Wnt Inhibitor Dickkopf-1 as a Target for Passive Cancer Immunotherapy

Nagato Sato1,2, Takumi Yamabuki1, Atsushi Takeano1,6, Junkichi Koinuma1, Masato Aragaki1, Ken Masuda1, Nobuhide Ishikawa1, Nobuoki Kohno3, Hiroyuki Ito2, Masaki Miyamoto2, Haruhiko Nakayama4, Yohei Miyagi5, Eiju Tsuchiya6, Satoshi Kondo2, Yusuke Nakamura1, and Yataro Daigo1,6

Abstract

Dickkopf-1 (DKK1) is an inhibitor of Wnt/β-catenin signaling that is overexpressed in most lung and esophageal cancers. Here, we show its utility as a serum biomarker for a wide range of human cancers, and we offer evidence favoring the potential application of anti-DKK1 antibodies for cancer treatment. Using an original ELISA system, high levels of DKK1 protein were found in serologic samples from 906 patients with cancers of the pancreas, stomach, liver, bile duct, breast, and cervix, which also showed elevated expression levels of DKK1. Additionally, anti-DKK1 antibody inhibited the invasive activity and the growth of cancer cells in vitro and suppressed the growth of engrafted tumors in vivo. Tumor tissues treated with anti-DKK1 displayed significant fibrotic changes and a decrease in viable cancer cells without apparent toxicity in mice. Our findings suggest DKK1 as a serum biomarker for screening against a variety of cancers, and anti-DKK1 antibodies as potential theranostic tools for diagnosis and treatment of cancer. Cancer Res; 70(13); 5326–36. ©2010 AACR.

Introduction

The concept of specific molecular targeting therapy has been applied to the development of innovative cancer-treatment strategies. At present, two main approaches are available in clinical practice: therapeutic monoclonal antibodies (mAb) and small-molecule agents (1). There is an increasing interest in the use of antibody-based immunotherapy for the treatment of malignant diseases, and some dramatic clinical responses have enhanced the activity in this field (1). For example, rituximab (Rituxan) is the chimeric anti-CD20 antibody that revolutionized lymphoma treatment (2). Trastuzumab (Herceptin) is the humanized Ab against the human epidermal growth factor receptor (HER)/ERBB2 for the treatment of metastatic breast cancer in which Trastuzumab (Herceptin) is the humanized Ab against the human epidermal growth factor (VEGF) used for the treatment of metastatic colorectal and lung cancers in combination with chemotherapy (4). The results were promising when compared with responses of advanced cancers to conventional cytotoxic agents, but the proportion of patients showing good response is still limited, and in some cases, the medication caused serious adverse effects including life-threatening ones. Hence, the development of new therapeutic antibodies targeting transmembrane and/or secreted proteins, which show a cancer-specific expression pattern and have an oncogenic function, is eagerly awaited.

To develop new diagnostics and therapeutics improving cancer treatment, we have been screening the candidate target molecules by the following strategy: (a) identifying genes upregulated in lung and esophageal cancers by genome-wide cDNA microarray system (5–11), (b) verifying the candidate genes for the absence of or very low level of expression in normal tissues (5), (c) validating biological significance of their overexpression by means of tissue microarray containing hundreds of archived lung cancer samples and functional assays for examining their activity in cell growth and/or invasive activity (12–33), (d) evaluating them for usefulness as a serum diagnostic/prognostic biomarker for lung cancer by ELISA (29–33), if they are tumor-specific transmembrane or secretory proteins, and (e) screening the epitopes recognized by human histocompatibility leukocyte HLA-A0201/A2402-restricted CTL, if they are categorized into cancer testis antigens and are dispensable for cancer cell growth/survival (34–36). Using this approach, we had identified Dickkopf-1 (DKK1) as a novel serologic and histochemical biomarker as well as a possible therapeutic target for lung and esophageal cancers (31). Our data indicated that DKK1 expression was associated with poor prognosis for patients with non–small cell lung...
cancer (NSCLC) or esophageal squamous cell carcinoma (ESCC), and that serum DKK1 levels were significantly higher in lung and esophageal cancer patients than in healthy controls; the proportions of the serum DKK1-positive cases was 74.1% for lung adenocarcinoma (83 of 112), 63.2% for lung squamous cell carcinoma (43 of 68), 69.4% for small cell lung cancer (39 of 85), and 63.0% for ESCC (51 of 81; ref. 31). In addition, we identified that the exogenous expression of DKK1 increased the invasive activity of mammalian cells. The evidence further prompted us to focus on DKK1 as a potential target for therapeutic antibodies as well as a serum biomarker for a wide range of human cancers.

