6．造血幹細胞の動員および自家造血幹細胞移植

化学療法後，自家造血幹細胞を採取する際，造血幹細胞動員の目的でG-CSFを使用する。また，自家造血幹細胞移植の際にも大量化学療法後にG-CSFを使用することは，今日一般的である。

7．放射線治療および放射線化学療法同時治療

頭頸部がん，食道がんなどでは放射線化学療法同時治療が行われるが，特に縦隔を含む病変の場合，G-CSFを使用すべきではない。放射線単独治療で好中球減少が遷延する場合は，G-CSF使用を考慮してもよい。

8．高齢者の悪性リンパ腫

Diffuse large B-cell lymphoma（DLBCL：びまん性大細胞型B細胞性リンパ腫）に代表されるような，いわゆるaggressive lymphoma（急速進行性リンパ腫）は化学療法によって治癒が期待できる。今日DLBCLの標準治療はR-CHOP療法であるが，65歳以上の高齢者の治療の際，FNおよびそれに伴う合併症を減少させる目的で予防的G-CSF投与が推奨される。また高齢者治療に際して抗がん剤用量を減量することは，FNのリスクを減らすかもしれないが，同時に治療成績をも低下させる可能性があり，それを回避するためにもdose intensityを保つ目的でG-CSF投与が推奨される。

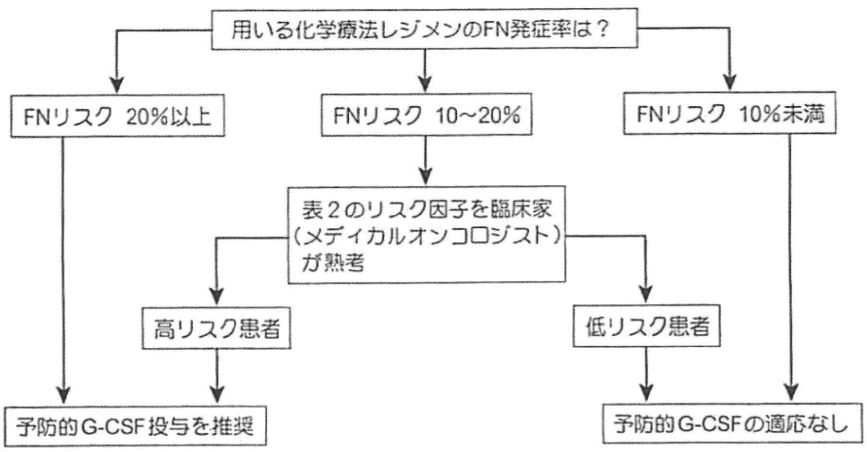

図　予防的G-CSF投与のアルゴリズム

FN発症率20%以上では予防的G-CSF投与を推奨し，10%未満では適応はない。10～20%では，患者のリスクに応じて臨床家（メディカルオンコロジスト）の判断が重要となる。

（文献1～3より改変引用）

おわりに

ASCO，EORTC，NCCNのガイドラインのエッセンスを抽出して，図に予防的G-CSF投与の概略を示したので参考にされたい。投与レジメンを十分理解した上で，患者の全身状態を把握し，メディカルオンコロジストとして適切なG-CSFが使用できること，そのことにより患者にとって安全で効果的な化学療法が遂行できることを期待したい。

（横山雅大）

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Dlk-1, a cell surface antigen on foetal hepatic stem/progenitor cells, is expressed in hepatocellular, colon, pancreas and breast carcinomas at a high frequency

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Delta-like 1 protein (Dlk-1), also known as preadipocyte factor 1 (Pref-1), is a transmembrane and secreted protein with epidermal growth factor (EGF)-like repeats. Dlk-1 is known to be expressed in foetal liver, but absent in neonatal and adult liver in mice and rats. Dlk-1 is also expressed in a subpopulation of hepatic oval cells, which are considered as stem/progenitor cells in rat adult liver. In this study, we generated monoclonal antibodies against human Dlk-1 (hDlk-1) and investigated hDlk-1 expression in human liver and hepatocellular carcinoma (HCC). Like rodent livers, hDlk-1 was detected in foetal liver, but not in adult liver. In HCC, hDlk-1 was positive for 20.5% of the cases examined and was localized in both cytoplasm and cell membrane, whereas hDlk-1 was undetected in viral hepatitis, nodular cirrhosis. Interestingly, hDlk-1 positive HCC was found more frequently in younger patients and its expression was correlated with alpha-fetoprotein expression. Furthermore, hDlk-1 was also detected frequently in colon adenocarcinomas (58%), pancreatic islet carcinoma (50%), and small cell lung carcinoma (50%). Thus, hDlk-1 is a cell surface protein expressed in many carcinomas including HCC and may be a potential target for monoclonal antibody therapy for carcinomas.

Keywords: cell surface protein/colon adenocarcinoma/hDlk-1/hepatocellular carcinoma (HCC)/small cell lung carcinoma.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence activated cell sorter; His, histidine; MEM, minimum essential medium; mRNA, messenger RNA; PBS, phosphate-buffered saline.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumours in the world (*1*). While the occurrence has been unusually high in Asia and Africa, it is recently increasing in United States and the incidence and mortality rates are anticipated to double over the next 10–20 years (*2*, *3*). HCC is often diagnosed at an advanced stage when curative therapies are of limited efficacy. In order to reduce morbidity and mortality of HCC, it is of prime importance to develop a system for early diagnosis, novel systemic therapies for the advanced disease as well as means to prevent HCC development.

Dlk-1 protein, also known as Pref-1, foetal antigen 1 (FA1), pG2 and ZOG, is a transmembrane and secreted protein, which is a member of the epidermal growth factor (EGF)-like family including Notch/Delta/Serrate (*4–8*). Dlk-1 is strongly expressed in foetal tissues such as liver, pancreas and skeletal muscle, but its expression is restricted in adult tissues such as placenta and adrenal grand (*9–12*). As there are many receptors and ligands in the EGF-like family proteins, which regulates cell fate and differentiation during development in many organisms, Dlk-1 may also play a role in development and differentiation (*12–14*). In fact, there are several reports showing the involvement of Dlk-1 in adipogenesis (*15*), hematopoiesis (*16*, *17*) and development of pancreas (*18*, *19*), placenta (*20*) and adrenal gland (*21*, *22*). In addition to the normal tissues, Dlk-1 was also shown to be expressed in several tumours, such as neuroblastoma (*23*), glioma (*24*), small cell lung carcinoma (*25*), myelodysplastic syndrome, acute myelogenous leukaemia (*26*), etc. (*27*, *28*). These results suggest that Dlk-1 may play an important role in tumourigenesis as well as organogenesis.

Previously, we demonstrated that Dlk-1 is strongly expressed on the cell surface of hepatoblasts in murine foetal liver from embryonic day (ED) 10.5–16.5 and Dlk-1+ cells isolated from foetal liver showed high-proliferative activity and bi-potentiality (*10*). Its expression is down-regulated in late gestation and completely absent after birth. In liver injury under conditions that limit proliferation of hepatocytes, immature cells with oval shaped nucleus called hepatic oval cells appear around the portal vein. As they are proliferative and express markers of hepatocytes and cholangiocytes, hepatic oval cells have been considered as adult liver progenitors (*29*). Similar cells were also shown to be present in severe hepatitis and implicated in tumourigenesis (*30*). The expression of Dlk-1 was also observed in a subpopulation of rat oval cells induced by the 2-acetylaminofluorene/partial hepatectomy model (*31*). These data suggest that Dlk-1 is a cell

surface antigen of foetal/adult hepatic stem/progenitor cells.

It has become clear that tumour, in many cases, is a heterogeneous cell population and only a small fraction of the cells possess the potential to self-renew. Cancer stem cell or tumour initiating cell, which was first documented in haematological malignancies, has subsequently been discovered in many solid tumours, including breast, brain, prostate, liver, lung, melanoma, pancreas and colon tumours (*32–36*). While it was shown that CD133, known as a stem cell marker, is expressed in cancer stem cells in many tumours including HCC (*37–41*), the relation between normal tissue stem cells and cancer stem cells is not clear in most of the cases.

In this study, we established many hybridoma clones which produced anti-hDlk-1 monoclonal antibodies (mAb). Among them, we selected three independent clones usable for immunohistochemistry and characterized these antibodies by flow cytometry. Using these mAbs which recognized a different epitope, we investigated the expression of human Dlk-1 (hDlk-1) during liver development by immunohistochemistry. The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. We then examined hDlk-1 expression in human neoplastic liver lesions. About a half of HCC specimens from under 40-years-old patients expressed hDlk-1, whereas the positive ratio of hDlk-1 over 50-years-old patients was ~10%. Our study indicates the possibility that hDlk-1 is a common cell surface antigen both in human foetal liver stem/progenitor cells and in a part of HCC. Moreover, hDlk-1 was also frequently expressed in colon, breast, pancreas and lung carcinoma. These observations suggest that hDlk-1 is a potential target for monoclonal antibody-based therapy in those carcinomas.

Materials and Methods

Plasmid constructs

Full length hDlk-1 and its derivatives (EGF1-3 and EGF 4-6) were amplified by PCR. The sequences of primers were as follows: Fw1: 5′-cgcgtccgcaaccagaagccc-3′, Rv1: 5′-aagcttgatctcctcgtcgccggcc-3′ (for full length hDlk-1), Fw2: 5′-gcggccgcgctgaatgcttcccggcc-3′, Rv2: tctagaggcccgaacatctctatcac-3′ (for hDlk-1 EGF1-3), Fw3: 5′-gcggccgcgcctgctcctcggcccccc-3′, Rv3: 5′-gcgtatagtaagctctgcgg-3′ (for hDlk-1 EGF4-6). All PCR products were verified by DNA sequencing. Full-length hDlk-1 cDNA was cloned in pcDNA3 vector (Invitrogen, Carlsbad, CA) with Flag tag. hDlk-1 EGF1-3 cDNA was subcloned in pME18SNeo carrying the signal sequence of CD8, His tag, and transmenbrane and cytoplasmic domains of FXYD5, which was kindly gifted by Dr Tanaka, M. (University of Tokyo, Tokyo, Japan). hDlk-1 EGF4-6 cDNA was subcloned in pME18SNeo containing the signal sequence of CD8, His tag.

