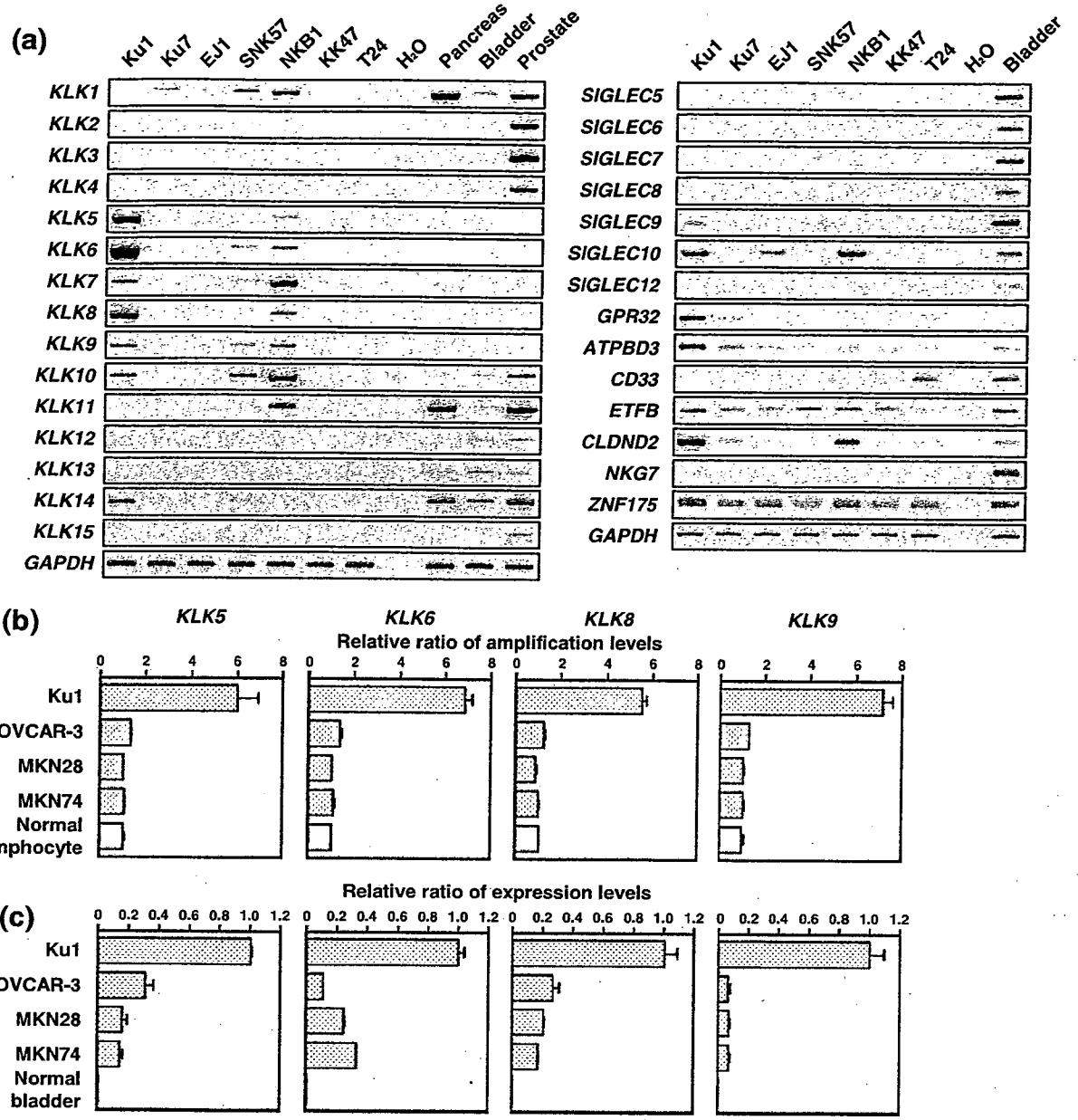

Fig. 3. Expression of genes located on the 19q13.3 amplicon in bladder carcinoma cell lines. (a) Expression of *KLK1–KLK15* and other genes located within smallest region of overlap in seven bladder carcinoma cell lines, one normal bladder as a normal control, and pancreas and prostate as positive controls detected by reverse transcription–polymerase chain reaction (PCR). Comparison of copy-number (b) and expression (c) of the *KLK5, KLK6, KLK8* or *KLK9* genes by quantitative real-time quantitative PCR. Genomic DNA of normal lymphocytes and total RNA of normal bladder were used as the normal counterpart of four cancer cell lines. These results were normalized to the copy number or expression levels of these *KLK* genes in each normal counterpart.

known to contain a chromosomal fragile site, and (ii) human *KLK* genes located at 19q13.3 had already been demonstrated to be a rich source of tumor biomarkers.

Fragile sites, especially common fragile sites, are highly unstable genomic regions. Although they are characterized utilizing an *in vitro* assay of chromosomal decondensation and breakage induced by inhibitors of DNA replication, their apparent *in vivo* significance is that they predispose chromosomes to breakage and rearrangement, especially in developing cancer cells.[30] It was reported that more than half of the chromosomal rearrangements found in bladder carcinomas were consistent with 77 of 118 common or rare fragile sites recognized in several types of human cancers, and that 55% of these fragile sites coincided with regions containing one or more genes associated with human cancers.[30] In bladder carcinoma, the most striking examples of these genes are the *MYC* oncogene at FRA8C/8E (8q24.1),[31] and the *CDKN2A/p16* tumor suppressor gene at FRA9A (9p21).[32] In our array-CGH analysis, five loci with high-level amplifications and three loci with homozygous deletions were consistent with fragile sites. Low-level gains or losses at these loci were also frequently observed in the cell lines used in this study without high-level amplifications or homozygous losses. These results strongly support the hypothesis that fragile sites contribute to carcinogenesis and cancer progression at least partly