**Materials and Methods**

**Cell lines and tissue samples**

The human cancer cell lines used in this study were as follows: four NSCLC cell lines (A549, LC319, NCI-H2170, and PC-14), 13 pancreatic cancer cell lines (Capan-1, Capan-2, HPAF-II, KLM-1, KP-1N, MiaPaCa-2, Panc02.03, Panc08.13, PK-1, PK-59, PK-9, PL45, and SUIT-2), four gastric cancer cell lines (MKN1, MKN45, MKN7, and MKN74), seven hepatocellular carcinoma (HCC) cell lines (HepG2, HUH-6, HUH-7, SNU-398, SNU-423, SNU-449, and SNU-475), four bile duct cancer cell lines (HuCCT1, TFK-1, RBE, and SSP-25), 14 breast cancer cell lines (BT-20, BT-474, BT-549, HCC1143, HCC1500, HCC1937, MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-453, MDA-MB-435S, SK-BR-3, T47D, and ZR-75-1), and two cervical cancer cell lines (C33A and HeLa; Supplementary Table S1). All cells were grown in monolayer in appropriate media supplemented with 10% FCS and were maintained at 37°C in humidified air with 5% CO₂. Surgically resected primary cancers from six pancreatic cancer patients, five gastric cancer patients, seven HCC patients, six bile duct cancers, six breast cancer patients, and five cervical cancer patients were obtained with informed consent at Kanagawa Cancer Center (Yokohama, Japan). Primary bile duct cancers were obtained with informed consent from six patients who had undergone surgery at Hokkaido University Hospital (Sapporo, Japan). Primary lung cancers in the same set of 18 lung cancer cases whose serum had been collected before surgery (nine patients with DKK1-positive tumors and nine with DKK1-negative tumors) were obtained at Hiroshima University Hospital (Hiroshima, Japan) and Kanagawa Cancer Center. This study and the use of all clinical materials used were approved by individual institutional Ethical Committees.

**Serum samples**

Serum samples were obtained with informed consent from 207 healthy control individuals (168 males and 39 females; median age of 50.3 y with a range of 31–61 y) and from 179 pancreatic cancer patients (114 males and 65 females; median age of 66 y with a range of 30–83 y), 101 gastric cancer patients (71 males and 30 females; median age of 62 y with a range of 29–82 y), 168 HCC patients (146 males and 22 females; median age of 70 y with a range of 32–84 y), 107 bile duct cancer patients (75 males and 32 females; median age of 67 y with a range of 45–75 y), 169 breast cancer patients (169 females; median age of 51 y with a range of 27–72 y), and 182 cervical cancer patients (182 females; median age of 48 y with a range of 31–77 y) who were registered in the Japanese Project for Personalized Medicine (BioBank Japan). These serum samples from a total of 906 cancer patients were selected for the study on the basis of the following criteria: (a) patients were newly diagnosed and (b) their tumors were pathologically diagnosed as cancers (stages I–IV). Serum was obtained at the time of diagnosis and stored at −150°C. The sample size of each cancer group (pancreas, stomach, liver, bile duct, breast, and cervix) and a healthy control group provided 90% statistical power (α = 0.05; β = 0.1) to detect group differences in mean levels of serum DKK1. Two-tailed P value of <0.05 was considered statistically significant.