Antibodies

Mouse monoclonal antibodies against hDlk-1 (clone DI-6, DI-2-20 and DI-4-22) were generated by the DNA immunization method (Nosan Corp., Kanagawa, Japan). To prepare purified monoclonal antibodies, hybridoma clones (3×10^6 cells) were intraperitoneally administered to BALB/c Slc-nu/nu mice (Japan SLC, Shizuoka, Japan), which received 2,6,10,14-tetramethylpentadecane (Sigma Aldrich Japan K.K., Tokyo, Japan) 7 days before injection of hybridoma. After collection of ascites, the antibodies were purified with a protein G column (GE Healthcare, Buckinghamshire, England). Rabbit polyclonal antibodies against hDlk-1 were prepared by immunizing with peptides containing the extracellular domain of hDlk-1 except for the putative signal sequence. Polyclonal antibodies were purified by affinity chromatography using columns conjugated with the peptides used for immunization.

Cell culture, transfection and flow cytometry

COS7 cells, HEK-293 cells, Huh-7 cells and SK-N-FI cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. HepG2 cells and C3A/HepG2 cells were maintained in MEM supplemented with 10% foetal bovine serum. COS7, HEK-293, Huh-7 and HepG2 cells were from Human Science Research Resource Bank (Osaka, Japan). SK-N-FI and C3A/HepG2 cells were purchased from American Type Culture Collection (Rockville, MD). Transfection was performed using Lipofectoamine and Plus reagent (Invitrogen). To establish HEK-293 cells stably expressing hDlk-1 (293-hDlk-1), HEK-293 cells were transfected with pcDNA3 vector containing full-length hDlk-1 cDNA and selected with G418 (Invitrogen). COS7 cells were transiently transfected with expression constructs containing either hDlk-1 EGF1-3 or hDlk EGF4-6, and two days after transfection, these cells were harvested and subjected to fluorescence activated cell sorter (FACS) analysis. 293-hDlk-1 cells were cultured to subconfluency and harvested for flow cytometry by FACSCalibur (Nippon Becton Dickinson, Tokyo, Japan).

Immunohistochemistry

Tissue arrays and sections of tumours used in this study were purchased from Cybrdi (Rockville, MD), Shanghai Outdo Biotech Co. (Shanghai, China), Super Bio Chips (Seoul, Korea), ISU ABXIS (Seoul, Korea), US Biomax (Rockville, MD). Clinical information of patients (age, sex, grade and pathology diagnosis) is described in their homepage and data sheets. Foetal liver specimens were purchased from Biochain (Hayward, CA).

Paraffin embedded tissue sections and arrays were deparaffinized, and then autoclaved for 5 min in citrate buffer (pH 6.0) or TE buffer (pH 9.0). Slides were treated with methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase activity, and incubated with anti-hDlk-1 mAbs (10 μg/ml) at 4°C over night. After washing with phosphate-buffered saline (PBS), sections were stained with Vectastain ABC Elite kit (Vector, Burlingame, CA) and then counterstained with haematoxylin (Wako, Osaka, Japan). HCC sections with more than 10% immunopositive cells, either cell membrane or cytoplasmic stainin, were considered as positive.

Result

Characterization of anti-hDlk1 monoclonal antibodies

We established over 100 hybridoma clones producing anti-hDlk-1 mAb. Among them, three independent clones usable for immunohistochemistry in paraffin-embedded tissue sections were selected. First, we evaluated the reactivity and specificity of these antibodies by flow cytometry. Three mAbs against hDlk-1, DI-6, DI-2-20 and DI-4-22, specifically recognized HEK-293 cells stably expressing hDlk-1 (Fig. 1B), but not parent HEK-293 cells (not shown). On the other hand, these antibodies failed to recognize mouse Dlk-1, which shares ~90% similarity with hDlk-1 at the amino acid level (data not shown). These results confirmed that anti-hDlk-1 mAbs, DI-6, DI-2-20 and DI-4-22, specifically recognize hDlk-1. We then mapped the region of hDlk-1 to which the antibodies bind using deletion mutants of hDlk-1 (Fig. 1A). As shown in Fig. 1D, DI-6 recognized the EGF repeats 1–3 (amino acid 24–129), whereas DI-2-20 and DI-4-22 recognized EGF repeats 4–6 (amino acid 126–382). DI-2-20 and DI-4-22 recognized the same epitope because they competed each other (results not shown).

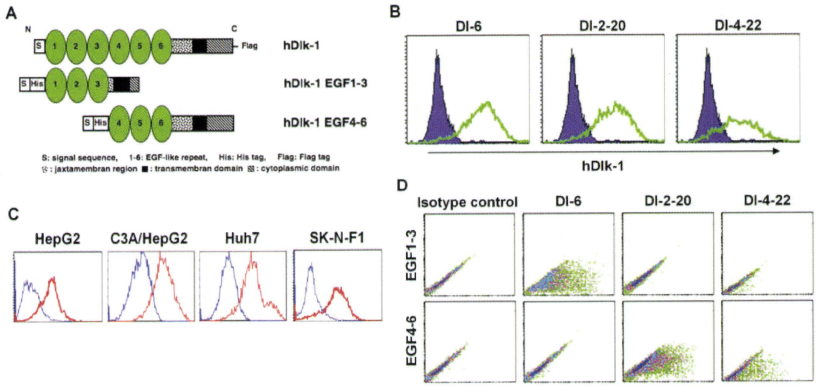

Fig. 1 Characterization of anti-hDlk-1 monoclonal antibodies by FACS analysis. (A) Schematic representation of various hDlk-1 constructs used in this study. (B) Three monoclonal antibodies used in this study specifically recognize hDlk-1 expressing cells. HEK-293 cells stably-expressing hDlk-1 were harvested, dispersed in a single cell suspension, and analysed by flow cytometry using monoclonal antibodies against hDlk-1, DI-6, DI-2-20, DI-4-22 (green line). Blue area: isotype control (mouse IgG1). (C) Anti-hDlk-1 mAb DI-2-20 also recognizes endogenous hDlk-1 in human cancer cell lines. Cancer cell lines indicated here were harvested and subjected to FACS analysis by using DI-2-20, respectively. Blue line: isotype control (mouse IgG1), red line: anti-hDlk-1 mAb (DI-2-20). Another monoclonal antibody, DI-6, showed similar result (data not shown). (D) Identification of the region of hDlk-1 to which anti-hDlk-1 mAbs bind. COS7 cells were transiently transfected with plasmids containing a various domain of hDlk-1 represented in (A). Two days after transfection, these cells were harvested and subjected to FACS analysis with anti-hDlk-1 mAb, DI-6, DI-2-20 and DI-4-22, respectively. DI-2-20 and DI-4-22 recognize EGF repeat 4–6, whereas DI-6 recognizes EGF repeat 1–3.

As reported previously, hDlk-1 was expressed early in liver development, but not in adult liver (*9*). To confirm that selected mAbs were suitable for immunohistochemistry in paraffin-embedded tissue sections, the expression pattern of hDlk-1 protein in liver was examined by immunohistochemical staining using DI-2-20 mAb (Fig. 2). A foetal liver tissue at 22 weeks (22w) showed membrane and cytoplasmic staining of hDlk-1 in hepatocytes. However, hDlk-1 was not detected in foetal liver at 38 weeks (38w) and adult liver. Northern blot analysis showed that hDlk-1 mRNA was strongly expressed in foetal liver from 6 to 12 weeks of gestation (not shown). The expression of hDlk-1 showed a pattern similar to mouse Dlk-1 during liver development, suggesting that hDlk-1 is also a marker of hepatic stem/progenitor cells in embryo. Similar results were obtained with either DI-6 or DI-4-22 mAb, though less sensitive than DI-2-20 (not shown). These results suggest that DI-2-20 was also useful for detecting endogenous hDlk-1 by immunohistochemistry. Therefore, we used mainly DI-2-20 mAb for further analysis.

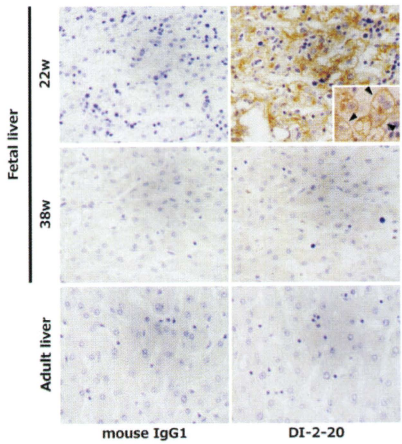

Fig. 2 Immunohistochemical analysis of hDlk-1 in foetal and adult liver. Each specimen was stained with anti-hDlk-1 mAb DI-2-20 (right panels). Foetal liver tissue at 22w (upper) shows membrane (arrow heads) and cytoplasmic staining with DI-2-20 monoclonal antibody. hDlk-1 staining was not observed in foetal liver at 38w (middle) and adult liver (lower). Mouse IgG1 used as isotype control was negative (left panels). Magnification is ×400.

