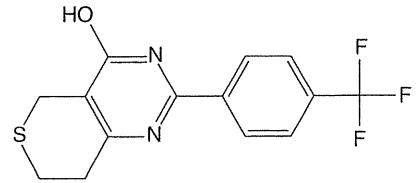
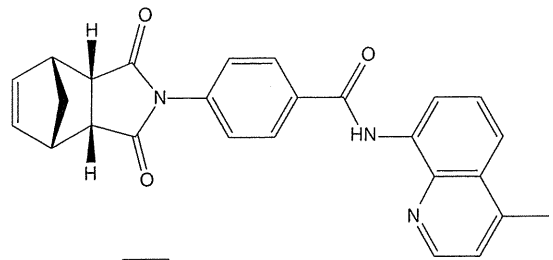
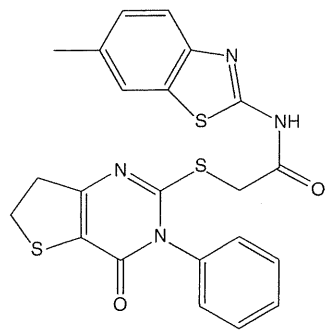
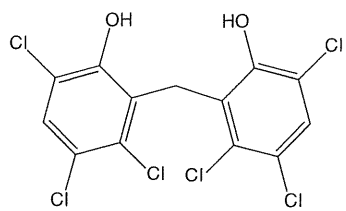
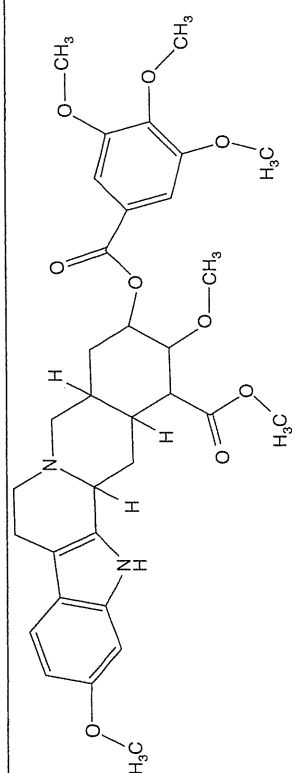
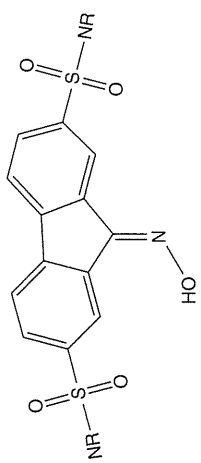
Table 2. Small-molecule inhibitors targeting the Wnt/β-catenin signaling pathway (continued).

Name	Structure	Action	Ref.
XAV939		Upregulation of axin protein levels by tankyrase inhibition	[80]
IWR-2		Stabilization of axin	[81]
IWP-2		Prevention of Wnt ligand secretion by Porcn inhibition	[81]
Hexachlorophene		Upregulation of ubiquitin ligase Siah-1	[82]

CBP: cAMP response element binding protein (CREB)-binding protein; Siah: Seven in absentia homolog; TCF: T-cell factor.

Table 2. Small-molecule inhibitors targeting the Wnt/β-catenin signaling pathway (continued).

Name	Structure	Action	Ref.
Isoreserpine		Upregulation of ubiquitin ligase Siah-1	[83]
LC-363		Degradation of β-catenin by ubiquitin-dependent proteolysis	[84,85]

CBP: cAMP response element binding protein (CREB)-binding protein; Siah: Seven in absentia homolog; TCF: T-cell factor.

The TCF/LEF family (TCF-1, TCF-3, TCF-4 and LEF-1) transcription factors are characterized by a DNA-binding high mobility group (HMG) box. In the absence of β-catenin, TCFs function as target gene transcriptional repressors by occupying the promoter. Stabilized β-catenin forms a complex with TCFs and converts them into transcriptional activators. The targeting of gene transcription by β-catenin/TCF requires co-activators such as CBP/p300 and brahma homolog 1 (Brg1), which interact with the C-terminus of β-catenin, and Bcl9/Pygopus, which interacts with its N-terminus. Lepourcelet *et al.* [72]described the interaction between β-catenin and TCF factors. Disruption of β-catenin/TCF complex formation blocks the expression of the Wnt/β-catenin signaling target genes. The N-terminal peptide of TCF binds to the groove formed by the armadillo repeats of β-catenin [73,74]. A binding assay to identify β-catenin/TCF complex inhibitors by high-throughput screening (HTS) was developed by attaching purified β-catenin, including the TCF binding site, onto a plate. Glutathione-S-transferase (GST)-fused TCF, anti-GST antibody, and an alkaline phosphatase (AP)-conjugated secondary antibody were then added to the plate, and disruption of the β-catenin/TCF complex was measured by the reduction in AP signals. After approximately 7000 purified natural compounds were screened, 6 compounds were identified as inhibitors. Biochemical assays and the Xenopus axis duplication assay were performed to validate the function of the inhibitors. Two fungal derivatives, namely PKF115-584 and CGP049090, were found to be effective antagonists of the β-catenin/TCF complex [72].

Sukhdeo *et al.* investigated the effects of PKF115-584 on human MM cells [75]. PKF115-584 blocks β-catenin/TCF transcriptional activity and induces cytotoxicity in MM cell lines and primary cells from MM patients *in vitro*. PKF115-584 treatment inhibits the growth of human MM cells *in vivo* and prolongs the survival of MM-cell-bearing mice. Similarly, Wei *et al.* evaluated the activity of the three inhibitors (PKF118-310, PKF115-584- and CGP049090) on human hepatocellular carcinoma (HCC) cells [76]. These inhibitors displayed dose-dependent cytotoxicity *in vitro* and suppressed tumor growth in xenograft models. A recent study showing that PKF115-584 and CGP049090 have growth inhibitory effects on human chronic lymphocytic leukemia cells was presented at the American Society of Hematology (ASH) meeting in 2009 [77].

4.6.2 *Cell-based reporter assay screening*

Wnt/β-catenin signaling activity can be assessed using the TOPFLASH reporter which contains TCF/LEF binding sites upstream of the luciferase open reading frame [78]. The reporter cells that stably harbor TOPFLASH show luciferase activity as β-catenin/TCF transcriptional activity. This assay has been used to screen small-molecule libraries for inhibitors of the Wnt/β-catenin signaling pathway (**Figure 2**).

Emami *et al.* identified a small molecule ICG-001, that showed activity in down-regulating the expression of β-catenin/TCF target genes [79]. These authors screened a small molecule library of 5000 compounds using a cell-based

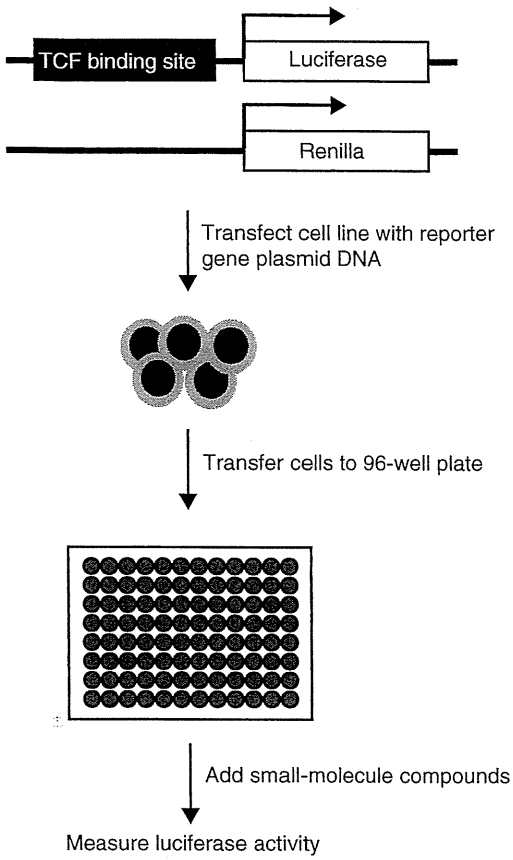


Figure 2. Cell-based reporter assay screening. A dual-luciferase system is used in this assay. Firefly luciferase is expressed in response to β-catenin/T cell factor (TCF) transcriptional activity. Renilla luciferase is expressed constantly.