**Semiquantitative reverse transcription-PCR**

A total of 3 μg aliquot of mRNA from each sample was reversely transcribed to single-stranded cDNAs using random primer (Roche Diagnostics) and SuperScript II (Invitrogen). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out as previously described (31), with the following sets of synthesized primers specific for DKK1 or with β-actin (ACTB)—specific primers as an internal control:

<table>
<thead>
<tr>
<th>DKK1</th>
<th>ACTB</th>
</tr>
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<tbody>
<tr>
<td>5′-TAGAGCTAGAGAAGGATCTC-3′</td>
<td>5′-CAAGGCATCATCACGTTCAAAAGGG-3′</td>
</tr>
<tr>
<td>5′-CAAGAGTCTCAGAGCTTCTCC-3′</td>
<td>5′-CTCTTTCTCTTCTTCTCTTCTCT-3′</td>
</tr>
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PCRs were optimized for the number of cycles to ensure product intensity to be within the linear phase of amplification.
Immunohistochemistry
To investigate the expression status of the DKK1 protein in clinical cancer tissues, we stained the tissue sections using a rabbit polyclonal antibody specific to human DKK1 (Santa Cruz) raised against the NH2-terminal portion (amino acids 1–120) of DKK1, as previously described (31). The specificity of anti-DKK1 antibody was confirmed by Western blot analysis using lysates of DKK1-expressing NSCLC and ESCC cells (31), and those that were transfected with small interfering RNA duplexes against DKK1 or control small interfering RNAs (Supplementary Fig. S1A and B).

ELISA
Serum levels of DKK1 were measured by an ELISA system that had been originally constructed (31). First, a rabbit polyclonal antibody specific to DKK1 (Santa Cruz) was added to a 96-well microplate (Nunc) as a capture antibody and incubated for 2 hours at room temperature. After washing away any unbound antibody, 5% bovine serum albumin was added to the wells and incubated for 16 hours at 4°C for blocking. After a wash, 3-fold diluted sera were added to the wells and incubated for 2 hours at room temperature. After washing away any unbound substances, a biotinylated polyclonal antibody specific for DKK1 using Biotin Labeling kit-NH2 (DOJINDO) was added to the wells as a detection antibody and incubated for 2 hours at room temperature. After a wash to remove any unbound antibody enzyme reagent, horseradish peroxidase-streptavidin was added to the wells and incubated for 20 minutes. After a wash, a substrate solution (R&D Systems, Inc.) was added to the wells and allowed to react for 30 minutes. The reaction was stopped by adding 100 μL of 2 N sulfuric acid. Color intensity was determined by a photometer at a wavelength of 450 nm, with a reference wavelength of 570 nm. The cutoff level in this assay was set to provide optimal diagnostic accuracy and likelihood ratios (minimal false-negative and false-positive results) for DKK1 (i.e., 14.7 U/mL), using receiver operating characteristic curves drawn with the data of 346 lung and esophageal cancer patients, and 207 healthy controls as previously described (31).

Matrigel invasion assay
NIH3T3 and PC-14 cells transfected either with p3XFLAG-tagged (COOH terminal) plasmids expressing DKK1 or with mock plasmids as well as lung cancer A549, NCI-H2170, and PC-14 cells were grown to near confluence in culture medium containing 10% FCS. The cells were harvested by trypsinization, washed in DMEM without addition of serum or proteinase inhibitor, and suspended in DMEM at concentration of 1 × 105 cells/mL. Before preparing the cell suspension, the dried layer of Matrigel matrix (Becton Dickinson Labware) was rehydrated with DMEM for 2 hours at room temperature. DMEM (0.75 mL) containing 10% FCS was added to each lower chamber in 24-well Matrigel invasion chambers, and 0.5 mL (5 × 104 cells) of cell suspension was added to each insert of the upper chamber. The plates of inserts were incubated for 22 hours at 37°C. After incubation, the chambers were processed; cells invading through the Matrigel were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