Expression of hDlk-1 in HCC cells

We then examined the cell surface expression of hDlk-1 in a number of cancer cell lines originated from HCC by flow cytometry by using DI-6 and DI-2-20 antibodies. Among them, significant cell surface expression of hDlk-1 was detected in HepG2, C3A/HepG2 and Huh-7 cells (Fig. 1C). These results suggest that hDlk-1 is the cell surface antigen of HCC

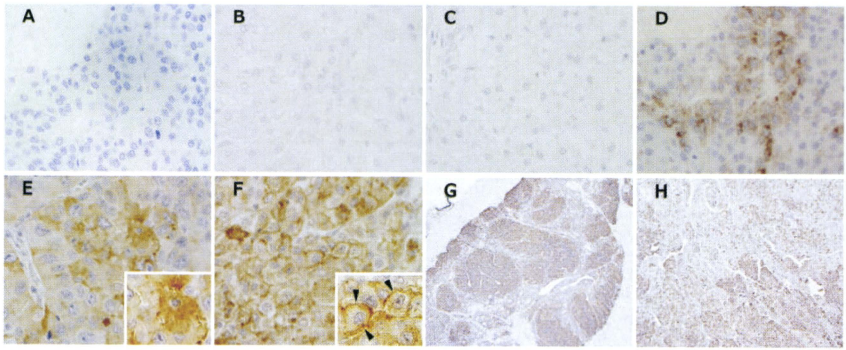

Fig. 3 hDlk-1 expression was observed only in hepatocellular carcinoma. Immunohistochemical staining with anti-hDlk-1 monoclonal antibody DI-2-20 was performed using tissue microarrays of liver tumour (388 malignant and 19 benign), nodular cirrhosis (40), viral hepatitis (11) and normal liver (26). The expression of hDlk-1 was detected only in HCC sections (D–F), not in adult liver (not shown), viral hepatitis (B), nodular cirrhosis (C), cavernous hemangioma (not shown) and intrahepatic cholangioma (not shown). hDlk-1 immunoreactivity in HCC cells was localized in either cytoplasm (E, inset) or cell membrane (F, inset). Membranous staining of hDlk-1 was shown by arrow heads. hDlk-1 expression was also examined in some samples of conventional tissue section corresponding to hDlk-1 positive spots in tissue array (G and H). Mouse IgG1 was used as negative control (A). Magnification is ×400 (A–F), ×1000 (inset in E and F) and ×40 (G and H).

cells. Additionally, hDlk-1 was also expressed in the cell surface of SK-N-F1 cells, a neuroblastoma cell line (Fig. 1C).

To evaluate the expression of hDlk-1 in non-neoplastic and neoplastic liver lesions, immunohistochemical analysis was performed by using tissue arrays. Typical staining profiles are shown in Fig. 3 and the results are summarized in Table I. hDlk-1 expression was undetectable at all in normal adult liver (0/26, not shown), non-neoplastic liver lesions, viral hepatitis (0/11, Fig. 3B) and nodular cirrhosis (0/40, Fig. 3C). hDlk-1 was also not found in cavernous hemangioma (0/19, not shown) and intrahepatic cholangioma (0/2, not shown). In contrast, hDlk-1 expression examined by DI-2-20 antibody was specifically observed in HCC and was positive for 79 out of 386 cases (20.5%, Fig. 3D–F). The pattern of hDlk-1 staining varied among individual tumours, e.g. hDlk-1 signal exhibited a uniform distribution within the tumour in one case (Fig. 3E, F), whereas it showed mosaic-like pattern in another case (Fig. 3D). Similar results were obtained with the same tissue array by using DI-6 antibody that recognizes a different epitope from DI-2-20 (not shown). Although hDlk-1 is a type I transmembrane protein, the immunoreactivity in HCC cells was mainly observed in the cytoplasm (Fig. 3E, inset), whereas hDlk-1 was expressed in cell surface in some cases (Fig. 3F, inset). We also examined some of conventional tissue slides corresponding to hDlk-1 positive spots in tissue arrays for hDlk-1 expression. The staining of hDlk-1 was not uniform, but covered more than 10% of the tumour in all tissue sections that we studied (Fig. 3G and H). No immunoreactivity was observed in normal tissues adjacent to the tumour (not shown).

Table I. Summary of immunohistochemical analysis.

	hDlk-1 staining	
	−	+
(A) *hDlk-1 expression in HCC (386 cases)*		
CS03-01-002 (Cybrdi)	40	15
CC03-01-001 (Cybrdi)	43	12
CC03-01-003 (Cybrdi)	46	10
CC03-02-001 (Cybrdi)	14	3
A204 (ISU ABXIS)	29	6
A204(II) (ISU ABXIS)	29	6
BC03013 (Biomax US)	49	10
OD-CT-DgLiv02-002 (Outdo bio.)	25	7
CS3 (HCC only) (Super Biochips)	32	10
Total	307 (79.5%)	79 (20.5%)
(B) *hDlk-1 expression in normal liver, non-neoplastic liver lesions, benign liver tumour and cholangiocarcinoma*		
Normal liver	23	0
Viral hepatitis	11	0
Nodular cirrhosis of liver	40	0
Cavernous hemangioma of liver	19	0
Intrahepatic cholangicarcinoma	2	0

As summarized in Table II, there was no clear correlation between hDlk-1 expression and pathological grade, gender, or aetiology such as HBV or HCV infection ($P>0.05$ by χ^2 test). In contrast, hDlk-1 expression was clearly correlated with age or expression of alpha-fetoprotein (AFP). Interestingly, hDlk-1 expression was detected at higher frequency in HCC under 50 years old (51 out of 162 specimens, 31.5%), whereas the hDlk-1 positive HCC was dramatically decreased over 50 years old (28 out of 220 specimens, 12.7%). Especially, the hDlk-1 positive HCC was 43.1% (22 out of 51 specimens) under 40 years old. AFP is a well-established marker for HCC and was

Table II. Relationship of hDlk-1 expression and clinical features.

	hDlk-1			
	−	+	hDlk-1+ ratio	
Grade				
I	46	9	16.40%	
II	158	54	25.50%	
III	50	14	21.90%	$P=0.347$
Gender				
Male	245	59	19.40%	
Female	58	20	25.60%	$P=0.225$
Age				
40>	29	22	43.10%	
40–49	82	29	26.10%	
50–60	99	17	14.70%	
>60	93	11	10.60%	$P<0.01$
AFP				
−	179	25	12.30%	
+	26	25	49.00%	$P<0.01$
Aetiology				
HBV+	44	12	21.40%	
HCV+	6	0	0.00%	
−	7	1	12.50%	$P=0.393$

present in 51 of 255 cases (20.0%). hDlk-1 was detected in 25 of 51 AFP-positive HCCs (49.0%), whereas 25 of 204 AFP-negative HCCs (12.3%) were positive for hDlk-1. These results indicated that the hDlk-1 was expressed more frequently in a patient under 50 years old and in AFP-positive HCC.

Expression of hDlk-1 in other carcinomas

We then examined the expression of hDlk-1 in various carcinomas and found that hDlk-1 was highly and frequently expressed in colon adenocarcinoma (58.6%), breast carcinoma (39.0%), pancreatic carcinoma (30.8%) and lung carcinoma (30.2%), but not in ovarian carcinoma (13.2%) and gastric carcinoma (3.33%). Interestingly, in pancreatic carcinomas, hDlk-1+ cells were found more frequently in islet carcinoma (50.0%) than duct carcinoma (28.3%). In the lung carcinoma, hDlk-1 was expressed in small cell lung carcinoma (52.5%), but only few non-small cell carcinoma (8.9%) expressed hDlk-1 (Fig. 4, summarized in Table III). These results suggested that hDlk-1 was expressed in various carcinomas.

As described above, hDlk-1 was more frequently expressed in AFP-positive HCC. Therefore we examined the expression of hDlk-1 in other AFP positive cancer, AFP-producing gastric cancer. hDlk-1 was rarely expressed in gastric carcinoma (3.33%), but was positive for two out of 10 cases in AFP-producing gastric cancer (20.0%, Fig. 4G and H, summarized in Table III). Previously, Dezso *et al.* (*42*) reported that hDlk-1 was highly expressed in hepatoblastoma, AFP-positive liver cancer occurring in childhood. Together with our result, it was suggested that hDlk-1 was frequently expressed in AFP-positive cancers.

Discussion

Previously, we demonstrated that Dlk-1 is strongly expressed in hepatoblasts in mouse foetal liver, down-regulated in late gestation, and completely disappeared in neonatal and adult liver. Single Dlk-1+ cell isolated from ED14.5 liver exhibited high proliferating activity and was able to differentiate into both hepatocyte and biliary epithelial cell lineages (*10*). These findings suggested that Dlk-1 is a cell surface antigen of foetal hepatic stem/progenitor cells in the mouse. In this study we prepared mAbs against hDlk-1 and showed that the expression pattern of hDlk-1 is similar to mouse Dlk-1/Pref-1 during liver development, i.e. hDlk-1 is expressed in foetal liver but not in adult liver and hDlk-1 is present in both cell membrane and cytoplasm (Fig. 2). Thus, hDlk-1 may be an excellent marker of foetal hepatic stem/progenitor cells in human as well.

In adult liver, hepatic progenitor cells (HPCs) appear around the portal vein when liver is severely injured. These cells are known as hepatic oval cells in rodents and express markers of both hepatocytes and biliary epithelium (*29*). HPCs in chronic liver diseases are suggested to contribute to liver regeneration as well as hepatocarcinogenesis (*43–45*). Dlk-1 is not expressed in normal liver and was found in a subpopulation of hepatic oval cells induced in rats treated with 2-acetylaminofluorene and partial hepatectomy, a well-established rat model of hepatic oval cell induction (*31*). However, in a mouse model of hepatic oval cell induction by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, Dlk-1 is not expressed in hepatic oval cells (*46*). In the present study, hDlk-1 immunoreactive cells were not found in the specimens of viral hepatitis and nodular cirrhosis. In contrast, it was expressed frequently in HCC, but not in intrahepatic cholangioma, cavernous hemangioma and non-neoplastic liver lesions. These results demonstrate that hDlk-1 is expressed in HCC at high frequency, but do not exclude the possibility that Dlk-1 is expressed in some of adult hepatic stem/progenitor cells in chronically injured liver, which can lead to tumourigenesis.

