J. Biochem. 2010;147(1):135–141 doi:10.1093/jb/mvp151

JB THE JOURNAL OF BIOCHEMISTRY

Generation of a recombinant single-chain variable fragment (scFv) targeting 5-methyl-2′-deoxycytidine

Received July 11, 2009; accepted September 8, 2009; published online October 8, 2009

Motohiro Ohshima, Tomomi Tadakuma, Hideki Hayashi, Kazuyuki Inoue and Kunihiko Itoh*

Department of Clinical Pharmacology and Genetics, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

*Kunihiko Itoh, Department of Clinical Pharmacology and Genetics, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan. Tel/Fax: +81-54-264-5673, E-mail: itohk@u-shizuoka-ken.ac.jp

We generated a single-chain variable fragment (scFv) against 5-methyl-2′-deoxycytidine (m^5dCyd) using phage display technology. The heavy and light chain variable region genes were amplified by the polymerase chain reaction (PCR) from hybridoma cell line FMC9 and assembled as an scFv fragment with a flexible linker $(Gly_4\text{-}Ser)_3$. The scFv DNA fragment was then cloned into pCANTAB-5E, and a phage displaying the scFv was produced. Antigen-positive phage clones were successfully selected by enzyme-linked immunosorbent assay (ELISA). The scFv was modified with FLAG and His tags for detection and purification. The scFv reacted strongly with m^5dCyd and weakly with 5-methylcytidine (m^5Cyd) but not with cytidine (Cyd) and 1-methyladenosine in a manner similar to the monoclonal antibody (MoAb). Although the specificities of scFv and MoAb were almost identical, the sensitivity of the scFv (IC_{50} 0.054 μg/ml) was ~80 times higher than that of the parent MoAb (IC_{50} 4.27 μg/ml), determined by inhibition ELISA. As a biochemical application of this scFv, we quantified the m^5dCyd content of genomic DNA by enzymatic hydrolysis using inhibition ELISA. The cancer cell lines HeLa, HeLa S3 and MDA-MB-453 contained ~1% of the methylated DNA in total genomic DNA, as did peripheral blood cell genomic DNA from healthy volunteers, but HT29 and T-47D showed hypomethylation compared with the HeLa, HeLa S3 and MDA-MB-453 cell lines. The scFv generated here may be applicable to the assessment of cellular DNA methylation levels and is more sensitive than the MoAb.

Keywords: DNA methylation/inhibition ELISA/monoclonal antibody/phage display/scFv.

Abbreviations: BSA, bovine serum albumin; CDR, complementarity-determining region; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; IC_{50}, 50% inhibitory concentration; LC–MS, liquid chromatography–mass spectrometry; PBS, phosphate-buffered saline; RNA, ribonucleic acid.

In the analysis covering genome, the expression of many genes is accompanied by DNA methylation in a directed or non-directed manner (*1*). DNA methylation is affected by DNA methyltransferase, which transfers a methyl group of *S*-adenosyl-L-methionine to the fifth carbon position of the cytosine; this is an important pathway in the repression of gene transcription (*2*, *3*). DNA methylation plays a critical role in important human diseases (*4–6*). Decreased genomic DNA methylation is a particularly important characteristic of cancer (*7*). Methyl deficiency of genomic DNA causes chromosome instability and tumour formation (*8*). Therefore, the quantification of global methylation could provide useful information for the detection and analysis of diseases (*9–11*).

In the quantification of DNA methylation, 5-methyl-2′-deoxycytidine (m^5dCyd) was measured by high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) using genomic DNA extracted from cells (*12*, *13*). The detection limit using HPLC is linear in the range from 0.1 to 1.6 μg/ml, while the detection limit using LC–MS is linear between 2 and 100 ng/ml. Instrumental methods can unequivocally identify analytic samples but are too expensive and requires a considerable skill for routine work. The immunoassay has several advantages, such as simplicity and cost-effectiveness. Mizugaki *et al.* (*14*) reported an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (MoAb) for the detection of DNA methylation; the antibody reacted with m^5dCyd to a greater extent than with 5-methylcytidine (m^5Cyd). The MoAb is available for selection, but its preparation is complicated and time-consuming, requiring expensive cell culturing facilities.

Recently, recombinant antibody technologies have provided a method to engineer low-cost antibodies with conserved specificity; of particular interest, single-chain variable fragment (scFv) technology was developed by McCafferty and co-workers (*15*,*16*). This approach relies on a phage display system in which antibody fragments, expressed as fusion proteins, are displayed on the phage surface (*17*). Using the polymerase chain reaction (PCR), immunoglobulin variable region genes were first amplified from a hybridoma or spleen (*18*). The variable heavy (V_H) and variable light (V_L) genes were fused with a flexible linker $(Gly_4\text{-}Ser)_3$ and then cloned into pCANTAB-5E. They were expressed as a fusion protein with a phage coat protein. The scFv-displaying phage was selected by the antigen and expressed the soluble scFv antibody. Though high selectivity was maintained, the scFv had high clearance (*19*) and membrane permeability (*20*) compared with whole antibody.

