Generation of a humanized anti-glypican 3 antibody by CDR grafting and stability optimization

Kiyotaka Nakano[a], Takahiro Ishiguro[b], Hiroko Konishi[c], Megumi Tanaka[c], Masamichi Sugimoto[b], Izumi Sugo[c], Tomoyuki Igawa[c], Hiroyuki Tsunoda[a], Yasuko Kinoshita[b], Kiyoshi Habu[a], Tetsuro Orita[a], Masayuki Tsuchiya[a], Kunihiro Hattori[a] and Hisafumi Yamada-Okabe[d]

Glypican 3 (GPC3), a glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan, is expressed in a majority of hepatocellular carcinoma tissues. The murine monoclonal antibody GC33 that specifically binds to the COOH-terminal part of GPC3 causes strong antibody-dependent cellular cytotoxicity against hepatocellular carcinoma cells and exhibits strong antitumor activity in the xenograft models. To apply GC33 for clinical use, we generated a humanized GC33 from complementarity-determining region grafting with the aid of both the hybrid variable region and two-step design methods. The humanized antibody bound to GPC3 specifically and induced antibody-dependent cellular cytotoxicity as effectively as a chimeric GC33 antibody. To improve stability of the humanized GC33, we further optimized humanized GC33 by replacing the amino acid residues that may affect the structure of the variable region of a heavy chain. Substitution of Glu6 with Gln in the heavy chain significantly improved the stability under high temperatures. GC33 also has the risk of deamidation of the -Asn–Gly- sequence in the complementarity-determining region 1 of the light chain. As substitution of Asn diminished the antigen binding, we changed the neighboring Gly to Arg to avoid deamidation. The resulting humanized anti-GPC3 antibody was as efficacious as chimeric GC33 against the HepG2 xenograft and is now being evaluated in clinical trials. *Anti-Cancer Drugs* 21:907–916 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2010, **21**:907–916

Keywords: antibody, antibody-dependent cellular cytotoxicity, deamidation, glypican 3, hepatocellular carcinoma, humanization, stabilization

Departments of [a]Genome Antibody Product Research, [b]Pharmaceutical Research, [c]Preclinical Research and [d]Research Planning and Coordination, Chugai Pharmaceutical Co. Ltd., Gotemba, Japan

Correspondence to Hisafumi Yamada-Okabe, PhD, Research Planning and Coordination Department, Gotemba Research Laboratories, 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan
Tel: +81 550 87 6730; fax: +81 550 87 3637;
e-mail: okabehsf@chugai-pharm.co.jp

Received 18 April 2010 **Revised form accepted** 13 August 2010

Introduction

Glypican 3 (GPC3) is a member of the glypican family of heparan sulfate proteoglycans that are attached to the cell surface by a glycosylphosphatidylinositol anchor [1]. Loss-of-function mutations of GPC3 in humans cause Simpson–Golabi–Behmel syndrome, an X-linked condition characterized by prenatal and postnatal overgrowth [2]. GPC3 regulates cell growth positively and negatively in a cell type-dependent manner. Overgrowth of Simpson–Golabi–Behmel syndrome and also of GPC3 knockout mice suggests a negative regulation of cell growth by GPC3 [3]. In contrast, GPC3 stimulates the growth of hepatocellular carcinoma cells (HCC) *in vitro* and *in vivo* by activating the canonical Wnt signaling [4].

GPC3 expression has been shown to be markedly elevated in HCCs at both the mRNA and protein levels [5–8]. GPC3 immunostaining was observed in more than 70% of the HCC patients. We recently found that GPC3 membrane expression might be involved in the increase of macrophages in human HCC [9,10]. In addition, Shirakawa *et al.* [11] reported that GPC3 expression is correlated with a poor prognosis in HCC patients. As there are detectable levels of GPC3 in the sera of HCC patients, soluble GPC levels may be used as a prediction marker for prognosis [12–14]. Furthermore, elevated levels of GPC3 have also been reported in melanoma [15,16] and gastric cancer [17].

Earlier, we generated a monoclonal antibody (mAb) against the COOH-terminal part of GPC3, which induced antibody-dependent cellular cytotoxicity (ADCC) against the GPC-positive human HCC cells but not the GPC3-negative cells [18]. The mAb, designated as GC33, exhibited marked tumor growth inhibition of subcutaneously transplanted HepG2 and HuH-7 xenografts that expressed GPC3 [19]. To apply GC33 for clinical use, we generated a humanized version. This study describes the humanization of GC33 in detail.

Methods

Construction of chimeric GC33

GC33 hybridoma was obtained in the earlier study by immunizing soluble GPC3 [18]. Total RNA was prepared from hybridoma cells using the RNeasy Plant Mini Kit

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833f5d68

(Qiagen, Chatsworth, California, USA). The variable regions of the heavy chain (VH) and the light chain (VL) cDNAs were obtained by rapid amplification of cDNA ends using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, California, USA) and the 3′-end primers designed for each chain of immunoglobulin (κ, γ2a). The PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, Wisconsin, USA) and the sequences of VH and VL for GC33 were determined. The framework regions (FRs) and complementarity-determining regions (CDR) were determined as reported [20]. To produce chimeric GC33, in which the mouse variable regions were linked to the human IgG1 and κ constant regions, the cDNA for the VH region was cloned at the HindIII/BamHI sites of HEF-VH-gγ1 [21] that carries the constant region of human IgG1, whereas the VL region was cloned at the HindIII/BamHI sites of HEF-VL-gκ [21] harboring the constant region of the human κ chain. The resulting plasmid DNA was transfected into CHO cells by electropolation. The mAbs in the culture media were purified by protein A-Sepharose affinity chromatography followed by dialysis against PBS.