It still remains unclear whether liver tumour is derived from hepatic stem/progenitor cells or mature hepatocytes. Recently, Lee *et al.* (*47*) reported that two subtypes (HB and HC) of HCC were categorized by analysis of gene expression patterns, and suggested that they may reflect the origin of tumour cells. The HB subtype shared a gene expression pattern with foetal hepatoblasts, whereas the HC subtype shared with adult hepatocytes, suggesting that the HB subtype may arise from hepatic stem/progenitor cells. The HB subtype of HCC showed poor prognosis compared to HC subtype. Interestingly, the HB subtype accounts for ~20% of HCC examined, similar to the frequency of hDlk-1 positive cells. In addition, the expression of hDlk-1 was more frequently found in HCC patients younger than 50 years old. Because HCC develops after a long latency period of chronic infection with HBV, HCV or both, the incidence of HCC is relatively high over 50 years old. Therefore, our finding that HCC patients under 50 years old expressed hDlk-1

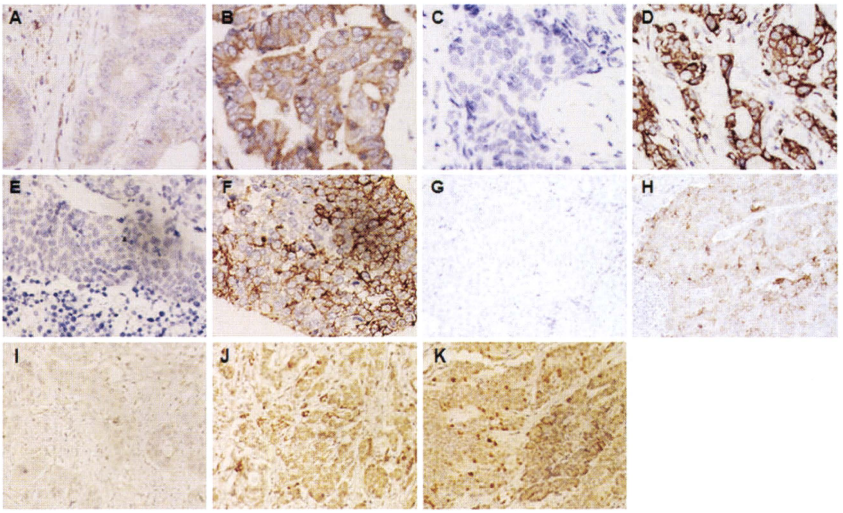

Fig. 4 hDlk-1 expression in various tumours. hDlk-1 expression in various tumours were examined by immunohistochemistry using anti-hDlk-1 mAb, D1-2-20. (A and B) Colon adenocarcinoma, (C and D) breast carcinoma, (E and F) small cell lung carcinoma, (G and H) AFP-producing gastric cancer, (I and J) pancreatic adenocarcinoma, (K) islet cell carcinoma. Tumour cells showed strong staining in cytoplasm (J and K), and cytoplasm and cell membrane (B, D, F and H). A, C, E, G and I showed hDlk-1 negative tumour cells. Magnification is ×200.

Table III. Summary of hDlk-1 staining in various tumours.

Tumour		Dlk−	Dlk+	Total
Colon adenocarcinoma		24 (41.4%)	34 (58.6%)	58
Ovarian carcinoma		59 (86.8%)	9 (13.2%)	68
Pancreatic carcinoma	Duct adenocarcinoma	33 (71.7%)	13 (28.3%)	46
	Islet cell carcinoma	3 (50.0%)	3 (50.0%)	6
Breast carcinoma		36 (61.0%)	23 (39.0%)	59
Lung carcinoma	NSCLC	51 (91.1%)	5 (8.9%)	56
	SCLC	19 (47.5%)	21 (52.5%)	30
Gastric carcinoma		29 (96.7%)	1 (3.33%)	30
AFP-producing gastric cancer	8 (80%)	2 (20%)	10	

more frequently is unexpected and intriguing. However, there was so far no clear correlation between hDlk-1 positive tumours in patients under 50 years old and specific aetiologies such as gender, pathological grade and stage. Recently, Huang *et al.* (*48*) also reported that hDlk-1 expression in HCC showed no significant correlation with HBV infection, tumour size and serology of AFP. Thus, our finding suggests that hDlk-1+ HCC develops in a relatively short latency period and may have an origin different from other HCC with a longer latency period. Alternatively, considering the recent finding that albumin positive hepatocytes can be converted into induced pluripotent stem cells (iPS) by transient expression of c-Myc, Sox2, Oct3/4 and Klf4 (*49*), conversion of mature hepatocytes to an immature stage with hDlk-1 expression may occur during chronic liver injury. Thus, it is tempting to speculate that hDlk-1 may be a hallmark of HCC originated from hepatic or cancer stem/progenitor cells. The origin and mechanism of tumourigenesis of HCC still need extensive investigation.

While Dlk-1, also known as Pref-1, was originally described as an inhibitor of adipogenesis (*8*), the precise function still remains unknown. In this study, we showed that hDlk-1 is expressed in not only HCC but also many carcinomas such as colon, breast, pancreatic and lung carcinomas. As previously reported, colony formation, cell growth and tumourigenicity of HCC cell lines were significantly decreased when the endogenous hDlk-1 was knocked down by RNAi (*48*), and hDlk-1 promoted proliferation of glioblastoma cell line (GBM cells) (*24*) and erythroid leukemia cell line (K562 cells) (*26*). Furthermore, Dlk-1 has been reported to interact with Notch 1, and modulate

Notch signalling as a negative regulator (*50*). Notch 1 signalling prevented HCC cells to proliferate by induction of cell cycle arrest and apoptosis (*51*). Thus, hDlk-1 may contribute to tumourigenesis by enhancing tumour growth. However, precise molecular mechanism of Dlk functions is still unknown, and requires further studies. Because hDlk-1 is a cell surface molecule expressed in many HCCs and also other carcinomas, but neither in normal adult liver nor most of the tissues, it may be an attractive target for antibody therapy. In this study, we established many monoclonal antibodies against hDlk-1, and now we are developing monoclonal antibodies against hDlk-1 that block proliferation of HCC in a xenograft model.

Conflict of interest
None declared.

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Prospective randomized phase II study determines the clinical usefulness of genetic biomarkers for sensitivity to primary chemotherapy with paclitaxel in breast cancer

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In patients with breast cancer, taxane as well as anthracycline play central roles in systemic chemotherapy. By evaluating the pathological response, we can gauge sensitivity to primary chemotherapy. However, biomarkers that would predict a response to taxane have not yet been established. We conducted a prospective randomized trial to evaluate whether selecting patients using sensitivity testing based on the gene expression of the tumor might enhance the probability of the pathological response. Five genes were identified as biomarkers derived from a microarray of DNA gene profiles from microdisected breast tumors. In the experimental arm (B1), 12 cycles of weekly paclitaxel, 80 mg/m^2, were preoperatively given when the sensitivity test was positive and therefore judged to be sensitive to paclitaxel. When the test was negative, meaning insensitive to paclitaxel, four cycles of FEC100 were given (arm B2). In the control arm (A), paclitaxel was administered weekly without the use of the sensitivity test. A total of 92 patients were enrolled and 86 patients were analyzed. The pathological response rate (pRR) of each arm was 36.4% in B1 (expected sensitive to paclitaxel), 21.1% in A (control) and 12.5% in B2, respectively. Weekly paclitaxel-treated patients selected by the sensitivity test did not enhance the pRR. The study failed to validate sensitivity testing using five gene expressions for primary chemotherapy with paclitaxel in patients with breast cancer. However, this study suggests that a randomized phase II study is a robust tool for obtaining a rapid conclusion on the usefulness of biomarkers and could be the foundation for further large clinical trials. (*Cancer Sci* 2011; 102: 130–136)

Trastuzumab, a molecular targeted agent, has greatly improved the survival rate in patients with breast cancer.[1] Trastuzumab binds human epidermal growth factor receptor type 2 (HER2) and downregulates cell proliferation signaling. Trastuzumab enriches its activity by selecting patients with HER2-overexpressed breast cancer. Biomarkers can both maximize activity and minimize toxicities. Cytotoxic agents such as taxane or anthracycline also play a crucial role in systemic chemotherapy for breast cancer.[2] To date, no specific biomarker of cytotoxic chemotherapeutic agents has been established.

Primary chemotherapy with anthracycline and taxane is standard care for patients with early-stage breast cancers to obtain breast conservation and survival benefit.[3] Primary chemotherapy informs us of its sensitivity by evaluation of the pathological response. The probability of a pathological complete response (pCR) from a single administration of taxane is no more than 20%.[4] In our experience, primary treatment with paclitaxel weekly produced a 7% pCR with complete disappearance of intraductal lesions and a 30% pathological response with more than two-thirds reduction in invasive lesions.[5] Taxane induces microtubule bundling, formation of multipolar spindles, mitotic arrest and apoptosis. Resistance to taxane derives from overexpression of ATP-binding cassette (ABC) transporter, for example, P-glycoprotein,[6] somatic mutation of β-tubulin,[7] βIII-tubulin isoform[8] or low expression of tubulin-binding protein tau.[9] However, the clinical usefulness of these biomarkers has not been determined. The DNA microarray provides a unique molecular portrait or signature regarding clinical behavior and drug responsiveness.[10–14] The expression pattern of selected genes, if found to be related to the sensitivity of cytotoxic agents, could yield a biomarker to predict the clinical response and outcome. We have developed a sensitivity test using quantitative RT-PCR of five selected genes to predict the response to paclitaxel. Commonly, retrospective studies have been used to find predictive biomarkers, but their level of evidence is low. To our knowledge, there have been few randomized trials directly addressing biomarkers in a prospective fashion.

Therefore, we have conducted a prospective randomized trial on whether the selection of patients using a sensitivity test to predict paclitaxel based on the gene expression of the tumor might enhance the probability of the pathological response. The current study aimed to validate the genetic diagnosis to predict sensitivity in primary chemotherapy with paclitaxel in women with breast cancer.