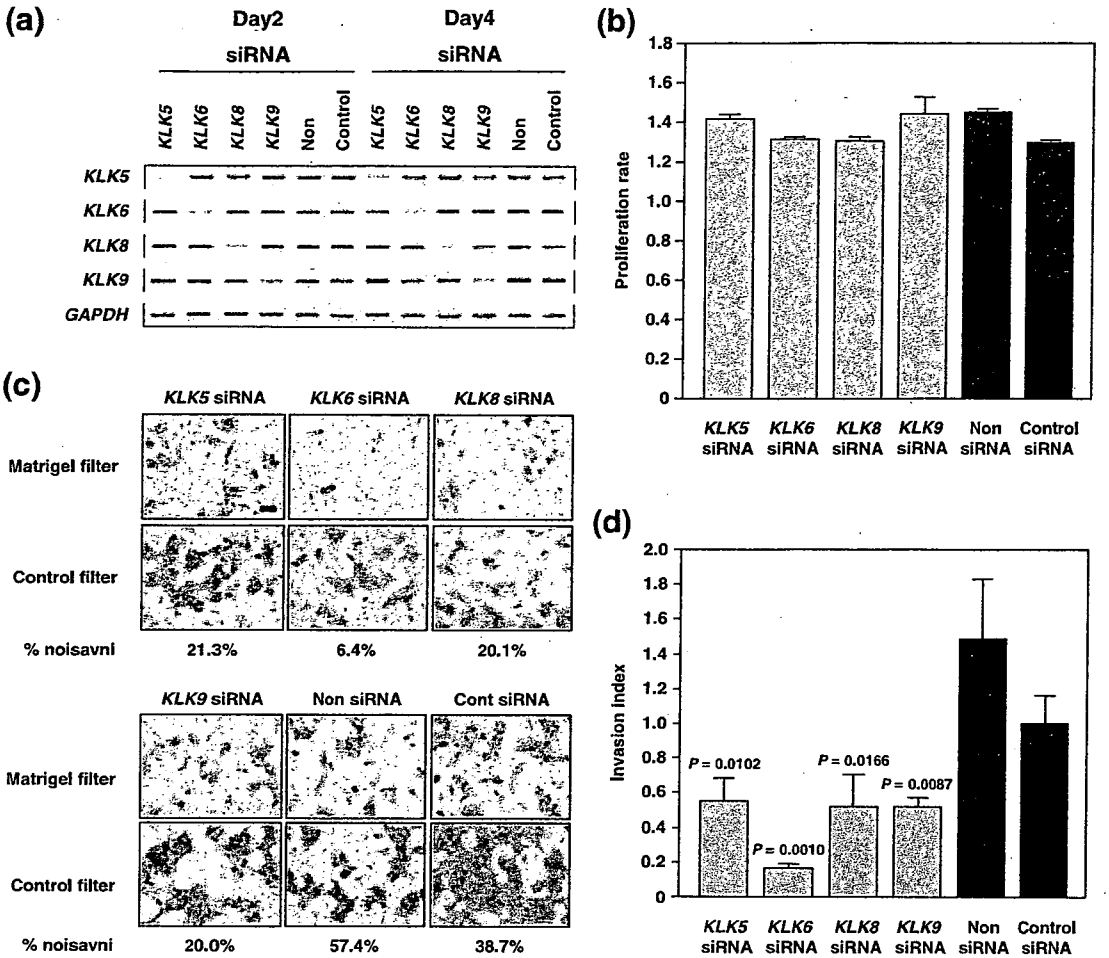

Fig. 4. Inhibition of the *in vitro* invasiveness of Ku1 bladder carcinoma cells by *KLK5, -6, -8* and *-9*-specific small interfering RNA (siRNA). (a) Reverse transcription–polymerase chain reaction analysis of *KLK5, -6, -8* and *-9* in siRNA-transfected Ku1 cells 2–4 days after transfection. Non, transfection of lipofectamine 2000 only; control, transfection of siRNA for the luciferase gene. (b) *In vitro* growth rate of *KLK5, -6, -8* or *-9*-suppressed cells in the presence of 5% fetal bovine serum. The cell number was counted 2–4 days after transfection. The proliferation rate was indicated as the ratio of the optical density at 450–650 nM on the second day. (c) Representative phase micrograph of Ku1 cells invading the Matrigel matrix and membrane (upper panel), and membrane only (lower panel). Invading cells were fixed and stained with the Diff-Quik stain. Scale bar = 100 μm. (d) Quantification of the cell invasion shown in (c). Percentage invasion: number of cells invading through Matigel insert membrane/number of cells migrating through control insert membrane ×100. Invasion index: % invasion test cell/% invasion control cell.

through activation or inactivation of target genes in some populations of bladder carcinomas.