Construction of humanized GC33

The mAb GC33 was humanized using the CDR-grafting method as described earlier [21–23]. The first versions (hGC33VHa, hGC33VLa) of the humanized GC33 VH and VL regions were generated using 14 overlapping oligonucleotides ranging from 36 to 50 bp, with 20 bp of complementarity, in a PCR-based protocol. The PCR primers for hGC33VLa and hGC33VHa are shown in Tables 1 and 2, respectively. The signal sequence of the accession number of S40357 that has a high homology with AB064105 was used as the signal sequence of the VL chain. The DNA fragments were cloned at the HindIII/BamHI sites of the HEF expression vectors. CHO cells expressing humanized GC33 were established and the humanized GC33 was purified by protein A-Sepharose affinity chromatography followed by size-exclusion chromatography (SEC). The hybrid variable regions, in which some FRs of the humanized variable regions had been replaced with mouse FRs, were constructed by overlap extension PCR. Additional changes of amino acids were introduced into the humanized GC33 VH and VL by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, California, USA).

Table 1 Sequence of the overlapping PCR primers for humanized antibody light chain variable region hGC33VLa

Primers	Sequence
hVLfhin	5′-ttcaagcttccaccatgaaatacctattgcctacggca-3′
hVLrbam	5′-ttgggatccactcacgtttgatctccagcttggtcc-3′
hVL1	5′-atgaaatacctattgcctacggcagccgctggattgttattactcgcggc-3′
hVL2	5′-tgagtcatcacaacatcggccatggccggctgggccgcgagtaataacaa-3′
hVL3	5′-gatgttgtgatgactcagtctccactctccctgcccgtcacccctggaga-3′
hVL4	5′-ctctgactagatctgcaggagatggaggccggctctccaggggtgacggg-3′
hVL5	5′-tgcagatctagtcagagccttgtacacagtaatggaaacacctatttaca-3′
hVL6	5′-tgtggagactgccctggcttctgcaggtaccaatgtaaataggtgtttcc-3′
hVL7	5′-ccagggcagtctccacagctcctgatctataaagtttccaaccgattttc-3′
hVL8	5′-gatccactgccactgaacctgtcagggaccccagaaaatcggttggaaac-3′
hVL9	5′-ttcagtggcagtggatcaggcacagattttacactgaaaatcagcagagt-3′
hVL10	5′-gagcagtaataaaccccaacatcctcagcctccactctgctgattttcag-3′
hVL11	5′-ggggtttattactgctctcaaaatacacatgttcctcctacgtttggcca-3′
hVL12	5′-tttgatctccagcttggtcccctggccaaacgtaggagg-3′

VL, light chain.

Table 2 Sequence of the overlapping PCR primers for humanized antibody heavy chain variable region hGC33VHa

Primers	Sequence
hVHfhin	5′-ttcaagcttccaccatggactggacctggaggttcctc-3′
hVHrbam	5′-ttgggatccactcacctgaggagacggtgaccaggg-3′
hVH1	5′-atggactggacctggaggttcctctttgtggtggcagcagctacaggtgt-3′
hVH2	5′-gctccagactccaccagctgcacctgggactggacacctgtagctgctgc-3′
hVH3	5′-ctggtggagtctggagctgaggtgaagaagcctggggcctcagtgaaggt-3′
hVH4	5′-tcggtgaaggtgtatccagaagccttgcaggagaccttcactgaggcccc-3′
hVH5	5′-ggatacaccttcaccgactatgaaatgcactgggtgcgacaggcccctgg-3′
hVH6	5′-ggatcaagagctcccatccactcaagcccttgtccaggggcctgtcgcac-3′
hVH7	5′-atgggagctcttgatcctaaaactggtgatactgcctacagtcagaagtt-3′
hVH8	5′-gattcgtccgcggtaatcgtgactctgcccttgaacttctgactgtaggc-3′
hVH9	5′-attaccgcggacgaatccacgagcacagcctacatggagctgagcagcct-3′
hVH10	5′-cgcacagtaatacacggccgtgtcctcagatctcaggctgctcagctcca-3′
hVH11	5′-ccgtgtattactgtgcgagattctactcctatacttactggggccaggga-3′
hVH12	5′-tgaggagacggtgaccagggttccctggccccagtaag-3′

VH, heavy chain.

Enzyme-linked immunosorbent assay

Soluble human GPC3 core protein (sGPC3 Δheparan sulfate, 1–563, mutated Ser495 and Ser509 to Ala) was prepared as described earlier [18]. Microtiter plates were coated with 1 μg/ml of sGPC3 HS and blocked with 1% BSA. The plates were then incubated with mAbs at the required dilutions. Bound mAbs were detected with alkaline phosphatase-labeled anti-human Ig VL κ (Sigma, Woodlands, Texas, USA). Absorbance at 405 nm was measured with a microplate reader and the results were analyzed using GraphPad Prism (GraphPad Software, San Diego, California, USA). Competition enzyme-linked immunosorbent assay (ELISA) was performed with biotinylated mouse GC33 that had been prepared using a Biotin Labeling Kit (Roche Diagnostics, Penzberg, Germany). sGPC3 ΔHS coated microtiter plates were incubated with biotinylated mouse GC33 in the presence of various concentrations of humanized GC33. After washing, the binding of biotinylated GC33 was detected using alkaline phosphatase-conjugated streptavidin (Invitrogen, Carlsbad, California, USA).