Materials and Methods

Patients. Eligible patients were women with histologically confirmed invasive carcinomas of the breast with a tumor size 3 cm or more in stages IIA, IIB, IIIA or IIIB (T1-4, N0-1 and M0). All patients were younger than 70 years and had performance status (Eastern Cooperative Oncology Group performance status) 0 or 1; life expectancy 6 months or more; adequate organ function; white blood cell count 4.0×10^9/L or absolute neutrophil count 2.0×10^9/L; hemoglobin 9 g/dL; platelets 100×10^9/L; blood urea nitrogen (BUN) and serum creatinine within normal limits; aspartate transaminase (AST), alanine transaminase (ALT) twice the upper limit of normal; total bilirubin 1.5 mg/dL; and electrocardiography (ECG) within normal limits. Excluded patients were those with

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Name of the trial register: Validation of genetic diagnosis to predict sensitivity in primary systemic chemotherapy with paclitaxel in women with breast cancer. Registration number: C000000413, UMIN Clinical Trials Registry.

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Table 1. Five genes identified as biomarkers

Gene ID	Affy probe	GenBank	UniGene	Gene Symbol	Uni-title
03921	223235	NM022138	Hs.487200	SMOC2	Secreted protein, acidic, cystein-rich related modular calcium binding 2
05918	NA	BG928645	Hs.494395	C9orf121	Chromosome 9 open reading frame 121
06334	205009	NM003225	Hs.162807	TFF1	Trefoil factor 1
19403	224968	NM080667	Hs.264208	CCDC104	Coiled-coil domain containing 104
20850	229580	BX097190	Hs.7413	NA	Transcribed locus

NA, not applicable.

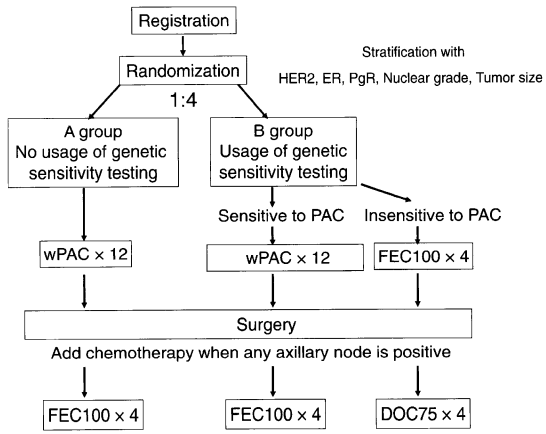

Fig. 1. Study design. Patients were stratified according to the status of human epidermal growth factor receptor type 2 (HER2), estrogen receptor (ER), progesteron receptor (PgR), nuclear grade and tumor size. Patients were randomly assigned to receive arm A or B with a ratio of 1:4. In arm A, patients received primary chemotherapy with paclitaxel without selection by genetic sensitivity testing. For patients in arm B, we used the genetic diagnosis for sensitivity to paclitaxel. In arm B1, patients diagnosed as sensitive to paclitaxel received paclitaxel. In arm B2, patients diagnosed as insensitive to paclitaxel received FEC100. When any axillary node was positive for cancer after curative breast surgery, additional chemotherapy was used. Patients pretreated with paclitaxel received FEC100. Patients pretreated with FEC100 received docetaxel. wPAC × 12, 12 cycles of weekly paclitaxel 80 mg/m^2; FEC100 × 4, four cycles of combination chemotherapy with fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2 and cycrophosphamide 500 mg/m^2 every 3 weeks; DOC75 × 4, four cycles of docetaxel 75 mg/m^2 every 3 weeks.

non-invasive or microinvasive breast cancer, stage IIIC or IV; inflammatory breast cancer; male gender; previous chemotherapy, hormone therapy or radiotherapy; active double cancer; serious complication with infection, cardiac disease, pulmonary fibrosis, interstitial pneumonitis, bleeding, hepatitis type B and its carrier; uncontrolled diabetes; heavy history of drug allergy, history of allergic reaction to drugs using the vehicle cremophor; pregnant, nursing or willing to become pregnant; or otherwise judged inadmissible by the investigators. The research ethics committee of Cancer Institute Hospital approved the study, and all patients gave written, informed consent.

Sensitivity testing. How the sensitivity testing was developed has been described in previous papers.[15,16] Basically, specimens were obtained by core needle biopsy before primary chemotherapy. To minimize the influence of stromal cells, pure populations of tumor cells were collected by laser captured microdissection. After RNA extraction, we performed gene expression profiling of 21 000 genes by DNA microarray to select the candidate genes. Surgically resected primary breast tumors were examined to determine the pathological response to chemotherapy. All clinical and genomic data were entered into an integrated database and analyzed to identify predictive factors. Differentially expressed genes were selected between the paclitaxel-resistant group and the paclitaxel-sensitive group. Then the expression of selected candidate genes was quantified by RT-PCR to confirm the array data and increase reliability. Furthermore, we narrowed the candidate genes down to establish a prediction system based on real-time RT-PCR. Finally, we identified a set of five genes predictive of patient response to paclitaxel in primary chemotherapy (Table 1). Before clinical application, the prediction system was validated retrospectively, revealing that in 51 patients the sensitivity testing using the expression of five genes produced 90% accuracy and a 9.8% error rate.

Study design. To validate the predictiveness of the pathological response by the sensitivity test in primary chemotherapy with paclitaxel, we conducted a prospective randomized trial, as shown in Fig. 1. Patients were stratified according to the status of HER2, estrogen receptor (ER), progesteron receptor (PgR), nuclear grade and tumor size. Participating patients were randomly assigned to receive arm A or B with a ratio of 1:4. For patients in arm A, we did not use the genetic diagnosis for sensitivity to paclitaxel, but they received primary chemotherapy with paclitaxel. For patients in arm B, we did use the genetic diagnosis for sensitivity to paclitaxel. When patients were diagnosed as sensitive to paclitaxel, they received primary chemotherapy with paclitaxel. Patients diagnosed as insensitive to paclitaxel received primary chemotherapy with FEC100.

Unless their disease progressed, patients were treated with 12 weeks of paclitaxel or four cycles of FEC100 and then underwent standard surgery. When any axillary node was positive for cancer, additional chemotherapy was used after surgery. Patients pretreated with paclitaxel received FEC100 and those pretreated with FEC100 (diagnosed as insensitive to paclitaxel) received docetaxel after surgery. In a partial resection of the breast, radiation was performed. If cancer was positive in four or more axillary nodes, prophylactic radiation was performed to the chest and regional nodes. Radiation was applied after completion of chemotherapy. In cases with positive estrogen receptor and/or progesterone receptor, appropriate endocrine

treatment of tamoxifen or aromatase inhibitors was used after completion of chemotherapy. Patients with HER2-overexpressed breast cancer received tri-weekly trastuzumab at a dose of 8 mg/kg followed by 6 mg/kg for 1 year after completion of surgery or postsurgical chemotherapy. Adjuvant trastuzumab was used subsequent to February 2008, which was the approval date in Japan.

Treatment. Paclitaxel was administrated at a dose of 80 mg/m^2 as an intravenous infusion over a period of 1 h every week for 12 weeks. Dexamethasone 10 mg, ranitidine 50 mg and granisetoron 3 mg were given intravenously 30 min before paclitaxel. Diphenhydramine 50 mg was given orally just before infusion. FEC100 consisting of fluorouracil (500 mg/m^2), epirubicin (100 mg/m^2) and cyclophosphamide (500 mg/m^2) was administered intravenously every 3 weeks for four cycles. Dexamethasone 20 mg and granisetoron 3 mg were given intravenously before FEC100. Docetaxel was intravenously administrated at a dose of 75 mg/m^2 for a 1 h infusion every 3 weeks for four cycles. Dexamethasone 8 mg was given as an intravenous infusion on day 1 followed by oral intake on days 2 and 3.

End-points. The primary end-point targeted improvement of the pathological response rate (pRR) as the percentage of patients with grade 2 and 3 as shown by sensitivity testing. The pathological response with grade 2 or 3 was defined as more than a two-third reduction in invasive lesions or complete disappearance of tumors, including intraductal lesions, respectively.[17] Secondary end-points examined the pathological complete response rate (probability of pathological response with grade 3), clinical response rate by the Response Evaluation Criteria in Solid Tumors guidelines (RECIST),[17] breast conservation rate, disappearance rate of axillary node metastasis, distant-metastasis-free survival, disease-free survival and overall survival. Adverse events and laboratory parameters were graded according to the National Cancer Institute, Common Toxicity Criteria, version 3.0.

Statistical analysis. Validity was defined as accuracy of the prediction system for sensitivity testing. Improvement of the pathological response was judged as high accuracy of the prediction system for sensitivity testing. The pathological response rates to paclitaxel in patients who were diagnosed as positive by sensitivity testing were compared with those in patients treated with paclitaxel who did not receive sensitivity testing. The difference in response rate in the two groups was assessed by the Fisher exact test for 2×2 contingency tables. The pathological response rate in the experimental arm was estimated as 80% compared with 30% in the control arm, which was calculated from 29% (15/51) of the pathological response rate in previous unpublished data. A sample size of 21 assessable patients in each arm (A and B1) was required to achieve 90% power with 5% error (two sided). A sample size of arm B (B1 + B2) required 72 ($21 \times 100/29$) patients. The number of cases that dropped out for any reason including inadequate sampling was estimated as 15%. A total of 109 patients were required in the current study. Patients were randomly assigned to receive arm A or B with a ratio of 1:4. An interim analysis was planned when at least 10 pathological assessable patients were obtained in arm B1. Disease-free survival and overall survival were calculated by the Kaplan–Meier method.