Antibody-based neutralizing assay
Cells were seeded onto 24-well plates (4.0 × 103 cells/well) for MTT assay (Cell Counting Kit-8; Dojindo) and Matrigel chambers (5 × 104 cells/well) for cell invasion assay. In the culture medium containing 0.1% fetal bovine serum, 50 or 100 nmol/L of an affinity-purified rabbit polyclonal anti-human DKK1 antibody (Santa Cruz) was added to analyze its neutralizing activity against DKK1. Normal rabbit IgG (Santa Cruz) was used as a negative control. To remove the complement, these antibodies were purified by overnight dialysis at 4°C using autoclaved PBS in Seamless Cellulose Tubing (Viskase Companies). Cell viability was determined by MTT assay after incubation for 10 days. Cell invasion activity after a 22-hour incubation was determined by Matrigel invasion assay as described (31). In this condition, the antibody was confirmed by MTT assay to have no suppressive effect on cell viability (data not shown). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (In situ Cell Death detection kit, Fluorescein, Roche Diagnostics), double staining with both Calcein-AM and propidium iodide (Cellstain-Double Staining kit, Dojindo), and immunofluorescent analysis using anti-cleaved caspase-3 (Cell Signaling Technology) were performed at 7 days after the antibody treatment according to the supplier’s protocol.

Mice model
The animal experiments were conducted according to the institutional and national guidelines for the care and use of laboratory animals, and approved by the institutional animal use committee. DKK1-positive A549 or DKK1-negative NCI-H2170 cells (6.0 × 106) were s.c. implanted into the right shoulder of 6-week-old male BALB/c nude mice (nu/nu). The six mice with tumor (50 mm2 volume on average) were randomized into two groups and i.p. administered with 100 μg/500 μL of a purified rabbit polyclonal anti-human DKK1 antibody (Santa Cruz) or 100 μg/500 μL of normal rabbit IgG (control; Santa Cruz) at days 1, 4, 8, 11, 15, 18, 22, 25, 29, and 32 (a total of 10 injections). Tumor volume was measured daily by using a caliper and applying the data to the formula [volume = 0.52 × (width)2 × (length)] to calculate the volume of a spheroid.

Results

DKK1 expression in cancers originated from various tissues
To examine a possible application of serum DKK1 levels as a diagnostic biomarker for a wide range of human cancers, we first investigated the expression level of DKK1 transcript in cancers derived from the pancreas, stomach, liver, bile duct, mammary gland, and uterus because our gene expression profile analysis implied the transactivation of DKK1 in these tumors. By means of semiquantitative RT-PCR experiments, we confirmed its elevated expression in five of six...
clinical pancreatic cancers, in three of five gastric cancers, in four of seven HCCs, in four of six bile duct cancers, in four of six breast cancers, and in three of five cervical cancers. As expected from the expression profile analysis, the DKK1 transcript was hardly detectable in their corresponding normal tissues (Fig. 1A). We also detected high levels of DKK1 expression in all of 13 pancreatic cancer cell lines, in 2 of 4 gastric cancer cell lines, in all of 7 HCC cell lines, in 2 of 4 bile duct cancer cell lines, 6 of 14 breast cancer cell lines, and 1 of 2 cervical cancer cell lines (Fig. 1B). To verify the overexpression of DKK1 in tumors, we performed semiquantitative RT-PCR and immunohistochemical analyses using another set of tumor tissue samples and anti-DKK1 antibody, and confirmed that DKK1 was highly expressed in various clinical cancer materials, but hardly detectable in their adjacent normal tissues examined (representative data of tumors were shown in Supplementary Fig. S2A and B).