reporter assay system. CBP is a transcriptional coactivator that binds to the C-terminal region of β-catenin and regulates its stability through acetylation. Emami *et al.* demonstrated that ICG-001 binds the CBP protein and competes for binding to β-catenin. The anti-apoptotic gene survivin and cyclin D1 were also downregulated by ICG-001 in colon cancer cell lines. ICG-001 increased caspase activity in colon cancer cell lines but not in normal colonic epithelial cells. In an *in vivo* study, ICG-001 reduced the number of intestinal polyps in Min micee, which carry a nonsense mutation in one APC allele. Moreover, ICG-001 reduced the tumor volume in a xenograft model of the colon cancer cell line SW620.

XAV939 is a synthetic compound that was first identified as a Wnt/β-catenin pathway inhibitor in a HTS using HEK293 cells stably harboring the Wnt reporter Super(8×) TOPFLASH [80]. XAV939 inhibits tankyrase1 and tankyrase2, and as a result, stabilizes Axin and subsequently degrades β-catenin. Tankyrases promote the ubiquitination of Axin, possibly through poly-ADP-ribosylation (PARsylation). XAV939 inhibits PARsylation by binding tightly to the poly-(ADP-ribose) polymerase (PARP) domain of tankyrases. XAV939 is a potent inhibitor of Wnt/β-catenin signaling through the inhibition of tankyrases.

Chen *et al.* identified potent inhibitors of Wnt/β-catenin signaling by multiple stage screening [81]. A 200,000-compound library was screened using mouse L cells stably harboring the Wnt reporter Super-(8×)TOPFLASH and a Wnt3A expression vector. After confirmation of the inhibitory activity, the compounds were categorized into two groups according to their site of action within the Wnt/β-catenin signaling pathway: inhibitors of Wnt production (IWPs) and inhibitors of Wnt response (IWRs). IWPs inhibit the secretion of the Wnt ligand through binding to porcupine (Porcn). Porcn is a member of the membrane-bound O-acyltransferase (MBOAT) family and it plays an important role in Wnt ligand secretion by adding a palmitoyl group to Wnt ligand proteins in the endoplasmic reticulum. Biochemical analysis revealed that IWRs bind directly to Axin and stabilize it, which leads to β-catenin degradation.

Park *et al.* identified hexachlorophene as a compound that decreased the level of intercellular β-catenin using cell-based reporter assay screening [82]. Hexachlorophene is able to degrade β-catenin by a mechanism independent of β-TrCP-mediated ubiquitination. Two types of ubiquitin ligases target β-catenin for destruction: the ubiquitin ligase β-TrCP recognizes GSK-3β-phosphorylated β-catenin, and the ubiquitin ligase seven in absentia homolog (Siah) targets β-catenin for degradation independently of its phosphorylation. Siah-1 expression is induced by p53, and Siah-2 is implicated in the regulation of the response to hypoxia. Siah forms a complex with Siah-interacting protein (SIP), S-phase kinase-associated protein 1 (Skp1) and Ebi, and interacts with the β-catenin/APC complex to induce β-catenin degradation. Hexachlorophene treatment caused an increase in the levels of Siah-1 mRNA. This conclusion indicated that the effect of hexachlorophene on β-catenin occurred through the Siah/APC pathway. Similar to hexachlorophene, isoreserpine, which was identified using the cell-based TOPFLASH reporter assay, was found to up-regulate Siah-1 and promote β-catenin degradation [83].

4.6.3 *Biomarker-based screening*

Biomarker-based screening is a new screening method based on transcriptional profiling. Transcriptional activity can correlate with the specific state of a disease. The application of transcriptional profiling to entire genomes is difficult due to cost and time requirements. However transcriptional profiling can be used in HTS when the cellular state can be monitored through the expression of selected genes. Transcriptional profiling techniques have progressed in terms of scale, cost, and ease of use. Biomarker-based screening focuses on specific transcriptional activities to identify the compounds of interest. In addition, transcriptional profiling enables the comparison of results and offers good reproducibility.

Avalon Pharmaceuticals (MD, USA) assessed the transcriptional response of a colon cancer cell line to treatment with β-catenin siRNA using full-genome microarray analysis [84]. In this study, nine biomarkers were selected for their potential as indicators for cancer therapy. A library of 90,000 individual compounds was screened to identify compounds that showed a similar expression pattern to the siRNA, and the compound LC-363 was detected based on its ability to mimic the effect of β-catenin knockdown (**Figure 3**). Interestingly, indomethacin and calphostin C, which are different type of Wnt/β-catenin pathway inhibitors, showed a similar expression pattern than that of the siRNA. The effect of LC-363 in promoting the degradation of β-catenin and inhibiting β-catenin/TCF transcriptional activity was validated in MM cells [85]. LC-363 decreased the expression of c-myc, cyclin D1 and survivin, which resulted in the inhibition of MM cell proliferation through the apoptotic pathway. LC-363 treatment prolonged the survival of MM-bearing mice. A clinical study with this compound series in solid and hematopoietic malignancies will be carried out in the future. These results demonstrated the effectiveness of biomarker-based screening in the identification of compounds of interest.

5. Conclusion

The effect of the aberrant activation of Wnt/β-catenin signaling in promoting tumor progression has been studied extensively. Because the inhibition of Wnt/β-catenin signaling activity is an effective approach to the treatment of human cancers, therapeutic agents that target for the protein components of the Wnt/β-catenin signaling pathway are being developed.

The identification of Wnt/β-catenin signaling inhibitors can be approached by different types of screening methods, including reporter assay screening for β-catenin/TCF-mediated transcriptional activity and binding assays for β-catenin-coactivators. Inhibitors that were identified using these methods were shown to have growth inhibitory effects on tumor cells *in vitro*. These inhibitors that target the Wnt/β-catenin signaling pathway need to be evaluated for their effects on other signaling pathways and on normal stem cells. Because the relationship between the components of the Wnt signaling pathway and those of other signaling pathways are not clear, inhibition of Wnt signaling might affect other pathways and therefore result in unexpected adverse effects.

The Wnt/β-catenin signaling pathway is important for not only for the developmental process but also for the maintenance of stem cells [86,87]. The potential effect of Wnt/β-catenin signaling inhibitors on normal somatic stem cell homeostasis and tissue maintenance therefore needs to be carefully evaluated.

Biomarker-based screening uses several parameters derived from full-genome microarray analysis to identify compounds of interest. LC-363 was identified by a screening study that used a biomarker driven approach, in which multiple parameters were designed from β-catenin siRNA [84]. LC-363 was identified and validated as a potential Wnt/β-catenin signaling pathway inhibitor. This result clearly shows that biomarker-based screening is a very effective method for the identification of compounds of interest.