Because the common criterion for a putative amplification target is that the amplification leads to consistent overexpression,[33] we compared the expression level of each positional candidate gene mapped to the SRO at 19q13.3 in bladder carcinoma cell lines. Overexpression of *KLK5*, *-6*, *-8* and *-9* was observed in three cell lines with copy number gains, which prompted us to focus on these four *KLK* genes as candidates for amplification at 19q13.3. Some *KLK* genes have been studied in terms of their diagnostic and prognostic value in prostate, breast and ovarian cancers.[11,14] However, the genetic alterations, overexpression and clinicopathological significance of *KLK* have never been reported in bladder carcinoma. Although a RT-PCR-based analysis with a panel of 42 primary bladder carcinoma cases (pTa, pT1, 23 cases; pT2–pT4, 19 cases) demonstrated that the frequency of *KLK5* expression alone was significantly higher in invasive tumors (pT2–pT4) (14.3%, 6/42) than in superficial tumors (pTa, pT1) (0%, 0/42) ($P = 0.0052$, Fisher's exact test), expression levels of *KLK5*, *-6*, *-8* and *-9* mRNA were markedly increased in invasive tumors compared with superficial tumors ($P < 0.0001$, $P = 0.0043$, $P = 0.0790$ and $P = 0.0037$, respectively, Mann–Whitney *U*-test). Our findings clearly indicate *KLK5*, *-6*, *-8* and *-9* to be the most likely targets of 19q13.3 amplification and suggest that they play an important role in the regulation of cancer-cell migration and invasion. Previously, hK5 in the serum of patients with breast and ovarian cancer was identified as a potential biomarker.[34] Thus, our data also suggest that *KLK5* (hK5) might be a novel biomarker of invasive bladder carcinoma.

We detected significant overexpression of *KLK5*, *-6*, *-8* and *-9* in the Ku1 cell line with the 19q13.3 amplification, and these expression levels were much higher than in the ovarian cancer cell line OVCAR-3, and the gastric cancer cell lines MKN28 and MKN74, which had already been reported to overexpress these *KLK*, without genetic alterations at 19q13.3.[15,17] However, to date, there is no report about amplification of these genes, except for *KLK6*, which had shown copy number gains in ovarian cancer.[27] Therefore, these findings as well as previous studies led us to speculate that upregulation of *KLK* expression occurs in a genomic copy number-dependent manner in each of these genes. However, as we could not detect copy number alterations of *KLK* genes in the primary tumors, a large-scale

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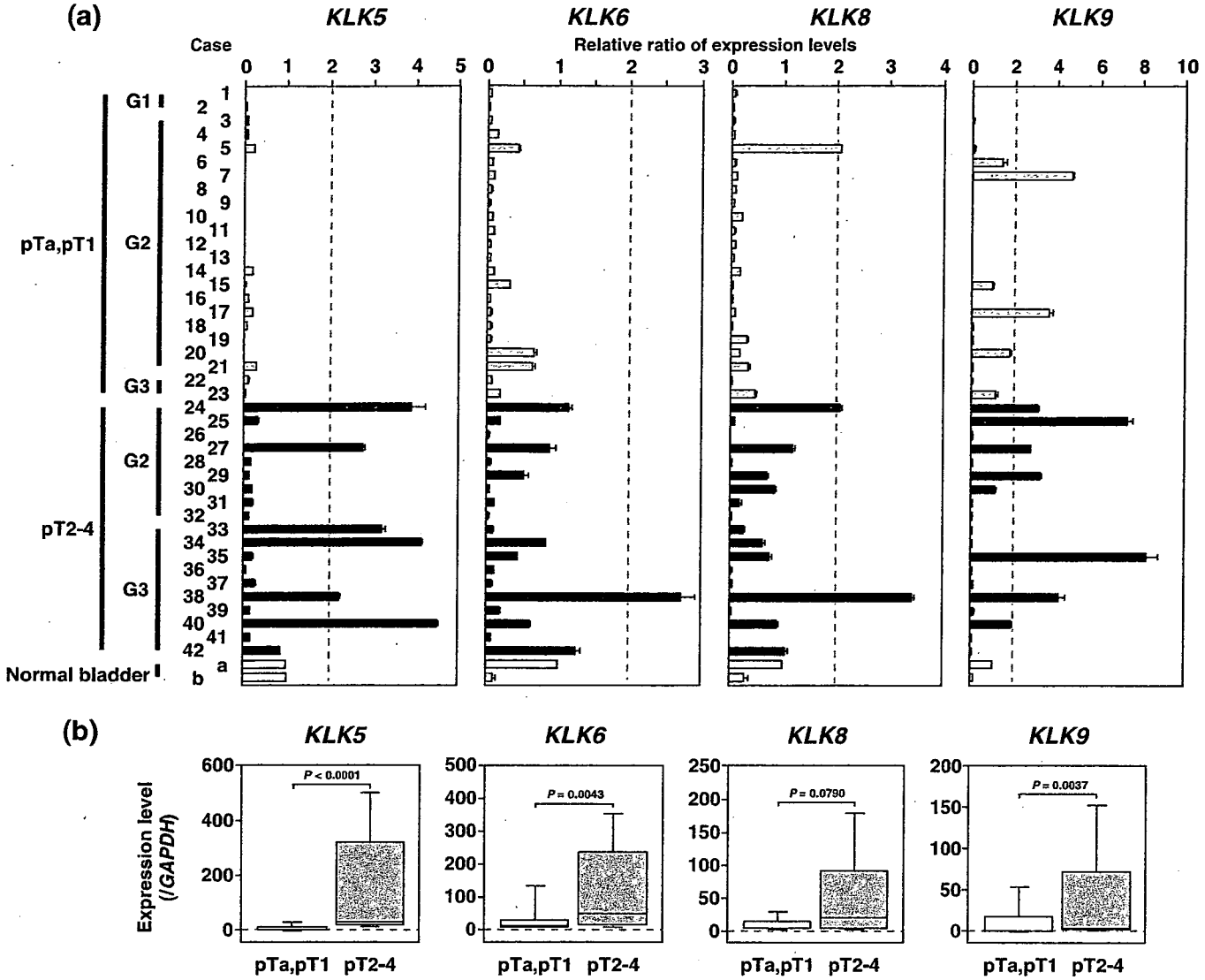

Fig. 5. Expression of *KLK5, -6, -8* and *-9* in primary bladder tumors. (a) Expression of *KLK5, -6, -8* and *-9* in primary tumors detected by reverse transcription–polymerase chain reaction. Cases 1–23, superficial tumor; cases 24–42, invasive tumor. The expression level of each transcript normalized to glyceraldehyde-3-phosphate dehydrogenase was quantified by MultiGauge (Fujifilm). Total RNA of normal bladder was used as the normal counterpart of primary tumors. The horizontal axis shows the relative ratio of *KLK* for the normal counterpart. (b) We compared the expression levels of *KLK5, -6, -8* and *-9* in superficial tumors with those in invasive tumors using a non-parametric Mann–Whitney *U*-test. Median values are indicated with horizontal bars in the boxes. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles.

analysis of the genetic alterations and expression of *KLK* genes is needed to make the relationship clear.