Serum levels of DKK1 in cancer patients

Because DKK1 protein was secreted into the sera of patients with lung or esophageal cancer (31), we measured by ELISA the levels of serum DKK1 in serologic samples from patients with either of these six types of primary cancers that overexpressed DKK1. The mean (± SD) of serum DKK1 was 23.8 ± 24.2 U/ml in 179 pancreatic cancer patients, 17.2 ± 18.0 U/ml in 101 gastric cancer patients, 18.3 ± 16.9 U/ml in 168 HCC patients, 12.5 ± 12.2 U/ml in 107 bile duct cancer patients, 27.0 ± 22.4 U/ml in 169 breast cancer patients, and 28.7 ± 28.0 U/ml in 182 cervical cancer patients (Fig. 1C). In contrast, the mean (± SD) of serum DKK1 in 207 healthy individuals were 6.1 ± 5.0 U/ml (31). The levels of serum DKK1 protein were significantly higher in cancer patients than in healthy volunteers (P < 0.001; Mann-Whitney U test; Fig. 1C). The proportions of the serum DKK1–positive cases was 50.8% for pancreatic cancer (91 of 179), 38.6% for gastric cancer (39 of 101), 53.0% for HCC (89 of 168), 29.9% for bile duct cancer (32 of 107), 65.1% for breast cancer (110 of 169), and 59.3% for cervical cancer (108 of 182). We further compared the serum levels of DKK1 protein with its expression levels in primary tumors in 18 lung cancer cases whose serum had been collected before surgery (nine patients with DKK1–positive tumors and nine with DKK1–negative tumors). The serum levels of DKK1 protein showed good correlation with the positivity of DKK1 staining in primary tumors (Supplementary Fig. S3), further implying that DKK1 was secreted from the tumor to blood. The results indicated the high specificity and the great potential of serum DKK1 as a biomarker for the detection of a relatively large proportion of common cancers.

Inhibition of cell growth and invasive activity by anti-DKK1 antibody

We previously showed that lung and esophageal cancer patients with DKK1–positive tumors showed shorter cancerspecific survival period than those with DKK1–negative tumors, and that DKK1 has cellular invasive activity in vitro (representative data of PC-14 cells was also shown in Supplementary Fig. S4; ref. 31). To examine the therapeutic potential of anti-DKK1 antibody, we investigated whether purified anti-DKK1 antibody (50 or 100 nmol/L) could inhibit the invasion of NIH3T3 cells transfected with DKK1-expressing plasmid. Expectedly, cellular invasion caused by DKK1 overexpression was suppressed by the anti-DKK1 antibody, and the number of DKK1-NIH3T3 cells that invaded through Matrigel became almost equivalent to that of parental NIH3T3 cells (Fig. 2A).

We then investigated the effect of anti-DKK1 antibody (50 or 100 nmol/L) on the lung cancer invasive activity through Matrigel using A549 cells, which showed high levels of endogenous DKK1 expression. The cellular invasive ability of A549 detected using Matrigel assays was suppressed by the addition of anti-DKK1 antibody into their culture media, in a dose-dependent manner (P < 0.0001 for 100 nmol/L, P = 0.0003 for 50 nmol/L; each paired t test; Fig. 2B, left), whereas that of PC-14 or NCI-H2170 cells with a hardly-detectable level of DKK1 was not affected (representative data of PC-14 was shown in Fig. 2B, right).

Similarly, we investigated the effect of anti-DKK1 antibody (50 or 100 nmol/L for 10 days) on the growth of A549 cells, which showed high levels of endogenous DKK1 expression. The growth of A549 cells was suppressed by the addition of anti-DKK1 antibody into their culture media, in a dose-dependent manner (P = 0.0005 for 100 nmol/L, P = 0.0022 for 50 nmol/L; each paired t test; Fig. 3A, left), whereas that of PC-14 or NCI-H2170 cells with a hardly-detectable level of DKK1 was not affected (representative data of NCI-H2170 was shown in Fig. 3A, right). To elucidate the molecular mechanism of growth inhibition of cancer cells by anti-DKK1 antibody, we microscopically examined the morphologic change of A549 cells treated with anti-DKK1 antibody and found that the cellular morphology was altered into oval shape (Fig. 3B). Propidium iodide staining suggested that most of them were dead cells (Supplementary Fig. S5). Additional microscopic analysis using anti–cleaved caspase-3 antibody and by TUNEL assay indicated that treatment of lung cancer cells with anti-DKK1 antibody induced apoptosis probably through proteolytic activation of caspase-3 (Fig. 3C and D).