Consequently, the scFv can be used for therapy and imaging (*21*).

In this study, we describe the cloning of anti-m^5dCyd antibody genes from hybridoma cells and the construction of the scFv. Similar to the MoAb, the scFv we obtained reacted strongly with m^5dCyd and weakly with m^5Cyd, but the scFv was more sensitive than the parent MoAb. We were able to use the scFv instead of MoAb for the immunochemical assessment of DNA methylation levels.

Materials and methods

Cell culture

FMC9 (IgG_{2a},λ)-producing hybridoma cells and the cell lines MDA-MB453, HT29, T-47D, HeLa S3 and HeLa were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, 100 μg/ml streptomycin and 2 mM glutamine. The cell lines were maintained in 95% air and 5% CO_2 at 37°C.

Cloning and sequencing of a gene encoding a MoAb against 5-methylcytidine

Total RNA from FMC9 hybridoma cells was extracted with the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The first-strand cDNA was synthesized with the AMV Reverse Transcriptase First-Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). PCR amplification of the heavy chain (HC) Fd region and the whole λ-light chain (LC) was performed with the GeneAmp PCR System 2400 (Perkin-Elmer, CA, USA) using the family-specific variable region and isotype-specific constant region primers (Table 1). The first round of PCR was performed for 35 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min. The amplified HC and LC fragments were cloned into pGEM T-Easy Vector (Promega, Madison, MA, USA). The nucleotide sequences of the HC and LC fragments were determined using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

scFv DNA construction

The V_H and V_L genes were amplified by PCR using primers as described (*22*). The V_H and V_L fragments were amplified separately using the PCR conditions of 35 cycles at 94°C for 1 min, 57°C for 1 min and 72°C for 1 min, followed by 72°C for 10 min. The scFv gene was constructed by assembling V_H, $(Gly_4\text{-}Ser)_3$ linker, and V_L fragments using overlap extension PCR. Finally, the full-length scFv gene containing *Sfi*I and *Not*I sites was amplified by PCR using the extension primers.

Table 1. Nucleotide sequence of primers used for amplification of mouse IgG_{2a}, λ.

Primers used for amplification of heavy chain Fd region	
Forward primer	
IA + IB	5′-SAG GTG CAG CTK CTC GAG TCA GGA CCT RGC-3′
IIA	5′-SAG GTY CAG CTG CTC GAG TCT GGA SCT GAG-3′
IIB	5′-CAG GTC CAR CTG CTC GAG YCT GGG GCT GAG-3′
IIC	5′-GAG GTT GAG GTG CTC GAG TGT GKG GCW GAG-3′
3A	5′-GAR GTG AAG GTG CTC GAG TCT GGA GGW GAG-3′
3B + 3C	5′-GAR GTG AAG CTT CTC GAG TCT GGA GGW GGC-3′
3D	5′-GAR GTG CAG CTG CTC GAG GGK GGG GGA GGA-3′
Reverse primer	
IgG_{2a}	5′-AGG CTT ACT AGT ACA GGG CTT GAT TGT GGG CCC-3′
Primers used for amplification of whole light chain	
Forward primer	5′-GCG CGC GAG CTC CAG GCT GTT GTG ACT CAG GAA TCT GCA-3′
Reverse primer	5′-GCG CGC TAT CTA GAA TTA GAG ACA TTC TGC AGG AGA CAG ACT-3′

Expression and screening of phage clones displaying scFv

The scFv gene was digested with the restriction enzymes *Sfi*I and *Not*I (TaKaRa, Shiga, Japan) and ligated into the phagemid pCANTAB-5E (GE Healthcare, Little Calflont, England). The ligation product was electroporated into competent *Escherichia coli* TG1 (suppressor strain) cells and streaked on an LB plate containing 50 μg/ml carbenicillin. Single colonies were picked and cultured with 200 μl of SB medium containing 20 mM glucose and 50 μg/ml carbenicillin for 5 h at 37°C; 20 μl of VSCM13 helper phage (1×10^{11} pfu/ml) was then added for 30 min without shaking, followed by incubation for 30 min with moderate shaking at 37°C. The culture was then centrifuged at 2500*g* for 5 min, and the bacterial pellet was resuspended with 200 μl SB medium containing 50 μg/ml carbenicillin and 70 μg/ml kanamycin; the culture was then incubated overnight with shaking at 30°C. The supernatant containing the scFv-displaying phage was collected by centrifugation at 9000*g* for 5 min.