KLK comprise a family of 15 genes encoding secreted serine proteases.[9,10] Recently it was reported that *KLK6*-downregulated gastric cancer cells lost invasive potential in an *in vitro* Matrigel invasion assay.[15] In the present study, we successfully demonstrated that *KLK5*, *KLK6*, *KLK8* and *KLK9* gene silencing clearly reduced the invasive activity of bladder cancer cells *in vitro*. Because hK5, hK6 and hK8 were capable of degrading *in vitro* the extracellular matrix (ECM), including fibronectin, laminin and type IV collagen,[12,13,29] our results suggest that hK9, as well as hK5, hK6 and hK8 expressed in bladder cancer cells, might have the capacity to degrade the ECM in Matrigel components.

In summary, we showed *KLK5, -6, -8* and *-9* to be the most likely targets of the 19q13.3 amplification in bladder carcinomas. This is the first report to present data on the amplification, overexpression and clinicopathological significance of these *KLK* in bladder carcinoma. Notably, we found a statistically significant association between *KLK5* overexpression and invasive potential in an analysis of primary bladder carcinomas. The *in vitro* and *in vivo* data strongly suggest *KLK* (hK) to be potential molecular markers in invasive bladder carcinoma.

Acknowledgments

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Supplementary Material

The following supplementary material is available for this article:

Fig. S1. The relative copy number of *KLK5*, *-6*, *-8* and *-9* genes in primary bladder tumors detected by a real-time quantitative polymerase chain reaction. Ku1 is a bladder carcinoma cell line with 19q13.3 amplification. Cases 1, 3, 5, 7, 9, 12, 13 and 17, superficial tumor; cases 24, 25, 29, 33, 34, 35, 38 and 40, invasive tumor; N1-6, lymphocytes derived from healthy individuals as a normal counterpart. The horizontal axis shows the relative ratio of the copy number of each *KLK* gene for the normal counterpart.

Table S1. Loci of high-level amplification ($\log_2$ ratio >2.0) detected in bladder carcinoma cell lines by array-based comparative genomic hybridization analysis using MCG Whole Genome Array-4500.

Table S2. Loci of homozygous deletion ($\log_2$ ratio <2.0) detected in bladder carcinoma cell lines by array-based comparative genomic hybridization analysis using MCG Whole Genome Array-4500.

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Lipoxygenase inhibitors induce death receptor 5/TRAIL-R2 expression and sensitize malignant tumor cells to TRAIL-induced apoptosis

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Lipoxygenases induce malignant tumor progression and lipoxygenase inhibitors have been considered as promising anti-tumor agents. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is one of the most promising candidates for new cancer therapeutics. Combined treatment with nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, and TRAIL markedly induced apoptosis in Jurkat T-cell leukemia cells at suboptimal concentrations for each agent. The combined treatment efficiently activated caspase-3, -8 and -10, and Bid. The underling mechanism by which NDGA enhanced TRAIL-induced apoptosis was examined. NDGA did not change the expression levels of anti-apoptotic factors, Bcl-x_L, Bcl-2, cIAP-1, XIAP and survivin. The expression of death receptor-related genes was investigated and it was found that NDGA specifically up-regulated the expression of death receptor 5 (DR5) at mRNA and protein levels. Down-regulation of DR5 by small interfering RNA prevented the sensitizing effect of NDGA on TRAIL-induced apoptosis. Furthermore, NDGA sensitized prostate cancer and colorectal cancer cells to TRAIL-induced apoptosis. In contrast, NDGA neither enhanced TRAIL-induced apoptosis nor up-regulated DR5 expression in normal peripheral blood mononuclear cells. Another lipoxygenase inhibitor, AA861, also up-regulated DR5 and sensitized Jurkat and DU145 cells to TRAIL. These results indicate that lipoxygenase inhibitors augment the apoptotic efficiency of TRAIL through DR5 up-regulation in malignant tumor cells, and raise the possibility that the combination of lipoxygenase inhibitor and TRAIL is a promising strategy for malignant tumor treatment. (*Cancer Sci* 2007; 98: 1417–1423)

Lipoxygenases convert arachidnic, linoleic and other polyunsaturated fatty acids into biologically active metabolites that influence cell signaling, structure and metabolism.[1] Various tumor cells overexpress 5- and 12-lipoxygenases.[2–7] These lipoxygenases induce tumor cell proliferation, metastasis and angiogenesis.[8–10] It has been reported that lipoxygenase inhibitors, such as nordihydroguaiaretic acid (NDGA), possess preventive effects on various types of mitogen-induced carcinogenesis and human cancer xenografts in *in vivo* animal models.[11–17] As the mechanism of anti-tumor effect, NDGA has been shown to inhibit tumor cell growth by the induction of apoptosis.[16–18] Thus, lipoxygenase inhibitors are promising agents for anticarcinogenesis and anti-tumor therapy.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells *in vitro* and *in vivo*, with little or no toxicity in normal cells.[19–21] Therefore, TRAIL is one of the most promising candidates for cancer therapeutics. Death receptor 5 (DR5 also called TRAIL-R2 or KILLER) is a receptor for TRAIL.[22–26] DR5 mediates TRAIL-induced apoptosis through the activation of caspases.[27] DR5 is a target gene of tumor suppressor p53.[26,28,29] Conventional anti-tumor agents, such as doxorubicin and 5-fluorouracil, also up-regulate DR5 expression in a p53-dependent manner.[26,28,30] Furthermore, silencing of DR5 by RNA interference leads to accelerated growth of tumor xenograft in mice and confers resistance to chemotherapeutic agents.[30] Taken together, the TRAIL–DR5 pathway is an attractive target for cancer therapy. However, some tumors remain tolerant to TRAIL-induced apoptosis.[31] Therefore, it is important to develop sensitizers that overcome the resistance to TRAIL-induced apoptosis in malignant tumors.