Inhibition of lung cancer cell growth by anti-DKK1 antibody in mice

We subsequently examined the effect of anti-DKK1 antibody on in vivo cancer growth using a mice model. A549 cells with a high level of DKK1 or NCI-H2170 cells with a hardly-detectable level of DKK1 were s.c. implanted into the right shoulder of 6-week-old male BALB/c nude mice (nu/nu). The six mice with tumor (50 mm² volume on average) were randomized into two groups and i.p. administered with 100 μg/500 L of a rabbit polyclonal anti-human DKK1 antibody or 100 μg/500 μL of normal rabbit IgG at days 1, 4, 8, 11, 15, 18, 22, 25, 29, and 32 (a total of 10 injections). We observed a significant A549-derived tumor growth suppression by anti-human DKK1 antibody, compared with mice treated with the same dose of control IgG (P = 0.0013; each paired t test; Fig. 4A and B). H&E staining using frozen section of the resected tumors detected a fibrotic change and...
Figure 1. DKK1 expression in primary cancers and serum levels of DKK1 in cancer patients. A, elevated expression of DKK1 transcript in clinical cancer tissues derived from six organs (pancreas, stomach, liver, bile duct, breast, and cervix). B, elevated expression of DKK1 transcript in cancer cell lines (pancreas, stomach, liver, bile duct, breast, and cervix). C, serum levels of DKK1 in patients with various types of cancer. Black lines, average serum level.
decrease of viable cancer cells in tumor tissues obtained at 35 days after the beginning of anti-DKK1 antibody treatment, and a stronger fibrotic change and more significant decrease of viable cancer cells at 60 days (Fig. 4C). On the other hand, in vivo growth of DKK1-nonexpressing NCI-H2170 cells was not significantly affected by anti-DKK1 antibody treatment (Supplementary Fig. S6A). In addition, we observed no obvious toxicity including weight loss during/after treatment or no histopathologic damage in six tissues (lung, heart, liver, kidney, bone marrow, and bone) in any of three mice administered with anti-DKK1 antibody (Supplementary Fig. S6B).

Figure 2. Inhibition of cell-invasive activity by anti-DKK1 antibody. A, Matrigel invasion assay evaluating the effect of anti-DKK1 antibody (50 or 100 nmol/L; Y-axis) on the invasion of NIH3T3 cells transiently transfected with DKK1-expressing plasmids. Cellular invasion caused by DKK1 overexpression was suppressed by addition of anti-DKK1 antibody into their culture media. Each experiment was done in triplicate. B, Matrigel invasion assay evaluating the effect of anti-DKK1 antibody (50 or 100 nmol/L; Y-axis) on the invasion of endogenous DKK1-overexpressing NSCLC cell line A549 (left) and a non-DKK1-expressing NSCLC cell line PC-14 (right). The cellular invasion of A549 cells detected using Matrigel assays was suppressed by addition of anti-DKK1 antibody into their culture media, in a dose-dependent manner, whereas that of PC-14 cells was not affected. Each experiment was done in triplicate.
Discussion