Antibody-dependent cellular cytotoxicity assay

ADCC from the humanized GC33 was determined by a ^{51}Cr-release assay [18]. SK-03, an SK-HEP-1 derived transfectant expressing human GPC3, was used as the target cell line [19]. As the effector cells, human peripheral blood mononuclear cells were purified from whole blood samples of healthy volunteers using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). Target cells were labeled with 0.1 mCi of ^{51}Cr-sodium chromate (New England Nuclear, Boston, Massachusetts, USA) at 37°C for 1 h. The ^{51}Cr-labeled cells were incubated with serial dilutions of antibodies in the presence of effector

cells at an E : T ratio of 50 : 1. After incubation at 37°C for 4 h, the supernatants were collected and the radioactivity of each supernatant was counted with a γ-counter. Specific cell cytotoxicity was calculated using the following formula: cell cytotoxicity (%) = (A–C)/(B–C), in which A, B, and C represent the radioactivities of the supernatants of the target cells incubated with the antibody plus effector cells, cells lysed with *2%* nonidet P-40, and nontreated cells, respectively.

Accelerated stability study

The humanized antibodies were diluted in the formulation buffer (20 mmol/l sodium acetate, 150 mmol/l NaCl at pH 6.0) at a concentration of 0.5 mg/ml and stored for 0–*2* weeks at 60°C. SEC was then carried out on an Alliance (2690/5) HPLC system (Waters, Milford, Massachusetts, USA) equipped with a TSKgel SuperSW3000 column (Tosoh, Tokyo, Japan). The column was equilibrated with 50 mmol/l sodium phosphate pH 7.0, 300 mmol/l NaCl. Samples were injected onto the column and eluted isocratically at a flow rate of 0.3 ml/min with the equilibration buffer. Protein elution was monitored at 280 nm. For quantification purposes, all species eluted before and after the main peak were integrated, and represent high molecular weight species and low molecular weight species, respectively.

Animals and drug administration

The human hepatoblastoma cell line HepG2 was obtained from ATCC and the cells were cultured in Eagle's minimal essential medium (Sigma) supplemented with 10% fetal bovine serum, nonessential amino acids, and 1 mmol/l sodium pyruvate. Six-week-old male, CB-17 severe combined immunodeficient mice were intraperitoneally injected with 200 μg of anti-asialo GM1 antibody (Wako, Osaka, Japan) and then subcutaneously inoculated with 5×10^6 cells of HepG2 suspended in 50% Matrigel (Becton Dickinson, San Jose, California, USA). When the tumor volume reached approximately 200 mm^3, the mice were intravenously administered the indicated doses of the anti-GPC3 antibody once a week for 3 weeks. The control mice received the same volume (10 ml/kg) of PBS. Tumor volume was estimated using the equation $V = ab^2/2$, in which a and b are the tumor length and width, respectively.

Results

Humanization of GC33

The cDNAs coding for the mouse GC33 VH and VL regions were isolated by PCR and 5′-rapid amplification of cDNA ends, respectively. The deduced amino acid sequences from the cDNAs showed typical mouse VL and VH variable regions belonging to κ VL subgroup II and VH subgroup II (A) (Fig. 1). A chimeric GC33, in which the VL region was linked to the human κ constant region and the VH region to the human IgG1 constant region, was cloned into an expression vector and used as a positive control for the evaluation of the humanized antibodies.

MAb GC33 was humanized using CDR grafting [21–23]. To select suitable human variable domains to serve as framework donors, the amino acid sequences of GC33 VH and VL were independently aligned against the entire repertoire of human antibody sequences contained in the Kabat Database (*ftp://ftp.ebi.ac.uk/pub/databases/kabat/*) and the ImMunoGeneTics Database (IMGT, *http://imgt.cines.fr/*). Canonical residues are key residues in the CDR and/or framework that determine the conformation of the CDR loop. Residues 24, 26, 27, 29, 71, 94 of the VH are part of the canonical structure [24], as are residues 2, 48, 64, 71 of the VL [25]. We searched the antibody sequences that matched these canonical residues with GC33. The FRs of the VH of human antibody DN13 [26] were 75% identical to the GC33 VH and the FRs of the human antibody K64 (GenBank accession number AB064105) were 89% identical to the GC33 VL. We selected the variable regions of these two human antibodies as frameworks for the humanized GC33 antibody VH and VL, respectively.

First, three CDRs from the GC33 VL or VH were directly grafted onto the human antibody VL or VH frameworks to generate humanized antibody genes. The CDR-grafted antibody VL (hGC33VLa) and VH (hGC33VHa) were synthesized using an overlap extension method. The resultant humanized *VH* gene and *VL* gene were cloned into expression vectors and were coexpressed with the GC33 chimeric antibody VH (cGC33VH) or VL (cGC33VL) expression vector in COS7 cells, yielding a humanized antibody (hGC33VHa/hGC33VLa), hemihumanized antibodies (hGC33VHa/cGC33VL, cGC33VH/hGC33VLa), and a chimeric antibody (cGC33VH/cGC33VL). These antibodies in the COS7 cell culture supernatant were quantified by sandwich ELISA and the binding activity was evaluated by soluble GPC3-coated ELISA. The results showed a threefold decrease in the antigen binding activity of the humanized GC33 VH compared with the chimeric GC33 VH (data not shown) and additional alterations to the human frameworks were required. The humanized GC33 VL bound to the antigen as effectively as the chimeric antibody (data not shown) and so no additional versions of the humanized VL regions were required.