Here, the authors show for the first time that lipoxygenase inhibitors sensitize malignant tumor cells to TRAIL-induced apoptosis through DR5 up-regulation.

Materials and Methods

Reagents. NDGA and AA861 were purchased from Sigma (St Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO). Soluble recombinant human TRAIL/Apo2L was purchased from PeproTech (London, UK). Human recombinant DR5/Fc chimera was purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture. Human Jurkat T-cell leukemia cells and prostate cancer DU145 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Human colon cancer SW480 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 4 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Human peripheral blood mononuclear cells (PBMC) were obtained as previously described.[32]

Detection of apoptosis. Cells were pretreated with the indicated concentration of lipoxygenase inhibitors for 24 h and then TRAIL was added to the culture medium. Cells were collected 12 h after addition of TRAIL. The collected cells were washed with phosphate-buffered saline (PBS). Jurkat cells and PBMC were fixed with 70% ethanol. DU145 and SW480 cells were suspended in a 0.1% Triton-X 100/PBS solution. The cells were treated with RNase A (Sigma) and the nuclei were stained with propidium iodide. The DNA content was measured using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). For each experiment, 10 000 events were analyzed. Cell Quest software (Becton Dickinson) was used to analyze the data.

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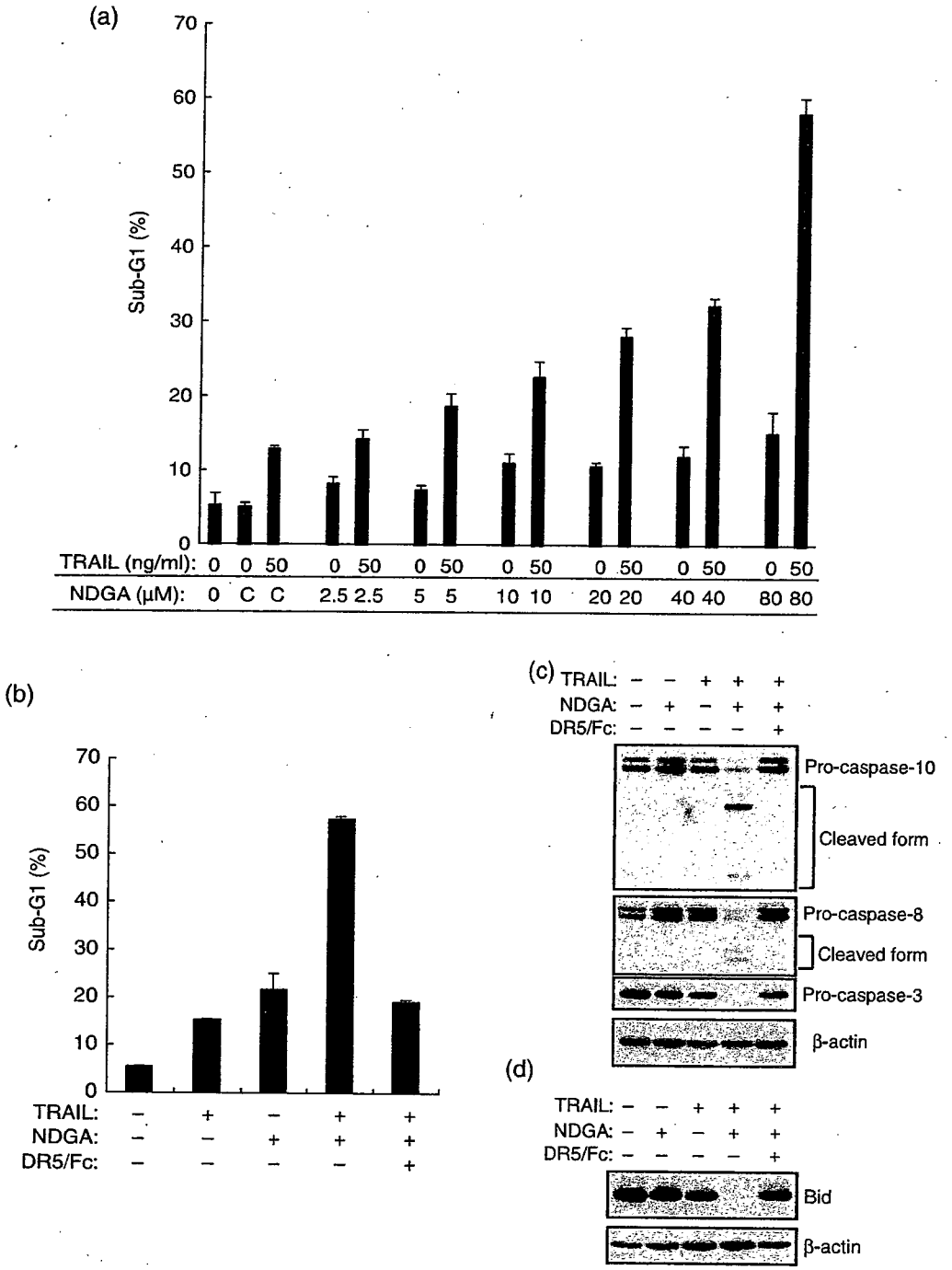