Despite the development of modern cancer therapy, cancer is still one of the major causes of death in the world. Over the last two decades, several candidate molecular targets and biomarkers for cancer therapy have been reported. However, suppression of some of the target molecules can provide survival benefits to a limited subset of the patients, and a few useful biomarkers are presently available. In order to identify molecules involved in pulmonary and esophageal carcinogenesis, and those useful as therapeutic targets and biomarkers for cancer, we have established an effective screening system and identified DKK1 as an oncoprotein whose upregulation is a frequent and important feature of the malignant nature of human cancers. In this study, we confirmed that DKK1 was highly expressed in cancers in various organs, but was hardly detectable in their adjacent normal tissues. To elucidate the possible mechanism of DKK1 overexpression in cancer cells, we cultured lung cancer A549 cells (high endogenous DKK1) and NCI-H2170 cells (hardly-detectable level of DKK1) in the presence or absence of 5-aza-deoxycytidine and examined DKK1 expression. Treatment of NCI-H2170 cells with 5-aza-deoxycytidine induced DKK1 mRNA expression in a dose-dependent manner, whereas that of A549 cells with 5-aza-deoxycytidine did not affect the level of DKK1 expression (Supplementary Fig. S7), implying that elevation of DKK1 expression in lung cancer cells is likely to be caused by some epigenetic alterations including the loss of promoter methylation of the DKK1 gene. In this study, we also found high levels of
DKK1 protein in serologic samples from 906 patients having cancers at an early or advanced disease stage in either of six organs. Although further validation using a larger set of serum samples covering various clinical stages will be required, our data presented here also indicate a potential clinical usefulness of DKK1 as a serologic biomarker for NSCLC that could be widely used in clinical practice, such as detection of cancer, prediction of the malignant potential of tumor, and monitoring the disease condition after any anticancer treatment. The evidence we showed here clearly imply that DKK1 is likely to be involved in critical steps of tumor growth/metastasis. We also found the possible inhibitory effect of anti-DKK1 antibody on cell invasion and growth. Moreover, systemic administration of anti-DKK1 antibody effectively suppressed growth of DKK1-positive tumors transplanted into nude mice without any obvious toxicity.

With the advances in understanding aberrant signaling pathways in various types of cancer, many pivotal regulators of malignant behavior of cancer cells have emerged as candidates for molecular target–based therapy. Both antibodies...
and small-molecule compounds are promising tools for the target-based cancer therapy (1). We in this study focused on the therapeutic application of anti-DKK1 antibody for cancer treatment. Recently, a few groups reported the usefulness of anti-DKK1 antibody for preventing bone absorption in some disease conditions. Another study showed that daily s.c. injection of a neutralizing anti-DKK1 antibody in the area surrounding myelomatous bone could suppress tumor-induced bone resorption and multiple myeloma growth in vivo without obvious toxicity by ameliorating bone turnover presumably through increased osteoblastogenesis and reduction of osteoclastogenesis (46, 47). The role of DKK1 in progression of bone lesions has also been studied in prostate cancer; transfection of DKK1 into the osteoblastic prostate cancer cell line C4-2B, which normally induces a mix of osteoblastic and osteolytic lesions, caused the cells to develop osteolytic tumors in severe combined immunodeficient mice (43). Current evidence indicated that anti-DKK1 antibody may be a less toxic therapeutic agent to inhibit cancer cell proliferation in certain types of human cancers overexpressing DKK1, although further preclinical studies to prove its efficacy and safety in individual cancer treatment are required to qualify anti-DKK1 antibody for the development of human clinical trials. Moreover, serum DKK1 could be a safe and less invasive biomarker for the selection of patients who should receive anti-DKK1 therapy.