6. Expert opinion

CSCs show different characteristics than bulk cancer cells. CSCs are resistant to conventional drugs and are associated with recurrent cancer and MRD [36,88]. The elimination of CSCs is therefore important for the successful treatment of cancer. Activation of Wnt/β-catenin signaling is found in several CSCs [38-42], which suggests that the Wnt/β-catenin signaling pathway is a potential therapeutic target in CSCs. However, the maintenance and proliferation of normal stem cells [86,89], including hematopoietic stem cells, are dependent of Wnt/β-catenin signaling. In addition, Wnt/β-catenin signaling is involved in bone formation through the regulation of osteoblast differentiation [90,91]. The possibility of adverse effects caused by the inhibitors of this pathway in normal stem cells and normal tissues is an issue of concern. Still, targeting Wnt/β-catenin signaling is an attractive possibility for cancer therapy because Wnt/β-catenin signaling is not only associated with CSCs, but also with EMT-mediated metastasis, and is therefore of prognostic value in cancer. Recent reports indicate that the accumulation of nuclear β-catenin is associated with EMT, which results in cell migration and metastasis. The inhibition of Wnt/β-catenin signaling is therefore a potential strategy for the prevention of cell migration and metastasis through its effect on EMT. The prognostic relevance of β-catenin in some cancers has been reported, and elevated β-catenin levels indicate a possible adverse prognosis for cancer patients [92-94]. As discussed above, CSCs play a significant role in the development and recurrence of several cancers, and Wnt/β-catenin signaling is important for the proliferation of CSCs. Inhibition of Wnt/β-catenin signaling is therefore a promising approach to the treatment of cancers.

The dosing and duration of the application of Wnt/β-catenin signaling inhibitors needs to be carefully determined to prevent adverse effects. In addition, the dependence of tumor growth on Wnt/β-catenin signaling varies according to the type of cancer; therefore, a Wnt/β-catenin-signaling-pathway inhibitor will be more effective in the treatment of cancers that are heavily dependent on the Wnt/β-catenin signaling pathway. In the treatment of cancers in which growth is associated with other signaling pathways, combination therapy consisting of Wnt/β-catenin-signaling-pathway inhibitors and other agents could improve the treatment efficacy. Wnt/β-catenin-signaling-pathway inhibitors possess anti-cancer properties and represent a promising approach for the treatment of cancer patients.

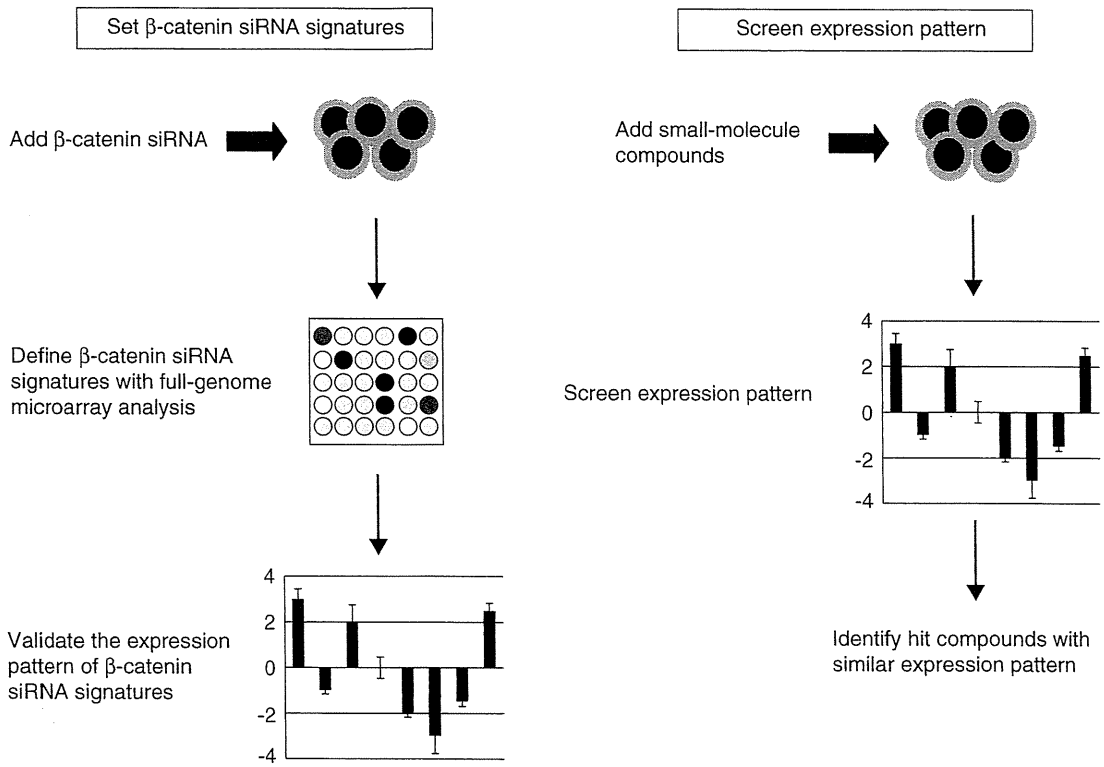


Figure 3. Biomarker-based screening. This assay proceeds in two steps: the first step consists of setting up the β-catenin siRNA signatures. The second step involves screening for compounds with similar expression patterns.

Target specificity is regarded as an important feature of a good inhibitor. An inhibitor that non-specifically suppresses other signaling activity is considered inadequate, even if it can suppress Wnt/β-catenin signaling activity. Generally, target specificity of hit compounds is confirmed by microarray analysis after the primary screening. If the primary screening considers the specificity and *in vivo* effects, it is possible to identify hit compounds at a more advanced stage for clinical application. Biomarker-based screening is therefore the preferred method of drug discovery. In the future, agents identified by these new screening methods will be ready for testing in clinical applications.

Declaration of interest

The authors declare no conflict of interest. This work was partly supported by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (EA and TM).

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REVIEW Open Access

RNA interference against polo-like kinase-1 in advanced non-small cell lung cancers

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Abstract

Worldwide, approximately one and a half million new cases of lung cancer are diagnosed each year, and about 85% of lung cancer are non-small cell lung cancer (NSCLC). As the molecular pathogenesis underlying NSCLC is understood, new molecular targeting agents can be developed. However, current therapies are not sufficient to cure or manage the patients with distant metastasis, and novel strategies are necessary to be developed to cure the patients with advanced NSCLC.

RNA interference (RNAi) is a phenomenon of sequence-specific gene silencing in mammalian cells and its discovery has lead to its wide application as a powerful tool in post-genomic research. Recently, short interfering RNA (siRNA), which induces RNAi, has been experimentally introduced as a cancer therapy and is expected to be developed as a nucleic acid-based medicine. Recently, several clinical trials of RNAi therapies against cancers are ongoing. In this article, we discuss the most recent findings concerning the administration of siRNA against polo-like kinase-1 (PLK-1) to liver metastatic NSCLC. PLK-1 regulates the mitotic process in mammalian cells. These promising results demonstrate that PLK-1 is a suitable target for advanced NSCLC therapy.

Introduction

Worldwide, approximately one and a half million new cases of lung cancer are diagnosed each year [1]. About 85% of lung cancer are non-small cell lung cancer (NSCLC), including adenocarcinoma, squamous cell, and large cell carcinoma [2], and NSCLC is the leading cause of cancer-related deaths. Surgery is generally regarded as the best strategy for lung cancers. However, only 30% of patients are suitable for receiving potentially curative resection [3], and it is necessary for other patients to be treated with chemotherapy. As we gain a better understanding of the molecular pathogenesis underlying NSCLC, new molecular targeting agents can be developed. Tyrosine kinase inhibitors (TKIs) targeting the epidermal growth factor receptor (EGFR), such as gefitinib and erlotinib, have shown remarkable activity in the patients with NSCLC, and particularly these TKIs are more effective to NSCLC with *EGFR* mutations in 19 exon (in-frame deletions) and exon 21 (L858R point mutation), which are found to be more prevalent in Asian patients [4,5]. However, despite the development of new TKIs, new mutations in *EGFR* exon 20, developing resistance to *EGFR* TKIs, have emerged in the treated NSCLC [6,7], and current therapies are not sufficient to cure or manage the patients with distant metastasis [8,9]. Therefore, novel strategies are necessary to be developed so that the patients with NSCLC can be cured.