To identify which mouse FR was required to create a functional antigen-binding site, hybrid variable regions were constructed [22]. When the FR3 of hGC33VHa was replaced by the mouse FR3, the binding activity was comparable with the chimeric antibody (data not shown). Thus, some amino acids in the mouse FR3 are thought to be critical in recreating a functional antigen-binding site. For the design of a second set of humanized GC33 VH regions, a structure model of the mouse GC33 variable regions was built using the molecular operating environment

Fig. 1

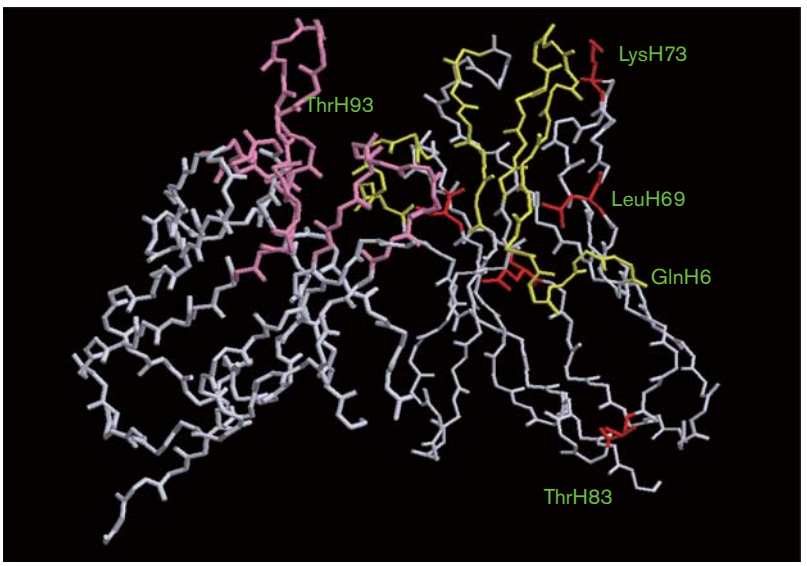
(a)

```
                FR1                                 CDR1  FR2             CDR2
                ---------1---------2---------3      -----  ----4---------  5----------6-----
                123456789012345678901234567890     12345  67890123456789  012a3456789012345
mGC33VH         QVQLQQSGAELVRPGASVKLSCKASGYTFT     DYEMH  WVKQTPVHGLKWIG  ALDPKTGDTAYSQKFKG
hGC33VHa        ----VE----VKK------V----------     *****  --R-A-GQ--E-M-  *****************
hGC33VHc        ----VE----VKK------V----------     *****  --R-A-GQ--E-M-  *****************
hGC33VHh        ----VE----VKK------V----------     *****  --R-A-GQ--E-M-  *****************
hGC33VHk        ----V-----VKK------V----------     *****  --R-A-GQ--E-M-  *****************

                FR3                                      CDR3      FR4
                ----7---------8------------9----        ----10--  ------11---
                67890123456789012abc345678901234        56789012  34567890123
mGC33VH         KATLTADKSSSTAYMELRSLTSEDSAVYYCTR        FYSY--TY  WGQGTLVTVSA
hGC33VHa        RV-I---E-T-------S--R---T-----A-        ********  ----------S
hGC33VHc        RV-----E-T-------S--R---T-------        ********  ----------S
hGC33VHh        RV-------T-------S------T-------        ********  ----------S
hGC33VHk        RV-------T-------S------T-------        ********  ----------S
```

(b)

```
                FR1                 CDR1                FR2
                ---------1---------2---  -----------3----  -----4---------
                12345678901234567890123  4567abcde8901234  567890123456789
mGC33VL         DVVMTQTPLSLPVSLGDQASISC  RSSQSLVHSNGNTYLH  WYLQKPGQSPKLLIY
hGC33VLa        ------S------TP-EP-----  ****************  ----------Q----
hGC33VLa_Arg    ------S------TP-EP-----  **********R*****  ----------Q----

                CDR2     FR3                                CDR3       FR4
                5------  ---6---------7---------8--------  -9-------  -10-------
                0123456  78901234567890123456789012345678  901234567  8901234567
mGC33VL         KVSNRFS  GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC  SQNTHVPPT  FGSGTKLEIK
hGC33VLa        *******  --------------------------V---Y-  *********  --Q-------
hGC33VLa_Arg    *******  --------------------------V---Y-  *********  --Q-------
```

(c)



Amino acid sequence of humanized GC33 heavy chain (VH) (a) and light chain (VL) (b) variable regions. mGC33VH and mGC33VL, respectively, indicate VH and VL variable regions of murine GC33 mAb. The VH of the human antibody DN13 was chosen as the framework for the humanized VH and the VL of human antibody K64 was chosen for the humanized VL. hGC33VHa, hGC33VHc, hGC33VHh, and hGC33VHk are different versions of the humanized VH variable region. hGC33VLa and hGC33VLa_Arg are different versions of the humanized VL variable region. Dashes represent amino acids that are the same as the corresponding residues in mouse GC33. Amino acids are numbered according to Kabat database.
(c) Structural model of GC33 antibody. Complementarity-determining regions (CDRs) of light chains are shown in dark gray. CDRs of heavy chains are shown in light gray. The five amino acid substitutions are highlighted in red. FR, framework region.

software (Montreal, Canada) (Fig. 1c), and five amino acid residues in FR3 (positions 66, 67, 69, 73, and 93) were selected to create a Vernier zone to adjust the CDR structure and fine-tune the fit to the antigen [21,27–30]. Additional versions were sequentially constructed by combining various amino acid residues of interest, and, as a result, two additional alterations, Ile69 to Leu (I69L) and Ala93 to Thr (A93T), were found to retain the full antigen binding activity with minimum alterations (Fig. 2a); the resultant humanized antibody was designated as hGC33VHc. In competitive binding assays, the humanized antibody hGC33VHc showed the same ability as chimeric GC33 to inhibit the binding of biotin-labeled murine GC33 to soluble GPC3 (Fig. 2b). The data were fitted to an equation for simple competition to provide the IC_{50}, yielding relative binding affinities (Fig. 2). To evaluate the antitumor activity, the humanized antibody and the chimeric antibody were subjected to ADCC assays using the GPC3-transfected cell line SK-03 with human peripheral blood mononuclear cells used as the target and effector cells, respectively. The chimeric antibody effectively induced ^{51}Cr release from the SK-03 cells in a dose-dependent manner. The humanized GC33 killed GPC3-expressing cells as effectively as the chimeric GC33 (Fig. 2c). These results indicate that the framework CDR in the humanized GC33 preserve the native CDR conformations.