Putative mechanisms of antibody-based cancer therapy can be classified into two categories (1). One is the direct action, which can be further subcategorized into three modes of action, (a) blocking function, (b) stimulating function, and (c) targeting function. In blocking function, therapeutic antibody blocks the function of target signaling molecules or receptors, by blocking ligand binding, inhibiting cell cycle progression or DNA repair, inducing the regression of angiogenesis, increasing the internalization of receptors, or reducing proteolytic cleavage of receptors (1). In stimulating function, mAb binds to membrane receptors and mimic the effect of their natural ligand, resulting in cell apoptosis (48). In targeting function, mAb conjugated with toxins, radioisotopes, cytokines, DNA molecules, or even small-molecule agents can selectively target tumor cells (1). The other mechanism of antibody therapy is the indirect action mediated by the immune system, including complement-dependent cytotoxicity and antibody-dependent cytotoxicity (1). Our data showed that the treatment of lung cancer cells with anti-DKK1 antibody alone induced apoptosis through the caspase-dependent pathway. Because DKK1 is a secretory protein, we may first speculate that anti-DKK1 antibody could bind to circulating extracellular DKK1 and neutralize its oncogenic function by inhibiting the ligand-receptor interaction, as is the case of bevacizumab (Avastin), a humanized recombinant mAb agent that targets the binding of secreted proangiogenic protein VEGF to its receptors (VEGFR-1 and VEGFR-2). Another possibility of the antitumor effect of anti-DKK1 antibody is that a ligand-antibody complex comprising DKK1 and anti-DKK1 antibody may overdrive the receptor ligand signals and change the balance of cell signals, which can induce cell death. DKK1 is known to enhance c-Jun-NH2-kinase phosphorylation that induces apoptosis in animal cap explants (49). However, our assay showed that the level of noncanonical Wnt signal such as JNK phosphorylation as well as the level and subcellular localization of the canonical Wnt signal protein β-catenin was not changed in DKK1-negative lung cancer NCI-H2170 cells after exogenous expression of DKK1 protein, or in DKK1-positive A549 cells after the treatment with anti-DKK1 antibody (Supplementary Fig. S8A–D).

To further examine the effect of exogenous DKK1 expression or anti-DKK1 antibody treatment on canonical Wnt pathway in lung cancer cells, we performed T-cell factor reporter assays using DKK1-negative lung cancer NCI-H2170 cells that were transfected with pTOP-FLASH or pFOP-FLASH vector as well as DKK1 expression plasmid, or using DKK1-positive A549 cells transfected with pTOP-FLASH or pFOP-FLASH vector, which were also treated with anti-DKK1 antibody. Although canonical Wnt signal activity detected as luciferase level was highly elevated in DKK1-negative colon cancer DLD-1 cells (control), it was significantly suppressed by exogenous DKK1 expression, which was concordant with a previous report that DKK1 is a negative regulator of Wnt signaling in colon cancer cells (Supplementary Fig. S9A and B; ref. 38). This suppressive effect of exogenous DKK1 expression on Wnt signaling was neutralized by 100 nmol/L anti-DKK1 antibody treatment (Supplementary Fig. S9C). On the other hand, canonical Wnt signal activity was not so high in DKK1-negative lung cancer NCI-H2170 cells compared with DLD-1 cells, and it was not significantly affected by exogenous DKK1 expression (Supplementary Fig. S9B). Furthermore, anti-DKK1 antibody treatment showed no detectable effect on Wnt signal activity in DKK1-positive lung cancer A549 cells (Supplementary Fig. S9D). The data implied that the induction of apoptosis of lung cancer cells by anti-DKK1 antibody is likely to be through the undefined signaling pathway. Because DKK1 receptors relevant to cancer growth and invasion still remains unclear, further investigation of this pathway is required to answer the questions about the mechanism of anti-DKK1 antibody efficacy. Interestingly, recent reports showed that DKK1 consists of conserved NH2-terminal (N1) and COOH-terminal (C1) cysteine-rich regions. Antagonism of canonical Wnt-signaling occurs through binding of C1 to LRPS/5/6 proteins on the surface of the cell and subsequent disruption of the cell surface Wnt/LRP5/6/Frizzled signaling complex (49). N1 lacks the Wnt-antagonizing activity, and the C1 and N1 domains in DKK1 are known to activate distinct signaling pathways. The early embryologic activity of DKK1 indeed requires a novel activity that resides within the N1 domain (50). In fact, our polyclonal antibody to DKK1 was immunized against an NH2-terminal portion of DKK1 protein (codon 1–120). The function of N1 may be associated with enhanced invasive activity and antiapoptotic signaling pathway that could be neutralized by anti-DKK1 antibody.

In summary, we have shown DKK1 as a biomarker for various kinds of common cancers and as a potential target for therapeutic antibodies. Anti-DKK1 antibody would be a promising tool for cancer immunotherapy.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Nagato Sato, Takumi Yamabuki, Atsushi Takano, et al.

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