RNA interference (RNAi) is a process of sequence specific post-transcriptional gene silencing induced by double-strand RNA (dsRNA) and this phenomenon was discovered in *Caenorhabditis elegans* (*C. elegans*) [10]. RNAi has been shown to function in higher organisms including mammals, and methods that exploit RNAi mechanisms have been developing. RNAi has now been well-established as a method for experimental analyses of gene function *in vitro* as well as in high-throughput screening, and recently, RNAi has been experimentally introduced into cancer therapy. To apply the RNAi phenomenon to therapeutics, it is important to select suitable targets for the inhibition of cancer progression and also to develop effective drug delivery systems (DDSs). Recently a lot of useful non-viral DDSs for small interfering RNAs (siRNAs) have been developed [11-17]. Besides selecting suitable targets, an important consideration for siRNA-mediated treatment is to predict and

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avoid off-target effects, which are the silencing of an unintended target gene, and potential immunostimulatory responses. To avoid those effects, the most specific and effective siRNA sequence must be validated. Modification of two nucleosides of the sense strand also completely co-inhibited the immunological activities of the antisense strand, while the silencing activity of the siRNA was maintained [18].

Polo-like kinase-1 (PLK-1) belongs to the family of serine/threonine kinases and regulates cell division in the mitotic phase [19,20]. PLK-1 is overexpressed in many types of malignancies and its overexpression is associated with poor prognosis of cancer patients [21,22]. In this review, we discuss possible RNAi strategies against PLK-1 in advanced lung cancers.

Mechanisms of RNAi

The precise mechanisms of RNAi are discussed in several reviews [23-25]. In the initiation phase of RNAi processes, following introduction of dsRNA into a target cell, dsRNA is processed into shorter lengths of 21-23 nucleotides (nts) dsRNAs, termed siRNAs, by the ribonuclease activity of a dsDNA-specific RNAse III family ribonuclase Dicer. Dicer consists of an N-terminal helicase domain, an RNA-binding Piwi/Argonaute/Zwille (PAZ) domain, two tandem RNAse III domains, and a dsRNA-binding domain [26,27]. Mammals and nematodes have only a single Dicer, which acts to produce both siRNAs and miRNAs [28-30], while other organisms have multiple Dicers which perform separate, specialized functions. *Drosophila* has two Dicers: *Drosophila* Dicer-1 is required for generating miRNAs, whereas *Drosophila* Dicer-2 produces siRNAs [25,31]. dsRNA precursors are sequentially processed by the two RNAse III domains of Dicer, and cleaved into smaller dsRNAs with 3' dinucleotide overhangs [26,32].

In the second effector phase, smaller dsRNAs enter into an RNA-induced silencing complex (RISC) assembly pathway [33]. RISC contains Argonaute (Ago) proteins, a family of proteins characterized by the presence of a PAZ domain and a PIWI domain [34]. The PAZ domain recognizes the 3' terminus of RNA, and the PIWI domain adopts an RNAse H-like structure that can catalyze the cleavage of the guide strand. Most species have multiple Ago proteins, but only Ago2 can cleave its RNA target in humans. The dsRNA is unwound by ATP-dependent RNA helicase activity to form two single-strands of RNA. The strand that directs silencing is called the guide strand, and the other is called the passenger strand. Ago2 protein selects the guide strand and cleaves its RNA target at the phosphodiester bond positioned between nucleotides 10 and 11 [32,35]. The resulting products are rapidly degraded because of the unprotected ends, and the passenger strand is also degraded [36,37]. The targeted RNA dissociates from the siRNA after the cleavage, and the RISC cleaves additional targets, resulting in decrease of expression of the target gene (Figure 1) [38].

Polo-like kinase-1

To develop RNAi therapy against cancers, it is essential that suitable gene targets are selected. Such targets include antiapoptotic proteins, cell cycle regulators, transcription factors, signal transduction proteins, and factors associated with malignant biological behaviors of cancer cells. All of these genes are associated with the poor prognosis of cancer patients. PLKs belong to the family of serine/threonine kinases and are highly conserved among eukaryotes. PLK family has identified PLK-1, PLK-2 (SNK), PLK-3 (FNK), and PLK-4 (SAK) in mammalians so far and PLKs function as regulators of both cell cycle progression and cellular response to DNA damage [19,39-41]. PLK-1 has an N-terminal serine/threonine protein kinase domain and two polo box domains at the C-terminal region. Polo box domains regulate the kinase activity of PLK-1 [21,42]. PLK-1 regulates cell division at several points in the mitotic phase: mitotic entry through CDK1 activation, bipolar spindle formation, chromosome alignment, segregation of chromosomes, and cytokinesis [19,43]. *PLK-1* gene expression is regulated during cell cycle progression, with a peak level occurring at M phase. Similar to its gene expression, PLK-1 protein expression and its activity are low in G0, G1, and S phases, and begin to increase in G2 phase with peak in M phase [44-47].

Whereas PLK-1 is scarcely detectable in most adult tissues [45,48,49], PLK-1 is overexpressed in cancerous tissues. Its expression levels were tightly correlated with

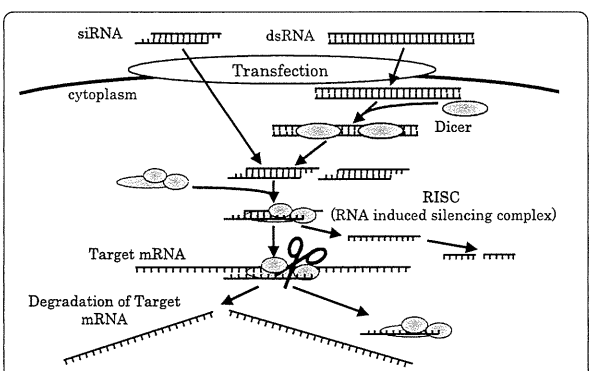

Figure 1 Mechanisms of RNA interference. After the introduction of dsRNA into a target cell, the dsRNA is processed into siRNA length of 21-23 nucletides by Dicer. siRNA then enters an RNA-induced silencing complex (RISC) assembly pathway. The dsRNA unwinds to form two single-strands of RNA. The passenger strand rapidly degrades and the guide strand binds and cleaves the target mRNA, resulting in mRNA degradation.

histological grades of tumors, clinical stages, and prognosis of the patients. PLK-1 mRNA levels were elevated in NSCLC tissues and this transcript levels were correlated with the survivals of cancer patients [50]. Moreover, the immunohistoligical study showed that PLK-1 protein was overexpressed in NSCLC tissues in patients at progressed stages of cancer (postsurgical stage ≥II) and in patients with poorly differentiated NSCLCs [51]. Patients with urinary bladder cancers expressing high levels of PLK-1 have a poor prognosis compared with patients with its low expression. Moreover, the histologically high-grade, deeply invasive, lymphatic-invasive, and venous-invasive bladder cancers demonstrated significantly higher PLK-1 expression [52]. As PLK-1 is overexpressed in other various cancers [21], PLK-1 overexpression is a prognostic biomarker for cancer patients.