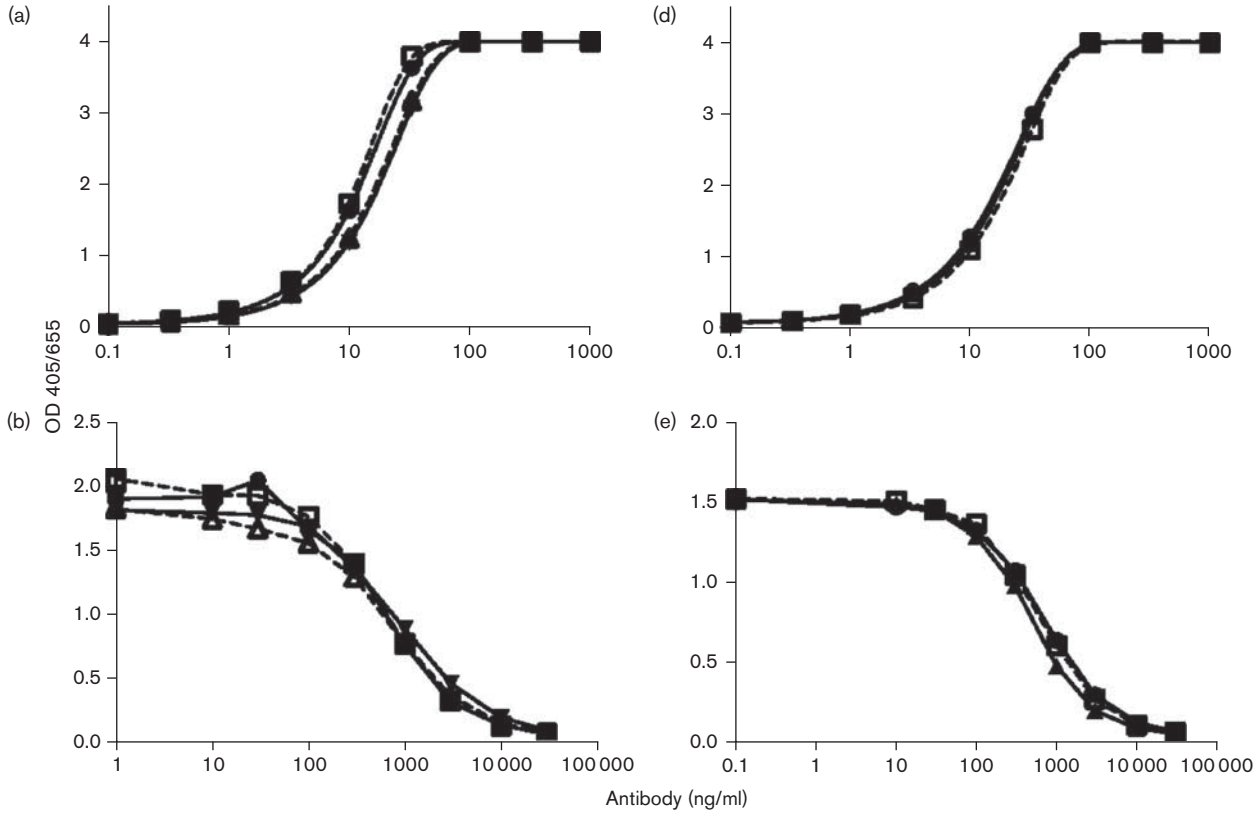
In contrast, to remove any artificial FR sequences in the humanized antibody, two-step design methods were carried out [23]. The hGC33VHc antibody was subjected to a homology search of the databases. The first half of the FR3 region (positions 66–82) of AB067018 and the last half of the FR3 region (positions 79–94) of AJ506422 showed higher similarity to humanized GC33VHc. Therefore, the humanized GC33 VH region was further engineered by introducing the alteration of Glu73 to Lys and Arg86 to Thr, which resulted in amino acid residues that matched the mosaic of the two FR3 sequences derived from AB067018 and AJ606422 (Fig. 1). This humanized antibody designed second, designated as hGC33VHh/hGC33VLa, and in which all the FRs were from naturally processed human FRs, bound to the antigen as effectively as the humanized antibody designed first (Fig. 2).