Phage ELISA was performed to screen the scFv-displaying phage clone for binding activity to m^5Cyd. The m^5Cyd was conjugated with bovine serum albumin (BSA) according to the method of Beiser and Erlanger (*23*). The m^5Cyd–BSA conjugate, dissolved in phosphate-buffered saline (PBS) (10 μg protein/ml), was fixed to wells of 96-well microtitre plates by overnight incubation at 4°C. The wells were then filled with 100 μl of 1% BSA in PBS for 1 h at 37°C. The solution was discarded and phage solution diluted with PBS was applied to each well, followed by incubation for 2 h at 37°C. After incubation, the wells were washed five times with 0.05% Tween 20 in PBS. Next, 1 : 1000 diluted HRP-conjugated rabbit anti-M13 antibody (GE Healthcare) was added, and the wells were incubated for 1 h at 37°C. After washing, the substrate solution of the Sumilon peroxidase assay kit (Sumitomo Berkuraito, Tokyo, Japan) was added to each well. Colour development was stopped by addition of stop solution, and absorbance at 450 nm was measured with a microplate reader (Model 550; Bio-Rad, Richmond, CA, USA).

Nucleic acid sequencing

Nucleic acid sequencing was performed on a Prism 310 Genetic Analyzer (Applied Biosystems). The HC Fd and whole LC nucleic acid sequences were determined by M13 universal primer. The scFv nucleic acid sequencing primers were used as the reverse primer for HC V_H region amplification, and the forward primer was used for LC V_L region amplification.

Introduction of FLAG and his tags into the scFv Gene

PCR was performed in two steps to introduce the FLAG and His tags into the scFv gene. In the first step, the scFv gene was amplified with scFv forward primer (5′-TTA TTA TTC GCA ATT CCT TTA GTT-3′) and scFv reverse-1 primer (5′-GTG ATG CCC TTT ATC ATC ATC ATC TTT ATA ATC TAG GAC AGT GAC CTT-3′) under the PCR conditions of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min for 35 cycles, followed by 72°C for 10 min. In the second step, the gel-purified first-step PCR product was amplified with scFv forward primer and scFv reverse-2 primer (5′-GATACA ATT GCG GCC GCC TAA TGG TGG TGA TGG TGA TGC CCC TTT ATC ATC-3′) under the same PCR conditions. The scFv DNA fragment was gel-purified, digested with the restriction enzymes *Sfi*I and *Not*I, ligated into pCANTAB-5E, then introduced into TG1. A tag-introduced scFv-displaying phage clone was expressed and screened by phage ELISA.

Preparation of soluble scFv fragments

The antigen-binding phage was infected with *E. coli* HB2151 cells of the non-suppressor strain to express soluble scFv fragment and applied to an LB agar plate containing 50 μg/ml carbenicillin. Colonies were randomly selected and then cultured in 50 ml SB with 50 μg/ml carbenicillin and 20 mM $MgCl_2$ until an OD_{600} of 0.8 at 30°C. Isopropyl β-D-thiogalactopyranoside (IPTG) (final concentration of 1 mM) was added, and the colonies were cultured overnight at 30°C. The bacterial cells were pelleted by centrifugation

at 2000*g* for 30 min, resuspended with 0.5 ml PBS containing 0.2 mM phenylmethylsulphonyl fluoride and lysed by four cycles of freeze-thawing. Cell debris was pelleted by centrifugation at 14,000*g* for 30 min, and the supernatant containing soluble scFv was purified using a Ni-NTA spin kit (Qiagen) under *non*-reducing conditions according to the manufacturer's instructions. The purity of the scFv preparation was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions. The scFv concentration was determined by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

ELISA

The wells of 96-well microtitre plate (Costar, Cambridge, MA) were coated with m^5Cyd–BSA (10 μg protein/ml) overnight at 4°C and blocked with 1% BSA in PBS. After removing the blocking solution, crude or purified scFv was added, and the plates were incubated for 2 h at 4°C. After incubation, the wells were washed five times with 0.05% Tween 20 in PBS. Mouse anti-FLAG antibody (diluted in the ratio 1 : 3000 in PBS) was added, and incubation was continued for 1 h at 4°C. After washing as above, the wells were incubated with 1 : 1000 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgG Fc antibody for 1 h at 4°C. After washing, *p*-nitrophenyl phosphate dissolved in 1 M diethanolamine buffer (pH 9.8) was added to each well, and absorbance was measured at 405 nm.