Inhibition of PLK-1 activity induces mitotic arrest and tumor cell apoptosis [53-55]. Depletion of *PLK-1* mRNA also inhibits the functions of PLK-1 protein in DNA damages and spindle formation and causes the inhibition of the cell proliferation in a time- and a dose-dependent manner. PLK-1 siRNA treatment induces an arrest at the G2/M phase in the cell cycle with the increase of CDC2/Cyclin B1 [51,52,56,57]. PLK-1 siRNA-transfected cells had dumbbell-like and misaligned nuclei, indicating that PLK-1 depletion induced abnormalities of cell division during M phase, and these cells were shown to yield to caspase-dependent apoptosis [51,52,56]. As mentioned above, the kinases of PLK family cooperatively act in mitosis. Quantitative real-time RT-PCR data showed that PLK-2 and PLK-3 transcripts were increased after PLK-1 siRNA treatment [51]. Unlike PLK-1, PLK-2 and PLK-3 play inhibitory roles. PLK-2 is regulated by p53 and PLK-3 is activated by the DNA damage checkpoint [40]. These observations suggest that PLK-1 depletion induced mitotic catastrophe and activation of spindle checkpoint and DNA damage checkpoint, resulting in increased transcription of PLK-2 and PLK-3. Consequently, these PLK family kinases cooperatively prevented G2/M transition and induction of apoptosis. Importantly, depletion of PLK-1 does not affect the proliferation of normal cells although PLK-1 plays an important role in cell division [51,53,58]. This suggests that some other kinases compensate loss of PLK-1 function during mitosis in normal cells [51,58]. Collectively, PLK-1 could be an excellent target for cancer therapy.

Atelocollagen

Although siRNA target molecules are overexpressed in cancer cells, most of them are essential to maintain homeostasis of physiological functions in humans. Therefore, siRNAs must be delivered selectively into cancer cells. Moreover, naked siRNAs are degraded by endogenous nucleases when administered *in vivo*, so that delivery methods that protect siRNAs from such degradation are essential. For these reasons, safer and more effective DDSs must be developed. DDSs are divided into two categories: viral vector based carriers, and non-viral based carriers. Viral vectors are highly efficient delivery systems and they are the most powerful tools for transfection so far. However, viral vectors have several critical problems in *in vivo* application. Especially, retroviral and lentiviral vectors have major concerns of insertional mutagenesis [59,60]. Consequently, non-viral DDSs have been strenuously developed [11-13].

Atelocollagen, one of powerful non-viral DDSs, is type I collagen obtained from calf dermis [61]. The molecular weight of atelocollagen is approximately 300,000 and the length is 300 nm. It forms a helix of 3 polypeptide chains. Amino acid sequences at the N- and C-termini of the collagen molecules are called telopeptide, and they have antigenecity of collagen molecules. As the telopeptide is removed from collagen molecules by pepsin treatment, atelocollagen shows low immunogenicity. Therefore, atelocollagen has been shown to be a suitable biomaterial with an excellent safety profile and it is used clinically for a wide range of purposes. Atelocollagen is positively charged, which enable binding to negatively charged nucleic acid molecules, and bind to cell membranes. Moreover, at low temperature atelocollagen exists in liquid form, which facilitates easy mixing with nucleic acid solutions. The size of the atelocollagen-nucleic acid complex can be varied by altering the ratio of siRNA to atelocollagen. Because atelocollagen naturally forms a fiber-like structure under physiological conditions, particles formed a high concentration of atelocollagen persist for an extended period of time at the site of introduction, which is advantageous to achieve a sustained release of the associated nucleic acid. Atelocollagen is eliminated through a process of degradation and absorption similar to the metabolism of endogenous collagen [61]. Alternatively, particles formed under conditions of low atelocollagen concentrations result in siRNA/atelocollagen complexes approximately 100-300 nm in size that are suitable for systemic delivery by intravenous administration. Atelocollagen complexes protect siRNA from degradation by nucleases and are transduced efficiently into cells, resulting in long-term gene silencing. For instance, Takeshita et al. demonstrated that the systemic siRNA delivery with atelocollagen existed intact for at least 3 days in tumor tissues using a mouse model [62].

Preclinical application of RNAi therapy against PLK-1 in a murine advanced lung cancer model

Here we introduce an application of PLK-1 siRNA against an advanced lung cancer. As described above,

PLK-1 is overexpressed in NSCLC tumors. Liver metastasis is one of the most important prognostic factors in lung cancer patients [8,9,63,64]. However, despite the development of new chemotherapeutic and molecular targeting agents, current therapies are not sufficient to inhibit liver metastasis. We investigated the effects of PLK-1 siRNA on the liver metastasis of lung cancers using atelocollagen as a DDS. We first established a mouse model of liver metastasis. Spleens were exposed to allow direct intrasplenic injections of Luciferase (Luc)-labeled A549 NSCLC cells. Ten minutes after injections of tumor cells, the spleens were removed. After Luc-labeled A549 cell engraftment was confirmed by using *In Vivo* Imaging System (IVIS) of bioluminescence imaging [65], PLK-1 siRNA/atelocollagen complex, nonsense siRNA/atelocollagen complex, or PBS/atelocollagen complex was administered by intravenous injection for 10 consecutive days following day 1 of transplantation. On day 35, mice treated with nonsense siRNA/atelocollagen complex or PBS/atelocollagen complex showed extensive metastases in the liver when compared to mice treated with PLK-1 siRNA/atelocollagen complex (Figure 2). Moreover, on day 70 after the inoculation of tumor cells, livers of mice treated with nonsense siRNA/atelocollagen or PBS/atelocollagen complex had numerous large tumor nodules, whereas the livers of mice treated with PLK-1 siRNA/atelocollagen complex showed a much lower number of smaller nodules. These findings indicate that PLK-1 siRNA/atelocollagen complex is an attractive therapeutic tool for further development as a treatment against liver metastasis of lung cancer [51]. Consequently, our preclinical applications suggest that PLK-1 siRNA is a promising tool for cancer therapy.

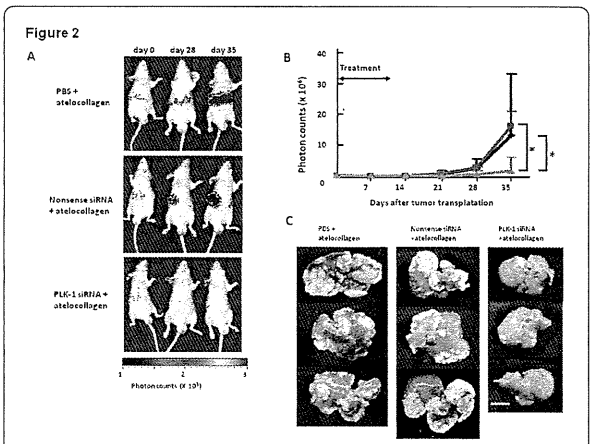

Figure 2 Application of PLK-1 RNAi therapy against liver metastatic NSCLC (cited from [51]). A. PBS/atelocollagen complex, nonsense siRNA/atelocollagen complex, or PLK-1 siRNA/atelocollagen complex was administered by intravenous injection. Representative mice showing bioluminescence after siRNA treatment. The photon counts of each mouse are indicated by the pseudocolor scales. B. Growth curves of inoculated Luc-labeled A549 cells measured by the IVIS (pink square, nonsense siRNA/atelocollagen complex (25 μg siRNA)-treated mice; blue diamond, PBS/atelocollagen complex-treated mice; orange triangle, PLK-1 siRNA/atelocollagen complex (25 μg siRNA)-treated mice; n = 5 for each group. On day 35 after inoculation, the luminesecence in the PLK-1 siRNA/atelocollagen-treated mice was significantly suppressed compared with that in other groups. * $p < 0.05$. Mean ± SD. C. Macroscopic analysis of mice livers after day 70 of inoculation. White nodules are metastatic liver tumors. Treatment with PLK-1 siRNA (25 μg) remarkably inhibited the growth of liver metastases compared with PBS or nonsense siRNA treatments (25 μg).