Results

Patient characteristics. Ninety-two patients were registered and assessed between February 2006 and February 2009 at the Cancer Institute Hospital. Six patients had too few tumor specimens to evaluate sensitivity testing. Eighty-six patients were randomized. In two patients, we were not able to assess the pathological response in the resected breast tumors, because of progression during primary chemotherapy and a withdrawal of consent to additional post-surgical chemotherapy. A total of 85 patients were assessed for pathological response at surgery. The median follow-up time of patients was 40.0 months, and the range was 17.0–49.8 months. All patients were Japanese women. The demographic characteristics of the present study population are presented in Table 2. The median age was 52.5 years (range, 31–68). Median size of tumor estimated as an invasive lesion was 3.75 cm (range, 3.0–9.9). While 81% of patients were T2, 41% of patients had no clinical axillary lymph node metastasis. Histology showed papillotubular carcinoma (8%), solid-tubular carcinoma (24%) or scirrhous carcinoma (65%). In 24% of patients, we found nuclear grade 3. Estrogen receptor or PgR was positive in 71% or 47% of patients, respectively. Positive HER2 status was defined as immunohistological (Hercep test) score 3+ (>10%) or FISH positive (ratio >2.0). Twenty-one percent of patients were HER2 positive. Intrinsic subtypes were divided as follows. Luminal A was defined as negative HER2 status with ER positive and/or PgR positive. Luminal B was defined as positive HER2 status with ER positive and/or PgR positive. HER2 subtype was positive HER2 status with both ER and PgR negative. Triple negative was HER2 negative, ER negative and PgR negative. Luminal A, Luminal B, HER2 subtype or triple negative was 64%, 8%, 13% or 15%, respectively. The background of arms A and B (B1 + B2) was mostly balanced except for a slight tendency towards more patients with papillotubular carcinoma, HER2 positive or luminal B, and fewer patients with grade 3 in arm A. The background in arms A and B1 was different because of selection by sensitivity testing.

Pathological response and clinical outcome. Interim analysis was performed after 11 patients were assessable for pathological response in arm B1. As shown in Table 3, the patients in arm B1, diagnosed as sensitive to paclitaxel, demonstrated 36.4% (4/11) of the pathological response rate, whereas patients who did not use sensitivity testing of paclitaxel showed 21.1% (4/19). The difference between arms A and B1 was not significant ($P = 0.627$). Since the pathological response rate (36.4%) of the experimental arm (B1) was far below the expected rate of 80% despite achievement with 82% (89/109) of the planned accrual number, the committee decided to terminate the study. In arm B2, the patients who were treated with FEC100 judged as insensitive to paclitaxel showed 12.5% (7/56) of the response rate. A pathological complete response was seen in 3.6% (2/56) of FEC100 (B2), but no complete response in the paclitaxel arms (A1 or B1). Pathological metastasis in resected lymph nodes at surgery was absent in 27% (3/11) of arm B1, in which the mean number of pathological positive nodes was 3.8. In one out of four pathological responders with grade 2 and 3, all axillary nodes disappeared. The clinical response rate of the paclitaxel-sensitive group (B1) was not improved at 55% (6/11) as compared with 53% (10/19) of the control arm (A) or 54% (30/56) in patients who were treated with FEC100 (B2). The breast conservation rate was not improved at 36% in arm B1, compared with arm A (32%) or arm B2 (52%). Disease-free survival and overall survival at 3 years in all patients ($n = 86$) were 81.2% and 94.6%, respectively (Fig. 2). Disease-free survival at 3 years in arms A, B1 and B2 was 72.3%, 62.3% and 87.6%, respectively. Adverse events are summarized in Table 4. One patient, who dropped out after five cycles of preoperative paclitaxel, was excluded to evaluate toxicity. A total of 85 patients were assessed for toxicity. Grade 3 or 4 of adverse events in the preoperative paclitaxel (A + B1) or FEC100 (B2) was 1.0% and 8.1%, respectively. There was no difference in the profile of adverse events between arms A and B (data not shown). No unexpected adverse events were observed.

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Table 2. Patient characteristics

Sensitivity testing	A	B1	B2	Subtotal of patients in B (B1 + B2)	All patients
	Not performed	Performed			
		Sensitive to paclitaxel	Insensitive to paclitaxel		
Treatment	Paclitaxel	Paclitaxel	FEC100		
No. randomized patients	19	11	56	67	86
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Median age	50	57.0	52.0	53.0	52.5
T					
T2	14 (74)	8 (73)	48 (86)	56 (84)	70 (81)
T3	4 (21)	3 (27)	5 (9)	8 (12)	12 (14)
T4	1 (5)	0 (0)	3 (5)	3 (4)	4 (5)
Median size (cm)	3.6	4.2	3.6	3.8	3.75
Range (cm)	3.0–5.7	3.0–5.8	3.0–9.9	3.0–9.9	3.0–9.9
N					
0	7 (37)	4 (36)	24 (43)	28 (42)	35 (41)
1	11 (58)	6 (55)	31 (55)	37 (55)	48 (56)
2	1 (5)	1 (9)	1 (2)	2 (3)	3 (3)
Stage					
IIA	7 (37)	4 (36)	25 (45)	29 (43)	36 (42)
IIB	7 (37)	4 (36)	23 (41)	27 (40)	34 (40)
IIIA	4 (21)	3 (27)	6 (11)	9 (13)	13 (15)
IIIB	1 (5)	0 (0)	2 (3)	2 (3)	3 (3)
Histology					
Invasive ductal carcinoma					
Papillotubular carcinoma	3 (16)	0 (0)	4 (7)	4 (6)	7 (8)
Solid tubular carcinoma	4 (21)	6 (55)	11 (20)	17 (25)	21 (24)
Scirrhous carcinoma	11 (58)	5 (45)	40 (71)	45 (67)	56 (65)
Others	1 (5)	0 (0)	1 (2)	1 (1)	2 (2)
Nuclear grade					
1	10 (53)	1 (9)	34 (61)	35 (52)	45 (52)
2	5 (26)	3 (27)	11 (20)	14 (21)	19 (22)
3	4 (21)	7 (64)	10 (18)	17 (25)	21 (24)
Undetermined	0 (0)	0 (0)	1 (2)	1 (1)	1 (1)
Estrogen receptor					
Positive	12 (63)	2 (18)	47 (84)	49 (73)	61 (71)
Negative	7 (37)	9 (82)	9 (16)	18 (27)	25 (29)
Progesterone receptor					
Positive	10 (53)	0 (0)	30 (54)	30 (45)	40 (47)
Negative	9 (47)	11 (100)	26 (46)	37 (55)	46 (53)
HER2					
Positive (IHC 3+ or FISH+)	6 (32)	4 (36)	8 (14)	12 (18)	18 (21)
Negative	13 (68)	7 (64)	48 (86)	55 (82)	68 (79)
Intrinsic subtype					
Luminal A	10 (53)	2 (18)	43 (77)	45 (67)	55 (64)
Luminal B	3 (16)	0 (0)	4 (7)	4 (6)	7 (8)
HER2 subtype	3 (16)	4 (36)	4 (7)	8 (12)	11 (13)
Triple negative	3 (16)	5 (45)	5 (9)	10 (15)	13 (15)

FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor type 2; IHC, immunohistochemistry.

Discussion

The current study failed to validate the sensitivity of testing using the expression of five genes. However, we became aware of the importance of deciding how to incorporate a new biomarker into clinical practice. Evidence levels of a biomarker are commonly derived from retrospective studies,[18,19] which harbor strong bias due to differing backgrounds. A large cohort or meta-analysis is mandatory to establish usefulness. Prospective trials to evaluate biomarkers have rarely been reported. Simon and simon *et al.* have proposed a refined guideline system for biomarker studies.[20,21] The guideline indicates that level 1 evidence may permit reproducible positive results from high-quality retrospective studies using archived specimens in the prospective trials addressing therapeutic questions, but not biomarkers. However, a prospective trial that would directly address biomarkers is still the gold standard to achieve level 1 evidence. Designing randomized trials for biomarkers presents several challenges.[21] One involves the therapeutic question of accommodation of biomarkers, such as the Tailor X trial of the 21-gene classifiers. The other involves the biomarker question, such as microarray testing of the 70-gene classifier. However,

Table 3. Response and clinical outcome

Sensitivity testing	A	B1	B2	Subtotal of patients in B (B1 + B2) (n = 67)	All patients (n = 86)
	Not performed	Performed			
		Sensitive to paclitaxel	Insensitive to paclitaxel		
Treatment	Paclitaxel (n = 19)	Paclitaxel (n = 11)	FEC100 (n = 56)		
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Pathological response Grade 2 + Grade 3	4 (21.1)	4 (36.4)	7 (12.5)	11 (16.4)	15 (17.4)
Pathologically free metastasis in resected lymph nodes	7 (36.8)	3 (27)	26 (46.4)	29 (43.2)	36 (41.8)
Mean no. pathological positive nodes	3.9	3.8	2.2	2.5	2.8
Pathological disappearance of axillary nodes in pathological responders (grade 2 + 3)	3 (75)	1 (25)	5 (71)	6 (55)	9 (60)
Clinical response (RECIST) CR + PR	10 (53)	6 (55)	30 (54)	36 (54)	46 (54)
Breast conservation	6 (32)	4 (36)	29 (52)	33 (49)	39 (45)

RECIST, response evaluation criteria in solid tumors.

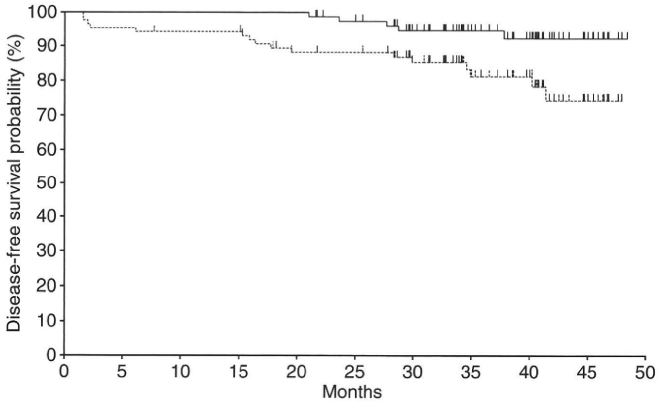

Fig. 2. Kaplan–Meier plot for disease-free survival (dashed line) and overall survival (solid line) in all randomized patients (n = 86).

these trials require a large number of patients to arrive at a definitive conclusion. It is difficult to conduct such a large trial for all possible biomarkers. A relatively smaller number of patients, approximately 100 like in this study, could be reasonable for evaluation in biomarker study design.