Optimization of humanized GC33

We performed further optimization of humanized GC33 using knowledge attained from earlier publications. First, we searched for amino acid residues with a risk of interfering with stable conformation. Jung *et al.* [31] reported that VH domain frameworks can be grouped into four distinct types, depending on the main chain conformation of framework 1. They established rules for the combinations of amino acids allowed at positions H6, H7, and H9 that maintain the stability of antibodies. The buried side chain of H6, which can be either a glutamate or a glutamine residue in almost all VH sequences, affects the stability of single chain variable fragment, and Glu in position H6 requires either a proline or a glycine residue in position H9. The VH domain of murine GC33 belongs to the structural subtype III (6Q7S9A), but the substitution of Gln6 to Glu occurred in the humanized GC33. This alteration causes a deviation from the combinations of amino acids allowed and may have a negative effect on the stability of the antibody. To assess this possibility, we made the alteration of Glu6 to Gln and the resulting hGC33VHk retained 100% of the antigen binding activity and ADCC activity (Fig. 2). Next, we compared the stability of humanized antibodies using an accelerated stability study. Three versions (hGC33VHc/hGC33VLa, hGC33VHh/hGC33VLa, and hGC33VHk/hGC33VLa) of humanized GC33 were formulated at a concentration of 0.5 mg/ml and stored at 60°C for 0, 1, and 2 weeks. The fragments and aggregates of the antibodies were detected by SEC. Figure 3a shows the rates of high molecular weight species and low molecular weight species. In the case of hGC33VHc/hGC33VLa, the rate of monomer antibodies decreased to 51% after incubation at 60°C for 2 weeks. In contrast, the monomer rate for hGC33VHk/hGC33VLa was greater than for either hGC33VHc/hGC33VLa or hGC33VHh/hGC33VLa. The test samples of the accelerated stability study were further analyzed for their binding activity against sGPC3. hGC33VHk/hGC33VLa retained the binding activity after incubation at 60°C for 2 weeks, whereas hGC33VHc/hGC33VLa and hGC33VHh/hGC33VLa showed about a two-fold decrease in the antigen binding activity (Fig. 3b). We concluded that the substitution of Gln6 with Glu improved the stability of the antibody.

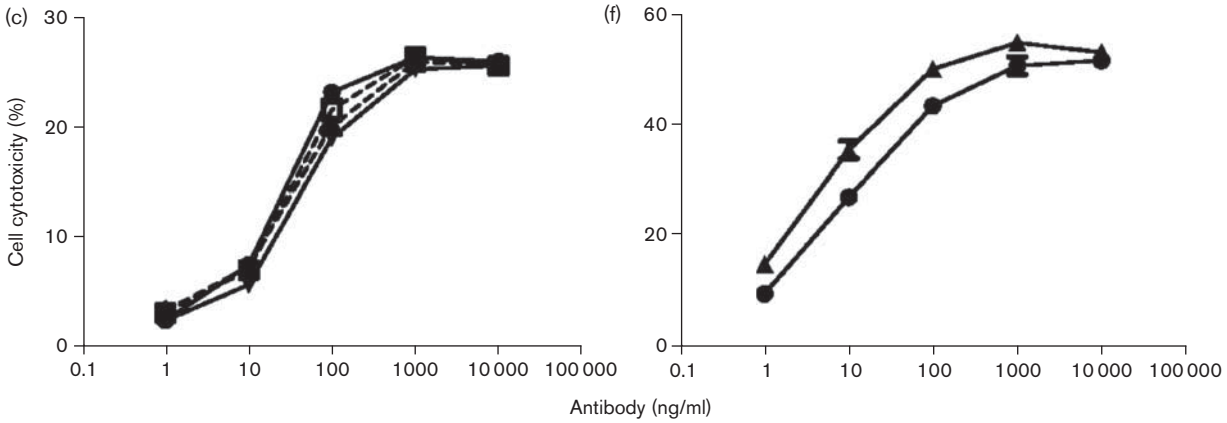
Next, we evaluated the risk of degradation from the amino acid residues of the antibodies. Nonenzymatic deamidation of Asn or Gln is a major degradation pathway of peptides and proteins and can occur spontaneously during manipulation, purification, and long-term storage. The reaction is initiated by deprotonation of the peptide bond nitrogen, followed by a nucleophilic attack on the side chain carbonyl, loss of ammonia, and the formation of succinimide. Succinimide is unstable and is hydrolyzed into a mixture of two products: isoaspartate and Asp [32]. Deamidation can cause structurally and biologically important alterations in proteins through the introduction of an unfavorable negative charge. Some mAbs have been reported to lose activity because of deamidation or isomerization in the CDR regions [33]. With regard to the primary sequence, deamidation rates depend on the amino acid residues adjacent to Asn in the peptide chain. Ser and Gly are found to be the most destabilizing C-terminal amino acids for the deamidation of Asn [34]. The humanized GC33 contains three Asn–Gly sequences. One of these sequences, Asn28 of the VL, is