Conclusion

Our preclinical studies demonstrated that RNAi therapy against PLK-1 using atelocollagen is effective against liver metastatic NSCLC cancers. Recently, several clinical trials for cancer therapy are ongoing (Additional file 1: Table S1, http://clinicaltrials.gov/ct2/home). Although RNAi shows excellent specificity in gene-silencing, several adverse effects including activation of immune reaction [66,67] and off-target effects (induction of unintended gene silencing) [68] are brought in *in vivo* application. Safer and more efficient DDSs for systemic delivery are warranted to be developed. Moreover, studies to establish the pharmacokinetics and pharmacodynamics of siRNAs on the administration are necessary steps in the potential approval of siRNA as a tool for cancer therapy. To maximize efficacy and to minimize adverse effects of RNAi, it should be determined whether siRNAs are best administered alone or in combination with chemotherapeutic agents [69,70], and whether it is better to administer a single specific siRNA or multiple specific siRNAs [57,71-73]. In conclusion, RNAi therapy represents a powerful strategy against advanced lung cancers and may offer a novel and attractive therapeutic option. The success of RNAi depends on the suitable selection of target genes and the development of DDSs. We anticipate that the continued development of effective DDSs and the accumulation of evidence further proving the success of siRNA treatment will advance RNAi as a promising strategy for lung cancer therapy.

Additional material

Additional file 1: Table S1 Clinical trials of RNAi.

Lists of abbreviations

Ago: Argonaute; DDSs: drug delivery systems; dsRNA: double-strand RNA; EGFR: epidermal growth factor receptor; IVIS: *In Vivo* Imaging System; Luc: Luciferase; NSCLC: non-small cell lung cancer; nt: nucleotide; PAZ: Piwi/Argonaute/Zwille; PLK-1: Polo-like kinase-1; RISC: RNA-induced silencing complex; RNAi: RNA interference; siRNA: small interfering RNA; TKI: Tyrosine kinase inhibitor

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Authors' contributions
EK carried out our all experiments concerning this review and drafted the manuscript. EA designed our all experiments, carried out *in vivo* experiments, and wrote this review. TM supervised our research and wrote this review. All authors read and approved the final draft.

Competing interests
The authors declare that they have no competing interests.

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CLINICAL DILEMMAS

ABO-incompatible living-donor lobar lung transplantation

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KEYWORDS:
lung transplantation;
ABO-incompatible;
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bone marrow transplantation

ABO-incompatible living-donor lobar lung transplantation was performed in a 10-year-old boy with bronchiolitis obliterans (BO) after bone marrow transplantation (BMT) for recurrent acute myeloid leukemia (AML). His blood type had changed from AB to O since he underwent BMT and he had no anti-A/B antibody, and received type B and AB donor lobar lungs. To our knowledge, this case represents the first successful living-donor lobar lung transplantation from ABO-incompatible donors.
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ABO-incompatible organ transplantation, especially kidney and liver transplantation, have been performed in an effort to overcome the donor organ shortage. However, very few cases have been reported involving ABO-incompatible lung transplantation, and there has been only one intentional lung transplant reported so far. Herein we report ABO-incompatible lung transplantation in a 10-year-old boy with bronchiolitis obliterans (BO) after bone marrow transplantation (BMT).

Case report

A 6-year-old boy was diagnosed with acute myeloid leukemia (AML) in 2005 and was treated with chemotherapy. In May 2008, at 9 years of age, he underwent BMT from an unrelated, HLA-identical, ABO-mismatched donor for recurrent AML. His blood type was originally AB(+) and, after receiving BMT from a blood type O(+) donor, his blood type changed to O(+). In early 2009, at 9 of age, he began complaining of dyspnea and was diagnosed with BO, with the presumption that the cause was pulmonary graft-vs-host disease (GVHD). Respiratory distress continued to worsen with respiratory *Pseudomonous aeruginosa* infection despite home oxygen therapy.

In January 2010, at 10 years of age, the patient was transferred to Kyoto University Hospital. On admission, his vital capacity was 0.72 liter (39.6% predicted), forced expiratory volume in 1 second (FEV_1) was 0.27 liter (16.3% predicted), and arterial blood gas showed pH 7.40, PaO_2 = 87.0 mm Hg and $PaCO_2$ = 55.8 mm Hg, with 2 liters/min oxygen administered via a nasal cannula.

Cadaveric lung transplantation was not a realistic option because brain death is accepted only for persons >15 years of age in Japan. His parents, a mother, 43 years old, ABO type AB(+), and father, 44 years old, ABO type B(+), each offered to be lung donors. The patient's ABO type had changed to type O according to ABO testing of red cells, but ABO serum test did not detect any anti-A/B antibody in his serum and tolerance to A and B antigens had been established. After careful discussion, we concluded that the risk of an ABO-incompatible lung transplant in this particular case would be equivalent to that of an ABO-compatible transplant, because the production of anti-A and anti-B antibody would be unlikely even if new A and B antigen was presented from the donor after lung transplantation.

In February 2010, the patient underwent living-donor lobar lung transplantation with a left lower lobe from his mother and a right lower lobe from his father. The surgical aspects of the donor lobectomy, donor back-table preservation technique and recipient bilateral pneumonectomy and lobar implantation have been described previously by Starnes and colleagues.[1] For peri-operative transfusion, type O red blood cells and type AB fresh-frozen plasma and platelets were used for the recip-

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ients. Post-operative immunosuppression included cyclosporine, mycophenolate mofetil and prednisone.

Post-operative course was relatively uneventful. The patient was completely weaned from the ventilator on post-operative day (POD) 3. There was transient, very weak detection of anti-A antibody (Table 1). However, there was no apparent acute cellular rejection (ACR) or antibody-mediated rejection (AMR) post-operatively. Because there was no clinical finding suggesting rejection, no lung biopsy was performed post-operatively.

He was discharged from the hospital on POD 75. At that time, arterial blood gas in room air showed pH 7.43, PaO_2 = 92.6 mm Hg and $PaCO_2$ = 37.6 mm Hg. FVC was 1.53 liters (83.2% predicted) and FEV_1 was 1.12 liters (67.9% predicted) (Table 2). Five months post-operatively, he returned to a normal life without oxygen inhalation and is able to perform daily activities.

Discussion

After bone marrow transplantation, if the patient has received marrow from a compatible but dissimilar ABO type, serum antibodies will not agree with red cell antigens. Our pediatric patient, who was originally type AB, received type O marrow, had circulating type O red cells, but produced no anti-A/B antibody in the serum at the time of lung transplantation. According to ABO testing of red cells, recipient (type O) donors (B and AB) ABO-type matching was incompatible; however, because the recipient had no anti-A/B antibody in serum, we could perform this surgical procedure with ABO-incompatible donors. Other possible hematologic changes that could occur in the recipient after lung transplantation were carefully discussed. Theoretically, the lymphocytes derived from the type B lung donor could produce anti-A antibodies in the recipient, and not only attack the recipient's other organs, which were originally type AB, but also attack contralateral type AB donor lung. However, there was only transient weak detection of anti-A antibodies and no AMR occurred post-operatively.