The current study failed to enrich responsive patients to treatment with paclitaxel. We expected that prediction of a pathological response would be more than 80% of sensitivity in the new testing. Unexpectedly, the pathological response of the experimental arm was as low as 36.4%. Since the pathological response rate, 21.1%, of the control arm was also lower than 30% as expected, performing the interim analysis in this study took a long time. We decided to terminate the study because we considered that the enrichment of response by sensitivity testing should be minimally more than 50% for clinically meaningful usage or further evaluation by a randomized large phase III study. We did not plan to address the specificity of gene testing, because the specificity could not be yielded from the data of arm B (B1 + B2). The reason was that patients in arm B2, who were judged as insensitive to paclitaxel, did not receive paclitaxel from an ethical point of view. However, we were able to examine the specificity of gene testing in arm A. In arm A (n = 19), 18 patients could be evaluated by gene testing, because of one sampling that contained no cancer cells. Twelve out of 15 patients who failed to obtain a pathological response exhibited as insensitive to paclitaxel by the gene testing. Therefore, the specificity resulted in 80% (12/15). One out of three patients who achieved a pathological response were revealed as sensitive to paclitaxel by the testing. The sensitivity of arm A resulted in 33.3% (1/3), which was similar to that of arm B1 (36.4%, 4/11). The present study aimed to examine whether the gene testing improved sensitivity, but not specificity. The number was too small to obtain a definitive result of specificity. The current study failed to show an enhanced response rate. However, if we conducted a phase II study with a single arm, we would not have been able to obtain such a clear conclusion as early as we did. Therefore, a small randomized study appears to be a robust tool in obtaining a rapid conclusion to evaluate the usefulness of biomarkers.

The methodology of this randomized trial might need further discussion. We wanted to determine whether the selection of patients by new testing could be useful. Thus, we considered that the selection by itself should be randomized. Namely, we compared the outcome for patients who were selected by testing with that of patients who were not selected. This is different from randomized trials that compare a new treatment with a standard therapy. Unbalanced randomization at a 1:4 ratio would minimize the number of patients in control arm A who were not selected by testing. Patients who wished to receive extensive, maximal primary chemotherapy did not enter this trial. One patient withdrew from this trial during her primary chemotherapy because she wanted to receive additional primary chemotherapy. Patients who wished to receive minimal chemotherapy were likely to participate in this trial. Patients with incomplete clearance of axillary tumors could receive additional chemotherapy after surgery. This ethical issue was discussed and approved by institutional review board.

In the current study, the clinical response rate and the conservation rate of the breast were 55% and 36%, respectively, with 27% of patients free from pathological metastasis in resected

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Table 4. Adverse events following preoperative chemotherapy

	Paclitaxel (n = 29)			FEC100 (n = 56)		
	Any grade	Grade 3	Grade 4	Any grade	Grade 3	Grade 4
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Anorexia	6 (21)	0	0	25 (45)	1 (2)	0
Fatigue	22 (76)	1 (3)	1 (3)	44 (79)	2 (4)	0
Nausea	7 (24)	0	0	36 (64)	0	0
Vomiting	2 (7)	0	0	23 (41)	4 (7)	0
Diarrhea	11 (38)	0	0	13 (23)	3 (5)	0
Constipation	15 (52)	0	0	31 (55)	1 (2)	0
Mucositis	0 (0)	0	0	13 (23)	1 (2)	0
Dysgeusia	2 (7)	0	0	4 (7)	0	0
Peripheral neuropathy	26 (90)	1 (3)	0	16 (29)	0	0
Alopecia	29 (100)	NA	NA	56 (100)	NA	NA
Hand-foot syndrome	0 (0)	0	0	2 (4)	0	0
Rash	3 (10)	0	0	1 (2)	0	0
Allergic reaction	1 (3)	0	0	0	0	0
Itching	1 (3)	0	0	0	0	0
Phlebitis	0 (0)	0	0	1 (2)	0	0
Myalgia	2 (7)	0	0	0	0	0
Infection	2 (7)	0	0	13 (23)	5 (9)	2 (4)
Febrile neutropenia	0 (0)	0	0	7 (13)	2 (4)	0
Leukopenia	19 (65)	1 (3)	0	53 (95)	28 (50)	15 (27)
Neutropenia	14 (48)	3 (10)	0	53 (95)	2 (4)	49 (88)
Anemia	15 (52)	0	0	37 (66)	2 (4)	0
Thrombocytopenia	0 (0)	0	0	3 (5)	1 (2)	0
AST elevation	15 (52)	0	0	23 (41)	0	0
ALT elevation	13 (45)	1 (3)	0	22 (39)	0	0
Total bilirubin elevation	3 (10)	0	0	3 (5)	0	0
Creatinine elevation	1 (3)	0	0	1 (2)	0	0
Hyperglycemia	3 (10)	0	0	14 (25)	0	0
All events	212 (27.0)	7 (0.9)	1 (0.1)	494 (32)	52 (3.6)	66 (4.5)

ALT, alanine transaminase; AST, aspartate transaminase; NA, not applicable.

lymph nodes. These results were not satisfactory. However, the study did not aim to improve breast conservation and clearance of axillary metastasis, but rather aimed to minimize exposure to cytotoxic chemotherapy for those sensitive to chemotherapy. Patients with axillary nodes involved were treated by adding adjuvant alternative chemotherapy with FEC100 or docetaxel. The results of disease-free survival and overall survival (81.2% and 94.6% at 3 years) were not assessable for further analysis. The safety profile of paclitaxel or FEC100 was similar to previous reports.[3–5] Both treatments were manageable.

In conclusion, the current study failed to validate sensitivity testing using five-gene expression for primary chemotherapy with paclitaxel in patients with breast cancer. However, a small prospective randomized study is useful for reaching a rapid conclusion on the usefulness of biomarkers. We consider that the present trial design is a prospective randomized phase II trial directly addressing the predictive biomarker question. The current compact trial could be a hallmark to proceed to further large clinical phase III trials.

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Disclosure Statement

The authors have no conflict of interest.

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Medical Oncology

Tumor Size Is a Potential Predictor of Response to Tyrosine Kinase Inhibitors in Renal Cell Cancer

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OBJECTIVES To investigate the correlations between the initial tumor size and size reduction rate in patients treated with targeted agents. To select the patients who can benefit the most from treatment with targeted agents, it will be necessary to find a tumor characteristic that predicts their effectiveness.

METHODS The data from 139 metastatic and 16 primary lesions treated with the targeted agents were retrospectively analyzed. They consisted of 86 sunitinib-treated and 69 sorafenib-treated lesions in 54 patients with metastatic renal cell carcinoma who had undergone treatment from April 2008 to July 2010. The relationship between the longest tumor diameter at baseline and the rate of reduction in tumor size was assessed using the Spearmancorrelation test.

RESULTS A linear, moderate to strong association between the initial tumor size and tumor size reduction rate was shown (correlation coefficient -0.441, $P < .001$). When these tumors were divided into 2 groups at the threshold value (23.95 mm), which was decided by the receiver operating characteristic curve analysis, the smaller tumors demonstrated a significantly greater size reduction than the larger tumors according to the Mann-Whitney U test ($P < .001$). Both univariate and multivariate linear regression analyses revealed that only the initial tumor size was associated with the rate of reduction in individual tumors ($P < .001$).

CONCLUSIONS The initial tumor size was a good predictor of the tumor size reduction. This simple observation could be useful for physicians who treat patients with metastatic renal cell carcinoma. In addition, in assessing clinical trials of targeted agents for metastatic renal cell carcinoma using the ResponseEvaluation Criteria in Solid Tumors, perhaps this association should be considered. UROLOGY xx: xxx, xxxx. © 2011 Elsevier Inc.

Surgical excision remains the standard and, indeed, the only curative therapy for patients with localized renal cell carcinoma (RCC). However, at the initial diagnosis, one third of patients with RCC will have visceral metastasis, and one half of the remainder will eventually develop distant metastases.[1] Previously, despite its limited clinical activity and significant toxicity, cytokine-based therapy was the mainstay treatment of metastatic RCC (mRCC).[1,2] A better understanding of the molecular biology of RCC has identified signaling pathways related to a hypoxia-inducible factor as rational targets, including the receptors of vascular endothelial growth factor and the mammalian target of rapamycin kinase for anticancer therapy for patients with mRCC.[3]

Because the agents aimed at these molecular targets have demonstrated significant objective responses with moderate and easily manageable toxic effects, a major breakthrough in the treatment paradigm for mRCC has occurred. Among them, sorafenib (Nexavar, Bayer Pharmaceuticals Corporation, West Haven, CT) and sunitinib (Sutent, Pfizer Inc., New York, NY) are tyrosine kinase inhibitors (TKIs), and target vascular endothelial growth factor receptors and platelet-derived growth factor receptors.[4,5]

As other new agents with alternative molecular targets emerge in RCC therapy, to select the patients who can benefit the most from these vascular endothelial growth factor receptor-targeted agents, it is necessary to find a biomarker or tumor characteristic that can predict their effectiveness. Because these agents function as angiogenesis inhibitors,[4,5] the initial tumor size and volume might be important in whether tumors can be expected to shrink using these treatments. Initially, we hypothesized

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that the initial tumor size would be inversely associated with the tumor size reduction rate. In the present study, we investigated the relationship between the initial tumor size and the tumor size reduction rate of patients treated with TKIs.