DNA extraction from peripheral blood cells and cancer cell lines and enzymatic digestion

Genomic DNA from peripheral blood cells and cancer cell lines was extracted with the DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. DNA digestion was performed as described (*14*). Briefly, boiled DNA was dissolved in 30 mM sodium acetate (pH 5.3) and incubated with 5 U Nuclease P1 (Wako) for 2 h at 37°C. Additional incubation with 0.4 U of alkaline phosphatase (TaKaRa), 10 mM $MgCl_2$ and 50 mM Tris–HCl (pH 9.0) for 2 h at 37°C was then performed. Finally, the pH was adjusted to 7.0 with 1 M Tris–HCl. Samples were kept at −30°C until analysis.

Results and discussion

PCR amplification of antibody gene fragment

The antibody HC Fd and LC genes were amplified by PCR with primer sets using cDNA synthesized from total RNA of anti-m^5Cyd MoAb (FMC9)-producing hybridoma cells (*14*). FMC9 HC gene fragments were amplified using the IIA-IgG2a primer set, and LC genes were amplified with the lambda primer set. The amplified products were cloned into pGEM T-Easy, the nucleotide sequences of V_H and V_L regions were then determined. The results are shown in Fig. 1. The computer-based structural and homology analysis revealed that the HC was 88.8% homologous with germline *3609.7.153*, and the LC was 97.8% homologous with germline *lambda 2*.

Construction and phage display of scFv gene

The V_H and V_L genes were amplified by PCR. As shown in Fig. 2, the reverse and forward primers used for amplifying-specific variable regions were designed based on the sequence data obtained. The amplification of V_H and V_L regions generated DNA fragments with an approximate length of 350 bp (lanes 2 and 3). The variable domain gene was assembled with a flexible linker $(Gly_4\text{-}Ser)_3$ using overlap extension PCR. The amplified gene, with an approximate length of 750 bp, was successfully amplified (lane 5), and then cloned into pCANTAB-5E. The plasmid DNA was transformed into *E. coli* TG1 cells. pCANTAB-5E contains a translational stop codon for the peptide tag followed by the amber codon (TAG) at the junction between the cloned scFv and the sequence for the gene 3-encoded adsorption protein (g3p). When a supE strain of *E. coli*, such as TG1, was transformed with the recombinant vector, the scFv was displayed as a g3p fusion protein on the surface of the phage virion. To screen for an antigen-binding scFv-displaying phage clone, we performed phage ELISA, which showed that one out of five clones reacted with m^5Cyd-BSA but not with BSA (Fig. 3).

Introduction of tag sequences and expression of scFv as a soluble protein

To facilitate the detection and purification of the expressed soluble scFv, the FLAG (*24*) and His tags were introduced into 5′-end of V_L. The scFv was expressed in a phage display form and then phage ELISA was performed. This showed that the scFv containing the tag sequence reacted with m^5Cyd-BSA, similar to scFv without the tag sequence (data not shown). To express them in soluble scFv form, the antigen-positive phages were infected with *E. coli* HB2151 cells of a non-suppressor strain. The expression of scFv protein was induced by IPTG, and *E. coli* HB2151 cells were lysed by four cycles of freeze-thawing. Soluble scFv secreted into the periplasmic space was purified with a Ni-NTA spin column (Fig. 4). The yield of scFv was 20 μg from 50 ml of culture.

Characterization of soluble scFv

The cross-reactivity of scFv was determined by inhibition ELISA. As shown in Fig. 5 and Table 2, scFv reacted with m^5Cyd and m^5dCyd but not with Cyd, 5-azacytidine and 1-methyladenosine in a manner similar to MoAb. This result implies that the scFv was actually derived from the parent MoAb. The IC_{50} of the scFv against m^5dCyd was 0.054 μg/ml, which was less than that of MoAb (4.27 μg/ml). The sensitivity of the scFv was higher than that of the parent MoAb. A similar result was obtained in our previous study (*25*). The increased sensitivity of the scFv fragment may be due to a difference in the number of antigen-binding sites between the scFv and MoAb. In inhibition ELISA, incubation of the bivalent MoAb with hapten at a low concentration would result in only one binding site being occupied, while another binding site would still be available for capture of the coating antigen. Consequently, the antigen-bound monovalent scFv fragment might be more sensitive than bivalent MoAb.

Measurement of DNA methylation levels in cancer cell lines and healthy peripheral blood cells

To establish the inhibition ELISA-based measurement system for the DNA methylation level, the detection limit and inter- and intra-assay variation of the assay system were determined. From the standard curve using authentic m^5dCyd, the detection limit was 3 ng/ml (Fig. 6). Good linearity was seen from 7 to 300 ng/ml. The inter- and intra-assay coefficients of variation were 5.6–11.7% and 1.9–5.5%, respectively. These results show that this ELISA system was suitable for the determination of m^5dCyd within the range of 7–300 ng/ml.