Fig. 2



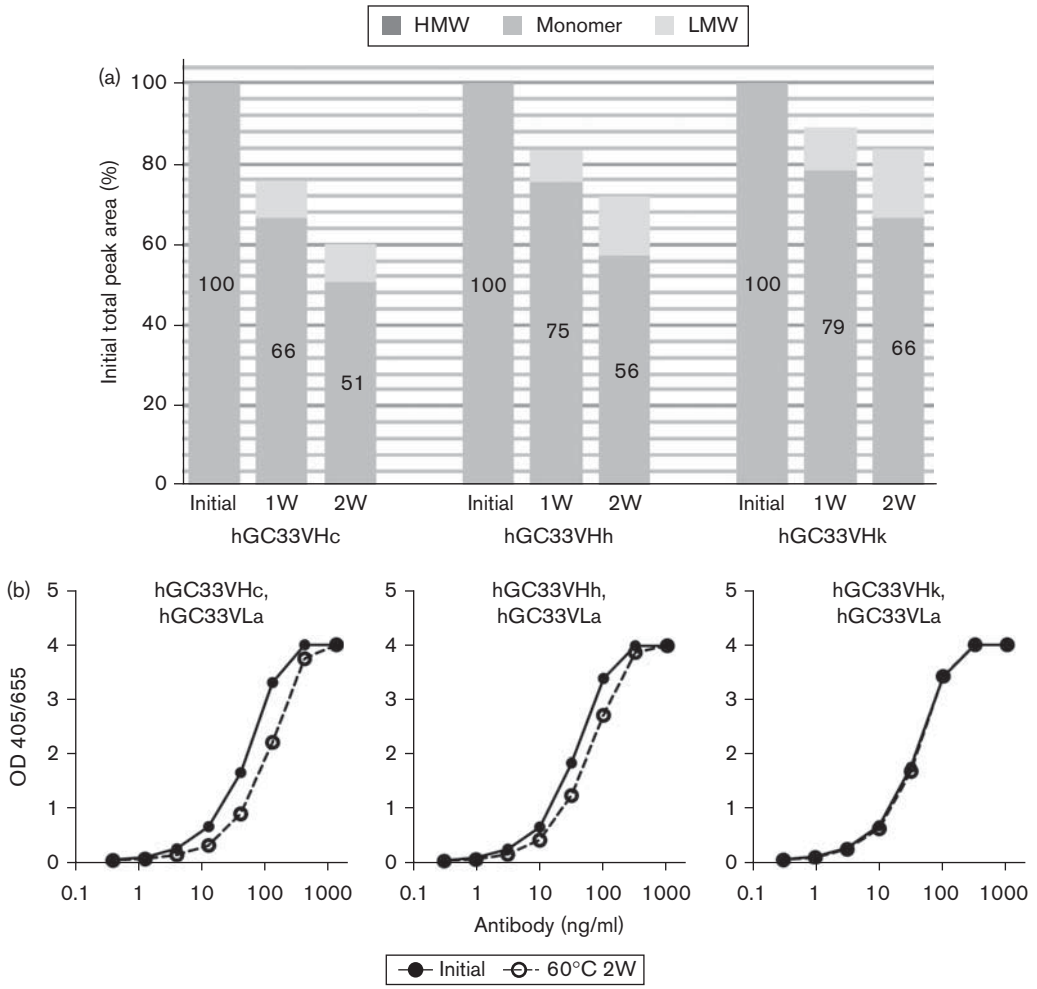
Antibody ID	IC_{50} (nmol/l)
mGC33VH/mGC33VL	4.10
hGC33VHc/hGC33VLa	5.86
hGC33VHh/hGC33VLa	4.85
hGC33VHk/hGC33VLa	4.18

Antibody ID	IC_{50} (nmol/l)
mGC33VH/mGC33VL	4.21
hGC33VHk/hGC33VLa	4.66
hGC33VHk/hGC33VLa_Arg	3.19



Analysis of chimeric and humanized GC33. (a and d) Binding of antibodies to soluble human glypican 3 (sGPC3) as measured by enzyme-linked immunosorbent assay. (b and e) Competitive binding assay measuring the ability of humanized and chimeric GC33 to compete with biotin-labeled murine GC33. The IC_{50} values are shown. (c and f) Antibody dependent cellular cytotoxicity. ^{51}Cr-labeled SK-03 was incubated with human peripheral blood mononuclear cells at an E : T ratio of 50 : 1 in the presence of the indicated concentration of the antibody. (a–c): hGC33VHc/hGC33VLa (▲), hGC33VHh/hGC33VLa (▽), hGC33VHk/hGC33VLa (●), mGC33VH/mGC33VL(□). (d–f): hGC33VHk/hGC33VLa (●), hGC33VHk/hGC33VLa_Arg (▲), mGC33VH/mGC33VL (□). OD, optical density; VH, heavy chain; VL, light chain.

Fig. 3



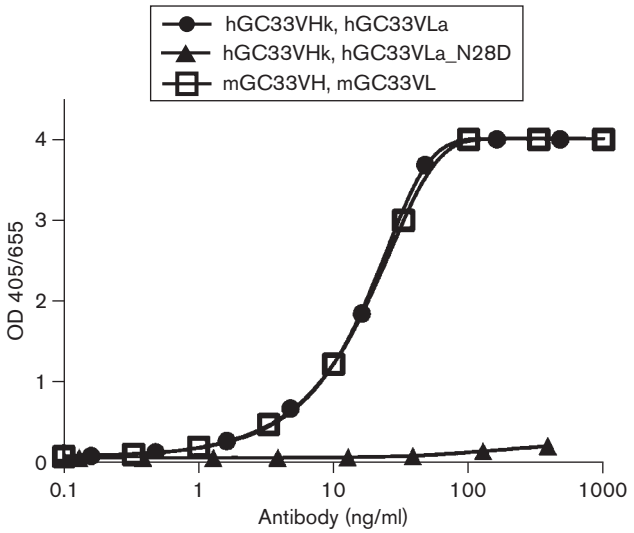
(a) Accelerated stability test of humanized GC33. Percentage of species with high molecular weight (HMW), low molecular weight (LMW), and monomer antibodies calculated from the peak of the size-exclusion chromatography of humanized antibodies (hGC33VHc/hGC33VLa, hGC33VHh/hGC33VLa, hGC33VHk/hGC33VLa) over 2 weeks of storage at 60°C, at 0.5 mg/ml formulated in 20 mmol/l sodium acetate, and 150 mmol/l NaCl at pH 6.0. (b) Binding activity of humanized GC33 after the accelerated stability test. (●): Binding activity after incubation at 4°C. (○): Binding activity after incubation at 60°C. OD, optical density; VH, heavy chain; VL, light chain.

in the binding region (CDR1), whereas the other two are in the immunoglobulin constant region.

To study the effect of deamidation on the activity of antibodies, we replaced Asn28 with Asp by site-directed mutagenesis (N28D), and found that mutant N28D greatly reduced the binding affinity to the antigen (Fig. 4). We concluded that Asn28 has a risk of deamidation that would result in the loss of binding activity. Site-directed mutagenesis to improve the stability of the mAbs by avoiding deamidation at 'hot-spot' residues such as Asn28 is not always feasible because this method may cause significant reduction in mAb potency. Therefore, we substituted the neighboring Gly29 by other amino acids to get rid of the deamidation. The mutant hGC33VLa_Arg, in which Gly29 of the VL was replaced with Arg, bound to the antigen as effectively as the chimeric antibodies (Fig. 2d and e) and showed ADCC activity as strong and as effective as hGC33VLa (Fig. 2f).