MATERIAL AND METHODS

Patient and Treatment

The data from 139 metastatic and 16 primary lesions treated with targeted therapeutics were retrospectively analyzed. They consisted of 86 sunitinib-treated and 69 sorafenib-treated lesions from 54 patients with mRCC who had undergone treatment at our hospital from April 2008 to July 2010. Each patient signed an institutional review board-approved protocol-specific informed consent form in accordance withnational and institutional guidelines. Sunitinib was administered orally at a dose of 50 mg/d, consistingof 4 weeks of treatment followed by a 2-week rest period. Sorafenib was administered orally at a continuous dose of 800 mg/d. Dose reductions of sunitinib (to 37.5 mg and then to 25 mg) and sorafenib (to 400 mg/d and then to 400 mg every other day) were performed, depending on the type and severity of the adverse events. All the target lesions were evaluated using multidetector computed tomography (CT) (Lightspeed Pro16, GE Healthcare Japan, Tokyo, Japan), which scans every 5 mm. The tumor measurements were performed by the physicians in charge of the respective patients in clinical practice and calculated separately for the response in the individual primary or metastatic sites. The response was assessed by multidetector CT at least every 2 cycles of treatment, according to the ResponseEvaluation Criteria in Solid Tumors, version 1.0 (RECIST).[6]

Statistical Analysis

To identify an optimal threshold for the prediction of >30% tumor reduction (partial response), receiver operating characteristics analysis was performed by incrementally increasing the cutoff values and recalculating the corresponding true-positive and false-negative rates. The relationship between the longest tumor diameter at baseline the tumor size reduction rate was assessed using the Spearmancorrelation test and the Mann-Whitney U test. Independent Student's t tests and analyses of variance were used in the univariate analysis for binomial variables, and correlation coefficient analyses were used for continuous variables. Multivariate linear regression analysis was used for the multivariate analysis. Statistical analyses were performed using the Statistical Package for Social Sciences, version 17.0, for Windows (SPSS, Chicago, IL). Two-tailed $P < .05$ was considered significant.

RESULTS

Patient Characteristics

The clinical and pathologic characteristics of the patients treated with TKIs are listed in Table 1. The median follow-up was 12.2 months (range 3.8-29.7). Overall, 16 patients (30%) demonstrated a partial response and 26 (48%) had stable disease according to the RECIST, indicating that 78% of the patients experienced a clinical benefit from these targeted agents. Progression was observed in 9 patients (17%) and early treatment failure before the initial assessment occurred in 3 patients (5%) owing to sorafenib-induced erythema multiforme.[7]

Table 1. Patient characteristics

Characteristic	Patients (n)
Total	54 (100)
Sex	
Male	43 (80)
Female	11 (20)
Age (y)	
Median	62
Range	25-80
ECOG performance status	
0	32 (59)
1	16 (30)
2	6 (11)
Tumor histologic type	
Clear cell	43 (80)
Clear cell plus sarcomatoid components	6 (11)
Papillary	2 (6)
Chromophobe	1 (2)
Xp translocation	1 (2)
Nephrectomy	
Yes	39 (72)
No	15 (28)
Cytokine therapy	
IL-2 and IFN	11 (20)
IFN	20 (37)
None	23 (43)
Tyrosine-kinase inhibitor	
Sunitinib	33 (61)
Sorafenib	21 (39)
Baseline serum laboratory findings	
Hemoglobin (g/dL)	
Median	11.8
Range	6.2-17.7
Corrected calcium (mg/dL)	
Median	9.3
Range	8.5-10.5
Lactate dehydrogenase (U/L)	
Median	173
Range	101-550
C-reactive protein	
Median	0.77
Range	0.03-19.4

ECOG, Eastern Cooperative Oncology Group; IL-2, interleukin-2; IFN, interferon.
Data in parentheses are percentages.

Response to Individual Targeted Lesions

We investigated the objective response of the individual primary or metastatic sites. A total of 155 tumors were examined, including 16 primary kidney lesions and 92 pulmonary, 26 lymph node, 10 liver metastatic, 6 adrenal gland, and 5 soft tissue sites. The mean ± standard deviation tumor size reduction rate was 23.8% ± 56.6%, and the tumor size was reduced by >30% in 103 tumors (66.5%) and >50% in 75 tumors (48.3%).

Correlation Between Initial Tumor Size and Tumor Size Reduction of Individual Targeted Lesions

We investigated the correlation between the initial tumor size and the tumor size reduction rate of the indi-

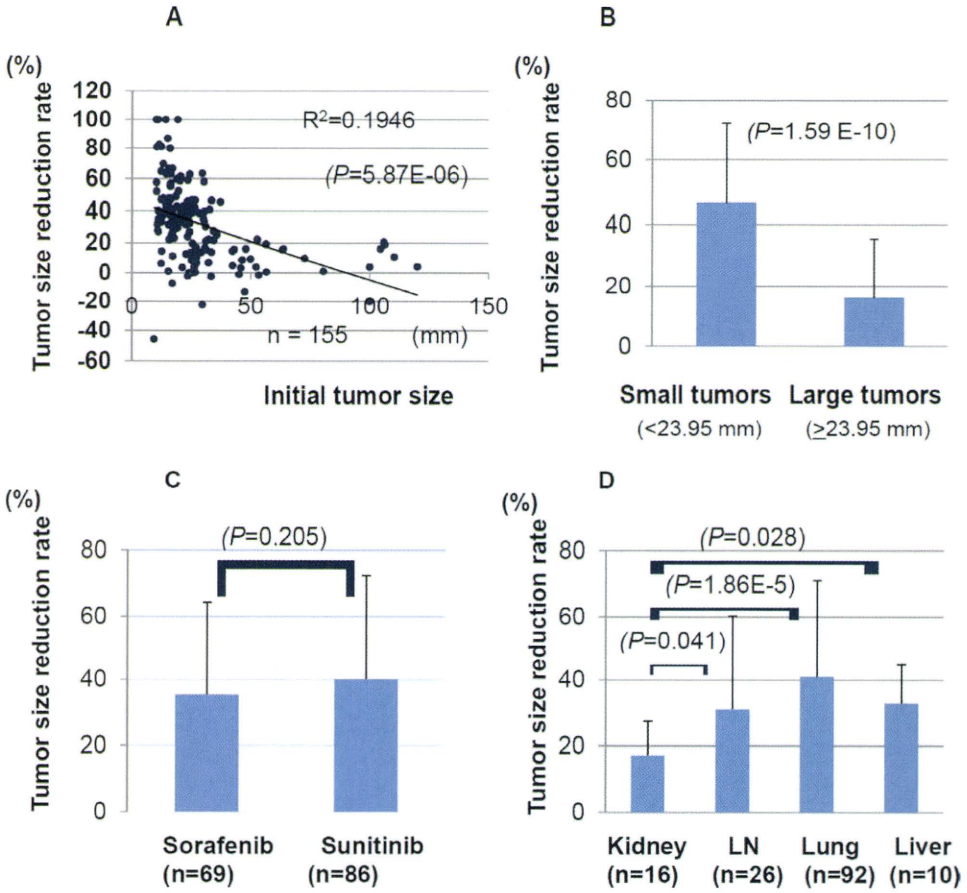

Figure 1. Association between primary tumor size and tumor size reduction. **(A)** Correlations between primary tumor size and tumor size reduction for individual tumor lesions. Smaller tumors demonstrated significant tumor size reduction compared with larger tumors. **(B)** Optimal threshold for prediction of >30% reduction (partial response) was 23.95 mm, as identified by receiver operating characteristics analysis. **(C)** No difference in tumor reduction rate demonstrated between sorafenib and sunitinib. **(D)** Primary kidney tumors demonstrated significantly small tumor size reduction compared with lymph node, metastatic lung, or liver lesions.

vidual targeted lesions. The linear association between the initial tumor size and the tumor size reduction is shown in Figure 1A. The correlation coefficient (r) was −0.441, indicating that a moderate to strong reverse association was confirmed between them ($P < .001$, Fig. 1A). Receiver operating characteristic curve analysis was performed using the clinical criteria of a partial response (>30% reduction) to separate those with and without a response. The area under the receiver operating characteristic curve was 0.814 ± 0.040, and the optimal detection threshold was 23.95 mm, with a sensitivity of 80.0% and specificity of 74.1%. When these tumors were divided into 2 groups at the threshold value (23.95 mm), the smaller tumors demonstrated a significantly greater size reduction than did the larger group ($P < .001$, Fig. 1B). In addition, among these patients, 16 had evaluable primary tumor and metastatic sites, when they were started with TKIs as induction therapy. The initial size of the 16 primary kidney tumor (77.8 ± 27.8 mm) was significantly larger than the metastatic lesions of the same patients (24.0 ± 12.7 mm, $P < .001$). Similarly, the tumor reduction rate of the primary tumor (16.1% ± 17.1%) was also significantly smaller than the metastatic lesions (43.2% ± 26.5%, $P < .001$).

Variables for Tumor Size Reduction

The relationship between the tumor reduction rate and the studied factors was investigated. The studied factors included initial tumor size, disease site, performance status, history of nephrectomy, history of cytokine therapy, TKI used (sunitinib or sorafenib), blood hemoglobin concentration, blood neutrophil count, blood thrombocyte count, serum calcium concentration, and serum lactate dehydrogenase concentration before the administration of TKIs. In the present study, no difference was found between the sorafenib-treated and sunitinib-treated lesions (Fig. 1C). In addition, the tumor reduction rate of the primary kidney was significantly smaller than that of the metastatic lymph node, pulmonary lesion, or liver lesion (Fig. 1D). However, no difference in the reduction rate was seen among the lymph node, lung metastatic, and liver metastatic lesions. On univariate analysis, the initial tumor size and the target organ were associated with the individual size reduction rate (Fig. 1A,B,D). Multivariate linear regression analysis revealed that only