Fig. 1. Combination treatment with nordihydroguaiaretic acid (NDGA) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in Jurkat T-cell leukemia cells. (a) Jurkat cells were treated with 50 ng/mL TRAIL and/or various concentrations of NDGA. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. C, treated with solvent dimethylsulfoxide (DMSO). (b) Jurkat cells were treated with 50 ng/mL TRAIL and/or 80 μM NDGA with or without 1 μg/mL death receptor 5 (DR5)/Fc chimera protein. Apoptosis (Sub-G1) was analyzed by flow cytometry. The values shown are means ($n = 3$); bars, SD. (c,d) The cell lysates were analyzed using western blotting with anti-caspase-10, -8 and -3 and anti-Bid antibodies. β-actin was used as a loading control.

RNA analysis. Total RNA from the cells was extracted using the Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. For northern blotting, total RNA (10 μg) was separated with electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Biodyne B; Pall, Pensacola, FL, USA). A full-length DR5 cDNA was used as a probe. Hybridization was carried out with a ^{32}P-labeled probe in PerfectHyb plus hybridization buffer (Toyobo, Osaka, Japan) at 68°C for 16 h and the membrane was washed at 68°C in 2 × sodium saline citrate (SSC) containing 0.1% sodium dodecyl sulfate (SDS). The blot was exposed to X-ray Film (Kodak, Chalon-sur-Saone, France). The RNase protection assay was carried out as described previously.[32]

Western blot analysis. Whole cell lysate containing 50 μg protein was separated on 10% SDS-polyacrylamide gel (PAGE) for electrophoresis, and blotted onto polyvinylidene difluoride

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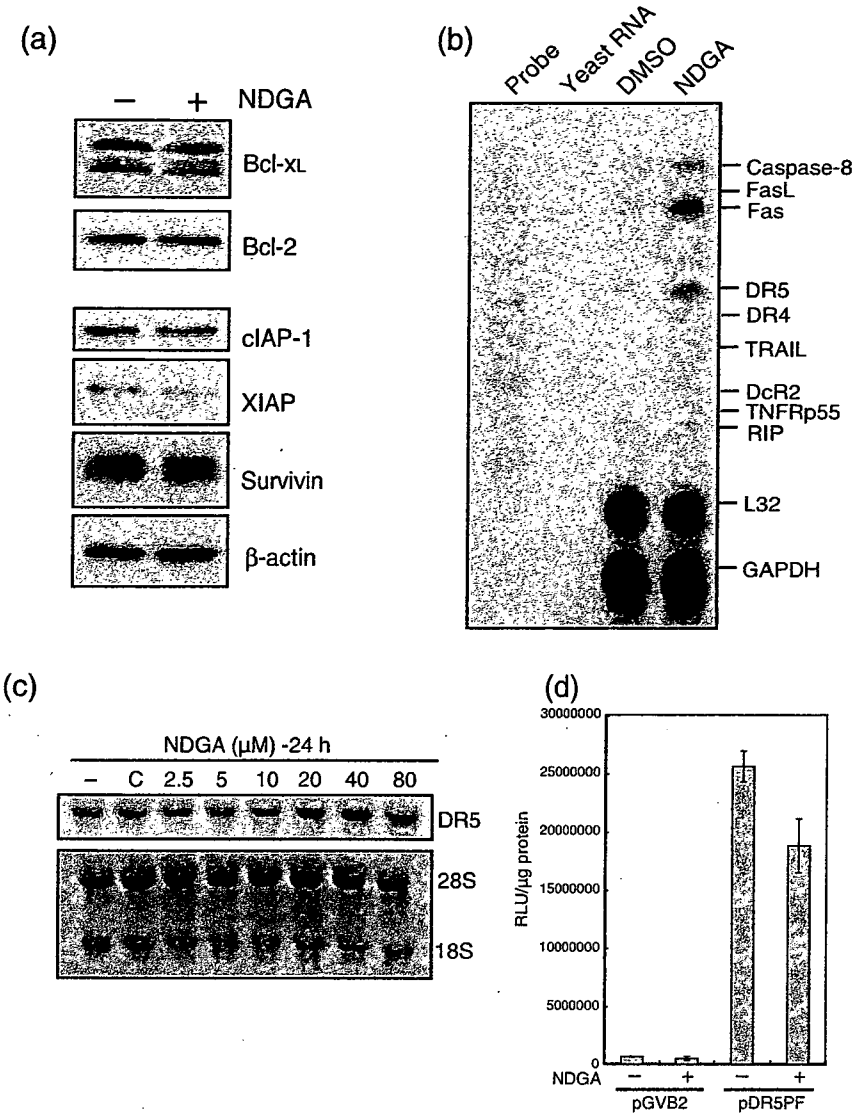

Fig. 2. Nordihydroguaiaretic acid (NDGA) up-regulates death receptor 5 (DR5) expression. (a) Whole cell lysates were prepared from Jurkat cells treated with dimethylsulfoxide (DMSO) or 80 μM NDGA for 24 h. The lysates were analyzed using western blotting of indicated proteins. β-actin was used as a loading control. (b) RNase protection assay for DR-related genes. Lane 1, Probes not treated with RNase. Lane 2, RNase-protected probes following hybridization with yeast tRNA. Lane 3 and 4, RNase-protected probes following hybridization with total RNA from Jurkat cells treated with DMSO (Lane 3) or 80 μM NDGA (Lane 4) for 24 h. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L32 are shown as loading controls. (c) Northern blot analysis shows the induction of DR5 mRNA by NDGA. Ethidium bromide staining of 28S and 18S rRNA are shown as loading controls. –, not treated; C, treated with DMSO. (d) Luciferase assay shows that NDGA does not increase DR5 promoter activity. Jurkat cells were transfected with a DR5-luciferase plasmid (pDR5PF) or a vacant plasmid (pGVB2). After 24 h, cells were treated with 80 μM NDGA or DMSO for 24 h. The values shown are means ($n = 3$); bars, SD.