Recently, many cases of ABO-incompatible organ transplantation, especially kidney and liver transplantation, have been performed to overcome the donor organ shortage. Japanese groups reported excellent patient and graft survival in ABO-incompatible kidney transplantation using regimens consisting of plasmapheresis, immunosuppression, immunoabsorption and splenectomy, showing similar outcomes to those of ABO-compatible donor transplants.[2,3] However, intentional ABO-incompatible lung transplantation was reported in only one case.[4] Pierson et al reported 42 instances (0.4%) of accidental ABO-incompatible lung transplantation among 9,804 primary lung transplants, according to the database of the Organ Procurement and Transplant Network in the USA,[5] and the outcomes were acceptable compared with those of ABO-compatible lung transplants when the intensive therapy was used as just described.

Although the present case showed a unique blood type background because of prior bone marrow transplantation, to our knowledge, this case represents the first successful living-donor lobar lung transplantation from ABO-incompatible donors. Although the short-term outcome was satisfactory, long-term follow-up is needed to determine whether this procedure is ultimately justified.

Table 1 Serologic Analysis of Anti-A and -B Antibody for Recipient

		Days post-transplant					
	Pre	7	12	19	26	54	82
Aggregation to type A RBC	0	W+[a]	0	W+	W+	0	0
Aggregation to type B RBC	0	0	0	0	0	0	0

RBC, red blood cells.
[a]Very weak aggregation.

Table 2 Time Trend of Pulmonary Function Test for Recipient

	Days post-transplant		
	Pre	82	188
Height (cm)	127.0	127.4	128.0
Body weight (kg)	24.0	25.0	28.0
VC (liters)	0.72	1.62	1.61
FVC (liters)	0.72	1.53	1.60
FEV_1 (liters)	0.27	1.12	1.09

Disclosure statement

The authors have no conflicts of interest to disclose.

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Research Article

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Single-step, label-free quantification of antibody in human serum for clinical applications based on localized surface plasmon resonance

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Abstract

The amount of antibody in blood is an important measure of health status for making critical decisions in clinical practice. Here, we demonstrated a single-step, label-free, molecular diagnositic method based on localized surface plasmon resonance (LSPR) using standard 96-well microtiter plates. We improved the LSPR biosensor so that it can measure antibodies to blood group antigens in human serum with a single-step operation. First, we employed the ampholytic polymeric surface modification technique to present an efficient molecular scaffold on the sensor surface. Second, we selected the combination of an appropriate reference molecule against the antigen and a blocking agent to significantly reduce the variability of signal due to nonspecific responses of the unknown in the sample. Finally, we overcame the analytical difficulty arising from serum and achieved a single-step "wash-free" measurement of the amount of target antibody in human serum.

From the Clinical Editor: In this paper, a novel, single-step, label-free, molecular diagnositic method is discussed for antibody detection based on localized surface plasmon resonance using standard 96-well microtiter plates.

Key words: Optical biosensor; Localized surface plasmon resonance; Immunoassay; Blood test

In clinical tests, antibody titers in blood are very important measures for diagnosis. Antibodies related to infectious diseases such as chickenpox or hepatitis are among the conventional targets of those tests. In autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus, autoantibodies play an important role in diagnosis as well. Other examples like blood group ABO-related immunoglobulin levels after living-donor organ transplantations are critically essential for treatments and prognosis of transplantees because antibody-mediated rejection is one of the primary causes of poor outcomes.[1] Several methods are used to measure antibody levels in serum. They include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence-activated flow cytometry (FACS). Moreover, among the various new types of chemical and biological sensors, the optical method using surface plasmon resonance (SPR) on planar gold films has made a particularly important contribution to affinity-based biosensing applications, including antigen-antibody reactions, during the past two decades. As mentioned in the reviews,[2-4] the SPR sensor has been widely used to monitor the change in the refractive index induced by a broad range of analyte bindings in a label-free manner. This direct detection method of biomolecules eliminates conventional signal transducers such as fluorophore and enzyme.

In our previous work,[5,6] we showed that the SPR method could be used to measure anti-human blood group A (anti-A) IgG titers in sera rapidly and quantitatively while avoiding the interference from IgM-type antibodies. This could be a promising tool because one of the clinical interests is the amount of IgG-type antibodies. Measurement of anti-A IgG titers in sera has been routinely conducted by the conventional test tube (TT) method. This method, however, has the intrinsic problem of interexaminer variability because it relies on visual observation.[7] Our study was the initial trial for clinical use of the SPR method.

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It improved the diagnostic operation by intermittently monitoring the level of the same patients' antibodies after clinical ABO-unmatched transplant.

However, because the SPR method is based on flow-cell type sensor chips, the resultant sequential operation is very complex and a more efficient procedure is desired in clinical practice. This instrumental characteristic possibly limits the reduction of the total assay time and the achievement of a high-throughput assay system. In addition, the SPR method detects unknown species, not only in the vicinity of the sensing planar gold surface, but at a relatively long distance from its surface. This can cause unpredictable and unnecessary responses of signals that affect the accuracy of the measurement and mandate the temperature control of the whole system to minimize the fluctuation of the refractive index. Therefore, signal compensations such as a reference flow cell or subsequent washing are necessary. Moreover, it requires us to regenerate a sensor chip surface before another test is to be done. It is quite a complex process to prepare the new sensor chip surface with the same sensor characteristics as previous ones. From the analytical viewpoint, these properties are not favorable to control the quality of each measurement in the long run.

In this article, inspired by the similarity to the SPR method and its less practical applicability, we further explore the possibility of the application of the localized surface plasmon resonance (LSPR) method[8] to this kind of measurement. As one of the promising technologies to acquire the information of molecular binding events, this method can be made with very simple set-up and assay procedures in comparison with those of SPR. Through the advance of nanofabrication techniques and nanophotonics, this particular label-free immunoassay has been widely investigated for many biological applications.[9-14] Previously, we have demonstrated its simple fabrication in a 96-well microtiter plate format and its applicability to a label-free immunoassay with the expectation that this method can be used in clinical tests.[15] We have found the density of immobilized gold nanoparticles (AuNPs) best to detect antibody in sample solution.

When metal nanoparticles are excited by light, their conduction electrons on the surface, known as surface plasmons, exhibit collective oscillations. These oscillations result in both absorption and scattering of incident light of a specific resonant wavelength. This phenomenon is called LSPR. The characteristic resonance energy of the surface plasmon is strongly dependent on the dielectric properties of the local environment around the nanometric particles.[16,17] Thus, small changes in the refractive index induced by surrounding liquid or analyte binding at or near the surface of nanometric particles can be measured as shifts in the LSPR spectra. We believe that the LSPR method has the benefits of both SPR and ELISA. These include a label-free simple assay like SPR and a high-throughput parallel operation like ELISA.

To our knowledge, our investigation is the first clinical application of the LSPR method with human blood samples. We measured anti-A IgG levels in human serum based on LSPR to compare with the previously established methods by SPR. Our study focused on simplicity, reproducibility and accuracy with respect to the clinical diagnosis. One of the difficulties in the label-free methodology is reducing the nonspecific responses from serum to increase the intensity of specific response. For this purpose, we employed novel zwitterionic copolymer to serve as a molecular scaffold on the surface of AuNPs. It can adsorb strongly onto the gold surface with static ionic interaction between its amino groups and gold and present efficient grafting points for further chemical modification on the surface of NPs, such as immobilization of antigen groups or blocking agents through its carboxyl groups, which would eventually lead to the improvement of signal intensity. Due to the improved signal intensity, the final measurements can be done even without removing and washing the serum before the measurements. We call this the "wash-free" measurement that simplifies the process as a single-step assay of a specific analyte in serum. Putting the test solution into a reaction well could be the only required operation to achieve accurate diagnostic results. Finally, for validation of our method, comparison of the results with several other diagnostic methods was also performed.