V_H chain sequence

```
          9         18         27         36         45         54         63         72
CAG GTC AAG CTG CAG GAG TCT GGG GCT GAG ATA TTG CAG CCC TCC CAG ACC CTC AGT CTG ACT TGT TCT TTC
 Q   V   K   L   Q   E   S   G   A   E   I   L   Q   P   S   Q   T   L   S   L   T   C   S   F

         81         90         99        108        117        126        135        144
TCT GGG TTT TCA CTG AGC ACT TCT GGT ATG GGT GTA GAG TGG ATT CGT CAG CCT TCA GGG AGG GGT CTG GAG
 S   G   F   S   L   S   T   S   G   M   G   V   E   W   I   R   Q   P   S   G   R   G   L   E
                                 CDR-H1
        153        162        171        180        189        198        207        216
TGG CTG GCA CAG ATT TGG TGG GAT GAT GAT AAG CGC TAT AAC CCA GTC CTG AAG AGC CGA CTG ACA ATC TCC
 W   L   A   Q   I   W   W   D   D   D   K   R   Y   N   P   V   L   K   S   R   L   T   I   S
                                 CDR-H2
        225        234        252        261        270        279        288        297
AAG GAT ACC TCC AGC AAC CTC AAG ATC GCC AAT GTG GAC AGT GCA GAT ACT GCC ACA TAC TAC TGT GCT CGA
 K   D   T   S   S   N   L   K   I   A   S   V   D   T   A   D   T   A   T   Y   Y   C   A   R

        306        315        324        333        342        351        360
ATA GCC TAT AAT CTC TGG TAC TTC GAT GTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
 I   A   Y   N   L   W   Y   F   D   V   W   G   Q   G   T   T   V   T   V   S   S
             CDR-H3
```

V_L chain sequence

```
          9         18         27         36         45         54         63         72
CAG GCT GTT GTG ACT CAG GAA TCT GCA CTC ACC ACA TCA CCT GGT GGA ACA GTC ATA CTC ACT TGT CGC TCA
 Q   A   V   V   T   Q   E   S   A   L   T   T   S   P   G   G   T   V   I   L   T   C   R   S

         81         90         99        108        117        126        135        144
AGT ACT GGG GCT GTT ACA ACT AGT AAC TAT GCC AAC TGG GTC CTA GAA AAA CCA GAT CAT TTA TTC ACT GGT
 S   T   G   A   V   T   T   S   N   Y   A   N   W   V   L   E   K   P   D   H   L   F   T   G
          CDR-L1
        153        162        171        180        189        198        207        216
CTA ATA GGT GGT ACC AGC AAG CGA GCT CCA GGT GTT CCT GTC AGA TTC TCA GGC TCC CTG ATT GGA GAC AAG
 L   I   G   G   T   S   K   R   A   P   G   V   P   V   R   F   S   G   S   L   I   G   D   K
                     CDR-L2
        225        234        243        252        261        270        279        288
ACT GAG GAT GAT GCA ATG TAT TTC TGT GCT CTA TGG TAC AGC ACC CAT TTC GTT TTC GGC GGT GGA ACC AAG
 A   A   L   T   I   T   G   A   Q   T   E   D   D   A   M   Y   F   C   A   L   W   Y   S   T
                                                                 CDR-L3
        297        306        315        324
GCT GCC CTC ACC ATC ACA GGG GCA CAG GTC ACT GTC CTA
 H   Y   V   F   G   G   G   T   K   V   T   V   L
```

Fig. 1 Nucleotide and deduced amino acid sequences of V_H and V_L regions of FMC9. CDR sequences are shown in underlined. These sequences are available from the DDBJ/GenBank/EMBL nucleotide sequence database under accession numbers AB500126 for the heavy chain and AB500125 for the light chain. The primers positions of PCR amplification are indicated by bold text.

To evaluate the ELISA system established here, we measured the m^5dCyd content in genomic DNA samples that were completely digested to nucleotide monomers with nuclease P1 and alkaline phosphatase. The results are shown in Fig. 7. HeLa (cervix carcinoma), HeLa S3 (cervix carcinoma), and MDA-MB453 (breast carcinoma) cells showed a methylation level of ~1% of the total genomic DNA, similar to healthy peripheral blood cells. On the other hand, the methylation levels of HT29

(colon carcinoma) and T-47D (breast carcinoma) were found to be lower than those of HeLa, HeLa S3 and MDA-MB453 cells.