(PVDF) membranes (Millipore, Bedford, MA, USA). Rabbit polyclonal anti-DR5 (Cayman Chemical, Ann Arbor, MI, USA), anti-caspase-3 (Cell Signaling Technology, Beverly, MA, USA), anti-Bcl-$x_{S/L}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-survivin and anti-cIAP-1 (R&D Systems) antibodies and mouse monoclonal anti-XIAP (R&D Systems) anti-Bcl-2-interacting domain (Bid), anti-caspase-8 and-10 antibodies (MBL, Nagoya, Japan), anti-Bcl-2 (Santa Cruz) and mouse monoclonal anti-β-actin antibody (Sigma) were used as the primary antibodies. The signal was detected with an enhanced chemiluminescence (ECL) western blot analysis system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Cell extracts were fractionated with ProteoExract Subcellular proteome extraction kit (Merck, Darmstadt, Germany).

Luciferase assay. A luciferase reporter plasmid containing a 2.5-kbp DR5 promoter, pDR5PF (= pDR5/SacI previously described[32]) or a vacant plasmid, pGVB2 (1.0 μg) was transfected into Jurkat cells (1×10^6 cells) using the DEAE-dextran method (CellPhect; Amersham Pharmacia Biotech). After 24 h, cells were treated with 80 μM NDGA or DMSO for 24 h. Cells were harvested and a luciferase assay was performed with luciferase assay reagents (Promega, Madison, WI, USA). Data were normalized by protein concentration measured with a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

Small interfering (si)RNA. The DR5, DR4 and LacZ siRNA were described previously[33] and synthesized by Proligo (Kyoto, Japan). One day prior to transfection, the cells were seeded into the medium without antibiotics at a density of 30–40%. The siRNA was transfected with Oligofectamine (Invitrogen, Carlsbad, CA, USA). 24 h after the transfection, the cells were treated with NDGA and/or TRAIL, and then the cells were harvested.

Results

NDGA sensitizes to TRAIL-induced apoptosis in Jurkat T-cell leukemia cells. The effect of the lipoxygenase inhibitor, NDGA, on TRAIL-induced apoptosis in Jurkat T-cell leukemia cells was investigated. Treatment with NDGA or exogenous recombinant TRAIL alone slightly induced apoptosis in Jurkat cells (Fig. 1a). However, interestingly, the combined treatment with TRAIL and NDGA markedly induced apoptosis. NDGA sensitized Jurkat cells to the apoptosis in a dose-dependent manner. Statistical analysis using factorial ANOVA showed significant interactions between the administrations of NDGA and TRAIL in Jurkat cells ($P < 0.001$), indicating that NDGA strongly sensitized Jurkat cells to TRAIL-induced apoptosis in a synergistic fashion. It is worth noting that the non-tagged form of recombinant TRAIL was used in the present study, because His-tagged TRAIL kills

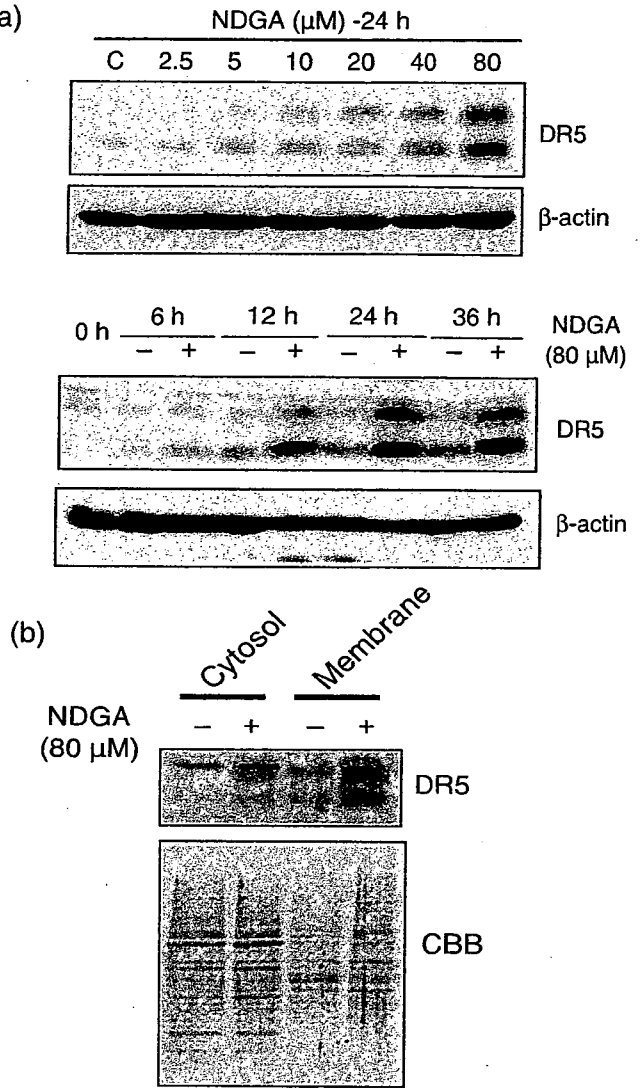

Fig. 3. Nordihydroguaiaretic acid (NDGA) increases death receptor 5 (DR5) protein in whole cell lysates and membrane fraction. (a) Jurkat cells treated with the indicated concentrations of NDGA for the indicated time were analyzed using western blotting with anti-DR5 antibody. β-actin is shown as a loading control. C or –, treated with dimethylsulfoxide (DMSO). (b) Cell lysates of Jurkat cells treated with 80 μM NDGA were fractionated and analyzed with western blotting with anti-DR5 antibody. Coomassie Brilliant Blue (CBB) staining of gel is shown as a loading control. –, treated with DMSO.

human normal hepatocytes.[21] These results indicate that NDGA acts as a sensitizer for TRAIL-induced apoptosis.