Methods

Functionalization of the surface of AuNPs

Ninety six-well polystyrene amine surface microtiter plates (Corning, Corning, New York) were used as the substrates on which the AuNPs (100 nmϕ; BBInternational, Cardiff, United Kingdom) were immobilized at the optimized density.[15] The surface of AuNPs were functionalized with polyampholite polymer PAS-410 (Nitto Boseki, Fukushima, Japan) and human blood group A trisaccharide antigen molecules (Carbohydrate Synthesis, Oxford, United Kingdom). Details in preventing nonspecific adsorption are described in the Supplementary Material, available online at http://www.nanomedjournal.com.

LSPR measurement of the levels of target antibody (anti-A)

Serum samples were taken from 41 healthy adult volunteers at Kyoto University Hospital, including 21 men and 20 women with informed consents according to the Declaration of Helsinki. Crude human serum was diluted by one third, using a diluent containing 0.26% PAS-410 and 0.01% BSA in phosphate buffered saline (PBS) (pH 7.0) to prepare the test solution. Without dilution, some samples were within the range of saturation of signal. Therefore, we decided to dilute all crude samples in this work. The diluent consisted of components similar to those on the surface of AuNPs, and we aimed to avoid unnecessary interaction between the sample and sensor surface. A 100 μL aliquot of the test solution was then incubated for 30 minutes at room temperature (24–25°C). Next, we measured its optical extinction spectrum in the "wash-free" condition. Subsequently, each well was washed and filled with 100 μL PBS. The optical extinction spectrum was measured again in the "wash" condition. The specific peak shift induced by the antibodies bound to immobilized antigens was obtained by subtracting the peak shift of the reference-well measured at the same point in time. With the purified anti-A IgG diluted in PBS, we created a plot of concentration-dependent shift of spectrum peak (λ_{max}) to estimate concentrations of the antibodies in the test solution. In addition, a

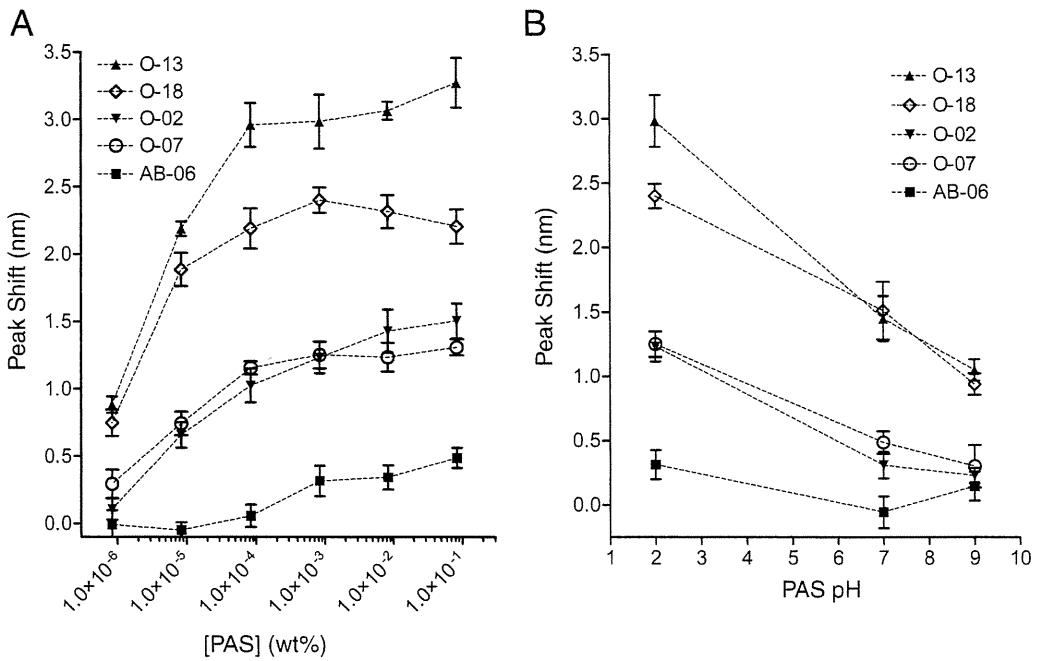

Figure 1. Effect of coating polymer concentrations and pH levels on sensor responses (spectrum peak (λ_{max}) shift). **(A)** Effect of concentration. **(B)** Effect of pH level. Data was acquired using the sera from 4 blood group O and 1 blood group AB volunteers. Error bars represent standard errors.

secondary analysis was performed to quantify bound IgG-type antibodies by adding a 100 μL aliquot of 100 μg/mL goat anti-human IgG monoclonal antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania) and incubating for 30 minutes at 37°C. The final spectra measurement was performed after being completely dried with compressed dried air. A plate reader (Varioskan; Thermo Fisher Scientific, Waltham, Massachusetts) was used to measure the optical extinction spectra of immobilized AuNPs on the plates. The spectra data were analyzed by fitting them to a Gaussian curve to identify a spectrum peak (IGOR Pro; WaveMetrics, Portland, Oregon).

Determination of antibody levels by the TT and SPR method and their correlation with those measured by the LSPR method

We employed the same procedure described elsewhere for the TT and SPR measurements.[5,6] For the SPR method, we used Biacore X system (GE Healthcare, Amersham, United Kingdom). The blood group A trisaccharide antigen molecules were immobilized on the sensor chip CM5 following the standard procedures. An anti-human IgG monoclonal antibody (Sanbio, Uden, The Netherlands) diluted with the running buffer HBS-EP (GE Healthcare) was used to measure the amounts of anti-A IgG associated with the blood group antigen A immobilized on the chip. All measurements were performed at 25°C. The deviations among different sensor chips were adjusted using the responses from the identical serum sample. We performed a statistical evaluation of correlation of the results from 3 methods. We computed linear correlations of both the TT and SPR methods with the LSPR method (SAS 9.1 software; SAS Institute, Cary, North Carolina).

Results

Sensor functionalization and assay optimization

Our sensor elements consisted of monodisperse AuNPs, which were immobilized on the surface of polystyrene microtiter plates as reported in our previous work.[15] Microtiter plates are very useful for multiplexing the assay on the same plate. Fundamentally, our LSPR method is characterized by determining shifts of λ_{max}, which are the measure of changes in the refractive index at or near the proximity of the surface of AuNPs. These shifts are induced by specific analyte binding to target elements on the surface of AuNPs and are to be converted into the actual amount of analyte molecules bound on the surface using the standard response curve. Spectrum measurement was done by standard plate reader. The surface of AuNPs was first coated with the polyampholytic polymer anchored on their surface by its amino groups. The polymer is a novel water-soluble zwitterionic copolymer composed of diallylamine hydrochloride salt and maleic acid. This polymer provides us with efficient grafting points for the target element of human blood group A trisaccharide antigen through its carboxyl groups. The characteristics of the polyampholytic polymer are strongly affected by pH levels, which in turn change the sensor properties. Thus, at first we evaluated the sensing properties in terms of signal intensity with various pH levels and concentrations of the polymer solution to achieve good selectivity (Figure 1). We used 5 representative human sera for this optimization, including 4 blood group O and 1 blood group AB. Although the variation of the concentration of anti-A in serum is relatively large even in healthy volunteers, it may due to the difference in individual exposure to the antigen. The serum of blood group AB works as a negative control