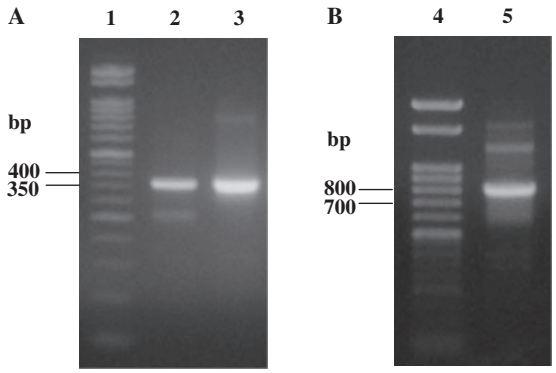

Fig. 2 PCR amplification of V_H and V_L genes (A) and assembly PCR for construction of scFv (B). Samples were electrophoresed on 2% agarose in 1× TAB buffer. Lanes 1 and 4: one STEP Ladder 50 (Nippon Gene); lane 2: V_H PCR product (about 350 bp); lane 3: V_L PCR product (about 350 bp); and lane 5: scFv PCR product (about 750 bp).

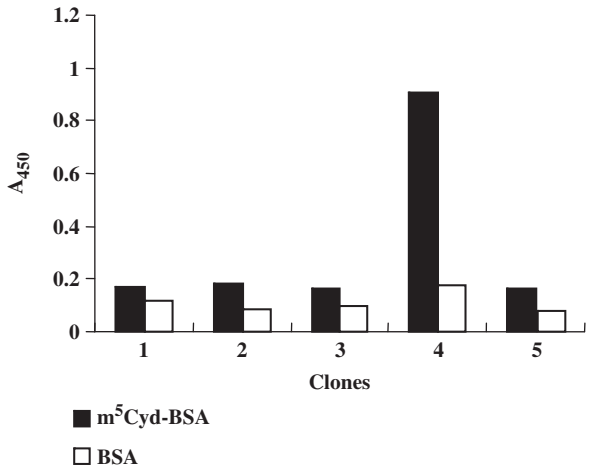

Fig. 3 Phage ELISA to determine the specificity of individual clones. Recombinant phages from five random clones were produced separately. Results are shown as means of duplicate assays. The clone of lane 4 reacted with m^5Cyd-BSA but not with BSA (negative control).

Conclusions

In this study, we cloned immunoglobulin genes from anti-m^5Cyd MoAb-secreting hybridoma cells. Using primer sets designed on the basis of the V_H and V_L sequences, an scFv phage display library was constructed. Phage-displayed scFv recognized m^5dCyd-BSA in the same manner as MoAb. The FLAG and His tag sequences were introduced into the scFv construct for purification and detection. Although the specificities of the scFv and MoAb were almost identical, the sensitivity of the scFv was approximately 80 times higher than that of the MoAb. To evaluate the usefulness of the prepared scFv, we measured the DNA methylation levels of genomic DNA from cancer cell lines and healthy peripheral blood cells by inhibition ELISA. The level of methylation of HeLa, HeLa S3 and MDA-MB-453 DNA was found to be 1% of the total genomic DNA, similar to peripheral blood

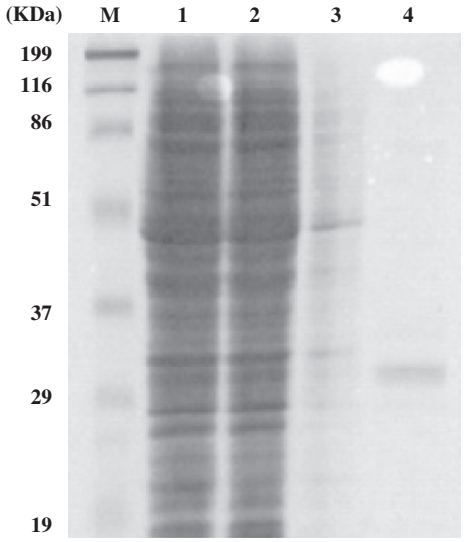

Fig. 4 Purification of scFv from lysate by Ni-chelate chromatography. The scFv was purified from crude bacterial lysates by Ni-NTA spin kit, then the purity was checked by 12% SDS–PAGE under a non-reducing condition followed by Coomassie brilliant blue staining. Lane 1: bacterial lysates; lane 2: non-adsorbed fraction; lane 3: 20 mM imidazole wash fraction and lane 4: 500 mM imidazole eluate fraction. M: molecular weight marker.