DR5/Fc chimera blocks apoptosis induced by the combination of TRAIL with NDGA. To investigate that NDGA sensitizes to apoptosis via a signaling pathway at the downstream of TRAIL, the DR5/Fc chimera protein, which is a dominant negative protein of a TRAIL receptor, was used. As shown in Fig. 1b, DR5/Fc chimera protein efficiently attenuated the apoptosis induced by the combination of TRAIL and NDGA. The cleavage and activation of caspases by combined treatment with NDGA and TRAIL was examined next. TRAIL or NDGA alone did not affected caspases; however, combined treatment with TRAIL and NDGA cleaved pro-caspase-10, -8 and -3 (Fig. 1c). The cleaved forms of caspase-10 and -8 were detected. DR5/Fc chimera protein also blocked the cleavage of caspases. Bid is a mediator of apoptotic signaling from the DR pathway to the mitochondrial pathway.[27] Bid is cleaved and activates downstream TRAIL signaling.[27] The combination of NDGA and TRAIL clearly cleaved Bid and the DR5/Fc chimera protein blocked the cleavage (Fig. 1d). These results indicate that NDGA enhances the apoptotic signaling performed by TRAIL.

NDGA up-regulates the expression of DR5, a receptor for TRAIL. To elucidate the mechanism of the sensitization of TRAIL-induced apoptosis by NDGA, whether NDGA regulates the expression of anti-apoptotic genes was examined. Western blotting of Bcl-x_L, Bcl-2, cIAP-1, XIAP and survivin was carried out. As shown in Fig. 2a, NDGA did not change the expression of these anti-apoptotic factors. Next, the expression of DR-related genes was investigated using RNase protection assay. As shown in Fig. 2b, NDGA up-regulated the expression of DR5, a receptor for TRAIL. In addition, caspase-8 and Fas were also up-regulated by NDGA. DR4 and DcR2 mRNA weakly increased but DcR1 was not detected. To confirm the DR5 up-regulation by NDGA, northern blotting of DR5 was carried out. NDGA increased DR5 mRNA in a dose-dependent manner (Fig. 2c). A luciferase assay with pDR5PF, a reporter plasmid containing an upstream promoter region of the *DR5* gene (~2.5 kbp), was carried out.[32] NDGA did not increase DR5 promoter activity. Next, western blotting was carried out to examine whether NDGA increases DR5 at a protein level. As shown in Fig. 3a, NDGA increased the DR5 protein level in a dose- and time-dependent manner. DR5 protein was markedly increased from 12 h after treatment with NDGA. The cell lysate was fractionated and western blotting was performed. NDGA increased the DR5 protein in the membrane fraction (Fig. 3b).

NDGA enhances TRAIL-induced apoptosis in prostate cancer DU145 and colon cancer SW480 cells, but not in normal PBMC. Whether NDGA enhances TRAIL-induced apoptosis in other malignant tumor cells was examined. Prostate cancer DU145 cells were used, because lipoxygenases are overexpressed and increase the metastatic potential in prostate cancer.[2,5] As shown in Fig. 4a, NDGA also enhanced TRAIL-induced apoptosis in DU145 cells in a dose-dependent manner. Western blotting showed that NDGA up-regulated the DR5 expression in DU145 cells (Fig. 4a). Moreover, NDGA up-regulated DR5 expression and enhanced TRAIL-induced apoptosis in colon cancer SW480 cells (Fig. 4b). TRAIL is attractive for cancer therapy, because TRAIL selectively induces apoptosis in cancer cells with little or no toxicity to normal cells.[19–21] Therefore, whether TRAIL-induced apoptosis was enhanced by NDGA in normal cells was investigated. Normal human PBMC derived from healthy volunteer were used as an experimental model. Interestingly, the combination of TRAIL and NDGA did not induce apoptosis in PBMC compared to malignant tumor cells (Fig. 4c). Moreover, NDGA did not up-regulate DR5 expression in PBMC (Fig. 4c). As shown in Fig. 4d, even high concentrations of TRAIL, such as 200 ng/mL, weakly induced apoptosis in Jurkat, DU145 and SW480 cells. These results indicated that when 50 ng/mL TRAIL was combined with 80 μM NDGA, the combination more strongly induced apoptosis than 200 ng/mL TRAIL treatment alone in Jurkat, DU145 and SW480 cells. Thus, NDGA reduced the threshold of TRAIL-induced apoptosis in malignant tumor cells.

Down-regulation of DR5 prevented apoptosis induced by the combination of NDGA and TRAIL. It was shown that NDGA up-regulated DR5 expression and sensitized malignant tumor cells to TRAIL-induced apoptosis. To confirm that DR5 up-regulation by NDGA is responsible for the sensitization to TRAIL, siRNA targeting DR5 were used and the induction of DR5 by NDGA was blocked. As shown in Fig. 5, DR5 siRNA efficiently down-regulated DR5 and prevented the sensitizing effect of NDGA to TRAIL-induced apoptosis. In contrast, down-regulation of DR4 did not block the apoptosis induced by the combination of NDGA and TRAIL. These results indicate that NDGA sensitizes malignant tumor cells to TRAIL-induced apoptosis due to the up-regulation of DR5.

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