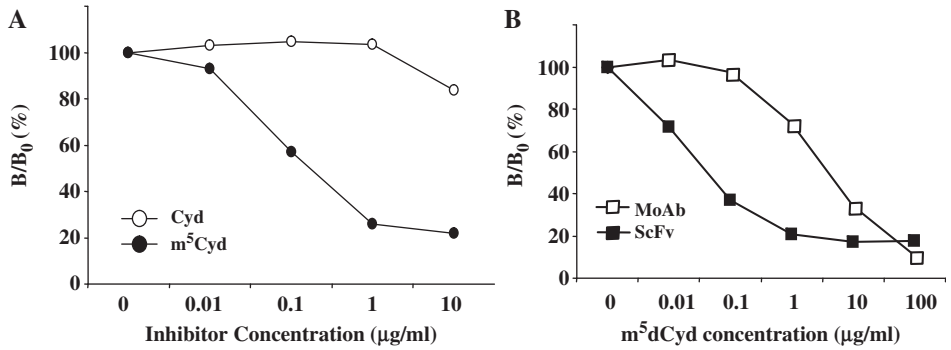

Fig. 5 Comparison of the specificity of scFv and MoAb. (A) The reactivities of the scFv against m^5Cyd and Cyd were determined by inhibition ELISA. The scFv reacted with m^5Cyd (closed circles) but not with Cyd (opened circles). (B) Comparison of the specificities of MoAb (open squares) and scFv (closed squares) against m^5dCyd. Each was reacted in duplicate. Results in (A) and (B) are shown as means. The IC_{50} of MoAb was 4.27 μg/ml and that of scFv was 0.054 μg/ml.

Table 2. Binding properties of MoAb and corresponding scFv as determined by inhibition ELISA.

Compound	Cross-reactivity (%)	
	scFv	MoAb
5-Methyl-2′-deoxycytidine	100	100
5-Methycytidine	31.76	29.01
Cytidine	0.10	0.15
5-Azacytidine	0.01	<0.01
1-Methyladenosine	<0.01	<0.01

Cross-reactivity (%) = IC_{50} (m^5dCyd)/IC_{50} (Analogs).

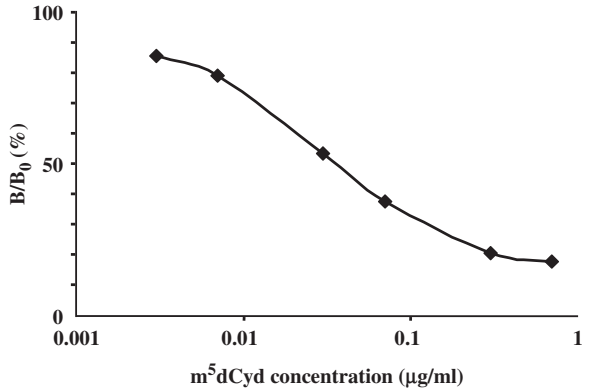

Fig. 6 Standard curve for the determination of m^5dCyd using an scFv-based inhibition ELISA system. Points represent means of duplicate assays.

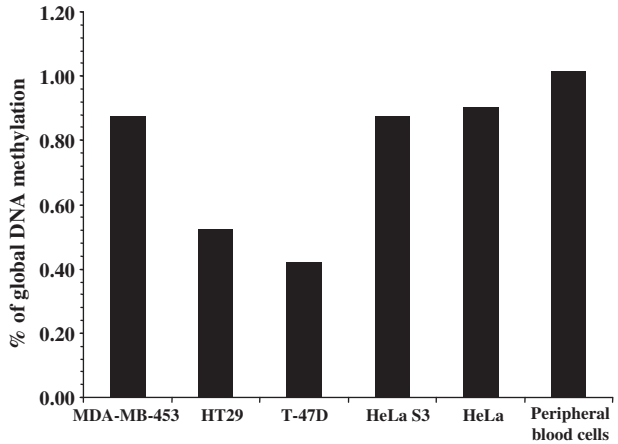

Fig. 7 Determination of DNA methylation levels in cancer cell lines and peripheral blood cells. The levels of 5-methyl-2′-deoxycytidine were calculated as their ratio to genomic DNA. Results are shown as means of duplicate assays.

cells, though HT29 and T-47D were found to be hypomethylated HeLa, HeLa S3 and MDA-MB453 cells. The scFv generated in this study may be useful for the immunochemical assessment of cellular DNA methylation levels in various specimens.

Acknowledgement

The authors would like to thank Prof. Takashi Masuko, Kinki University of Pharmaceutical Sciences, for providing cancer cell lines.

Funding
This work was supported in part by a Grant-in-Aid for Scientific Research from the University of Shizuoka.

Conflict of interest
None declared.

References

1. Bird, A. (2002) DNA methylation patterns and epigenetic memory. *Genes Dev.* **16**, 6–21
2. Herman, J.G. and Baylin, S.B. (2003) Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* **349**, 2042–2054
3. Issa, J.P. (2004) CpG island methylator phenotype in cancer. *Nat. Rev. Cancer* **4**, 988–993
4. Jones, P.A. and Baylin, S.B. (2002) The fundamental role of epigenetic events in cancer. *Nat. Rev. Genet.* **3**, 415–428
5. Kato, C., Petronis, A., Okazaki, Y., Tochigi, M., Umekage, T., and Sasaki, T. (2002) Molecular genetic studies of schizophrenia: challenges and insights. *Neurosci. Res.* **43**, 295–304
6. Petronis, A. (2003) Epigenetic and bipolar disorder: new opportunities and challenges. *Am. J. Med. Genet. C. Semin. Med. Genet.* **123C**, 65–75
7. Chung, W., Kwabi-Addo, B., Ittmann, M., Jelinek, J., Shen, L., Yu, Y., and Issa, J.P. (2008) Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. *PLoS ONE* **3**, e2079
8. Gaudet, F., Hodgson, J.G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J.W., Leonhardt, H., and Jaenisch, R. (2003) Induction of tumors in mice by genomic hypomethylation. *Science* **300**, 489–492
9. Moore, L.E., Pfeiffer, R.M., Poscablo, C., Real, F.X., Kogevinas, M., Silverman, D., Garcia-Closas, R., Chanock, S., Tardon, A., Serra, C., Carrato, A., Dosemeci, M., Garcia-Closas, M., Esteller, M., Fraga, M., Rothman, N., and Malats, N. (2008) Genomic DNA hypomethylation is a biomarker for bladder cancer susceptibility in the Spanish bladder cancer case-control study. *Lancet Oncol.* **9**, 359–366
10. Smith, I.M., Mydlarz, W.K., Mithani, S.K., and Califano, J.A. (2007) DNA global hypomethylation in squamous cell head and neck cancer associated with smoking alcohol consumption and stage. *Int. J. Cancer* **121**, 1724–1728
11. Guerrero-Preston, R., Santella, R.M., Blanco, A., Desai, M., Berdasco, M., and Fraga, M. (2007) Global DNA hypomethylation in liver cancer cases and controls: a phase I preclinical biomarker development study. *Epigenetics* **2**, 223–226
12. Magana, A.A., Wrobel, K., Caudillo, Y.A., Zaina, S., Lund, G., and Wrobel, K. (2008) High-performance liquid chromatography determination of 5-methyl-2′-deoxycytidine, 2′-deoxycytidine, and other deoxynucleosides and nucleosides in DNA digests. *Anal. Biochem.* **374**, 378–385
13. Liu, Z., Liu, S., Xie, Z., Blum, W., Perrotti, D., Paschka, P., Klisovic, R., Byrd, J., Chan, K.K., and Marcucci, G. (2007) Characterization of *in vitro* and *in vivo* hypomethylation effects of decitabine in acute myeloid leukemia by a rapid, specific and sensitive LC-MS/MS method. *Nucleic Acids Res.* **35**, e31
14. Mizugaki, M., Itoh, K., Yamaguchi, T., Ishiwata, S., Hishinuma, T., Nozaki, S., and Ishida, N. (1996) Preparation of a monoclonal antibody specific for 5-methyl-2′-deoxycytidine and its application for the

detection of DNA methylation levels in human peripheral blood cells. *Biol. Pharm. Bull.* **19**, 1537–1540
15. McCafferty, J., Griffiths, A.D., Winter, G., and Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348**, 552–554
16. Clackson, T., Hoogenboom, H.R., Griffiths, A.D., and Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature* **349**, 293–299
17. Fuh, G. and Sidhu, S.S. (2000) Efficient phage display of polypeptides fused to the carboxy-terminus of the M13 gene-3 minor coat protein. *FEBS Lett.* **480**, 231–234
18. Carson, D.A. and Freimark, B.D. (1986) Human lymphocyte hybridomas and monoclonal antibodies. *Adv. Immunol.* **38**, 275–311
19. Milenic, D.E., Yokota, T., Filpula, D.R., Finkelman, M.A., Dodd, S.W., Wood, J.F., Whitlow, M., Snoy, P., and Schlom, J. (1991) Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res.* **51**, 6363–6371
20. Yokota, T., Milenic, D.E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* **52**, 3402–3408
21. Yazaki, P.J., Kassa, T., Cheung, C.W., Crow, D.M., Sherman, M.A., Bading, J.R., Anderson, A.L., Colcher, D., and Raubitschek, A. (2008) Biodistribution and tumor imaging of anti-CEA single-chain antibody-albumin fusion protein. *Nucl. Med. Biol.* **35**, 151–158
22. McCafferty, J., Hoogenboom, H. R., and Chiswell, D.J. (1996) *Antibody Engineering*. pp. 1–40, Oxford University Press, Oxford, NY
23. Beiser, S.M. and Erlanger, B.F. (1966) Antibodies which react with nucleic acids. *Cancer Res.* **26**, 2012–2017
24. Brizzard, B.L., Chubet, R.G., and Vizard, D.L. (1994) Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques* **16**, 730–735
25. Itoh, K., Suzuki, K., Ishiwata, S., Tezuka, T., Mizugaki, M., and Suzuki, T. (1999) Application of a recombinant Fab fragment from a phage display library for sensitive detection of a target antigen by an inhibition ELISA system. *J. Immunol. Methods* **223**, 107–114