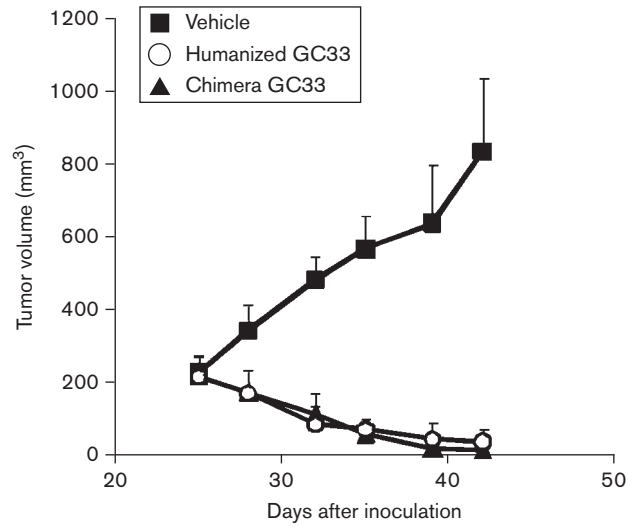
The final version of humanized GC33 (hGC33VHk, hGC33VLa_Arg) specifically bound to GPC3 and induced ADCC as effectively as chimeric GC33. The binding affinity of humanized GC33 was determined by Scatchard analysis, in which increasing concentrations of radioactive iodine-labeled antibodies were incubated with human hepatocellular carcinoma HuH-7 cells. The dissociation constant (or K_d value) of humanized GC33 for GPC3 was calculated to be 0.673 ± 0.024 nmol/l (mean ± standard deviation) from three independent experiments. When administered to the severe combined immunodeficient

Fig. 4



Antigen binding assays for humanized GC33 and N38D mutant that mimic the effect of deamidation. Serial dilutions of chimeric GC33 (mGC33VH/mGC33VL), humanized GC33 (hGC33VHk/hGC33VLa), and the deamidation mimic mutant (hGC33VHk/hGC33VLa_N28D) were added to soluble human glypican 3-coated wells. Bound antibodies were detected with alkaline phosphatase-labeled anti-human Ig light chain κ. OD, optical density; VH, heavy chain; VL, light chain.

Fig. 5



Antitumor activity of humanized GC33 and chimeric GC33 in HepG2 xenograft models. Severe combined immunodeficient mice were inoculated subcutaneously with 5×10^6 cells of HepG2. When the tumors reached an average volume of 200 mm^3, the mice were administered 5 mg/kg of humanized GC33 (○), chimera GC33 (▲) or only PBS (vehicle, ■). GC33 and PBS were administered once a week for 3 weeks. Tumor volume is indicated. Each data point is the mean value of five animals; bars, standard deviation. Arrows indicate the days on which GC33 was administered.

mice transplanted with HepG2 cells, 5 mg/kg of the humanized GC33 significantly inhibited tumor growth. The antitumor activity of the humanized GC33 was almost the same as chimeric GC33 (Fig. 5).

Discussion

To reduce the immunogenicity of murine mAbs, humanized antibodies have been developed by grafting the CDRs of a mouse antibody onto the corresponding regions of a human antibody. However, the transfer of murine CDRs alone usually results in a significant loss of antigen binding activity because certain FRs are critical for the preservation of the CDR conformation. A number of alterations in human FRs are required to recreate a functional antigen-binding site. The number of changes in the human FRs depends heavily upon human FR selection and should be minimized to avoid immunogenicity in humans. As a general approach, key residues in FRs are identified by comparing canonical structures [35,36] with packing residues and consensus amino acid sequences for the subgroups of the variable regions [30,37] and by carefully examining a structural model of the variable regions. In many cases, however, a random mutation approach is still required.

To assist in this process, we have devised a useful method based on the construction of mouse–human hybrid variable regions [22]. In the case of the humanized GC33, the use of a hybrid of variable region enabled us to identify the importance of the Ile69 and Ala93 in FR3, which was found to be critical for FR residues to yield 100% antigen binding activity. To remove the artificial FR sequences in the humanized antibody, we have also devised a useful method based on redesigning the modified FRs [23]. In the case of the humanized GC33, the FR3 of hGC33VHc, for which two amino acid substitutions were required for optimal antigen bindings, was redesigned. We constructed hGC33VHh, which comprised all the sequences found in the naturally processed human FRs. Although naturally processed human variable regions have somatic mutations with respect to the germ-line segments, their immunogenicity is apparently less than that of artificially generated FR sequences.

In addition, using a knowledge-based approach, we optimized the stability of humanized GC33. From data of earlier humanized antibodies obtained in-house and from public sources, we realized that certain residues were important for stability and therefore selected some amino acid residues for modification. According to Jung *et al.* [31], the marked structural variability of the VH framework 1 region is caused by three residues (H6, H7, H9), and deviation from the allowed combinations of these amino acids may lead to severely reduced stability. They generated six mutants of single chain variable fragment forms, and the thermodynamic stability was

compared after equilibrium denaturation with urea. In the case of GC33, we found the rules can be applied to the stability of IgG forms. Our data may not directly indicate long-term stability with normal storage conditions; however, we can conclude that substitution of amino acids is effective in improving the stability of antibodies.

Next, we substituted the amino acid residue at risk of deamidation in CDR1 of the VL. We found that the substitution of Asn28 with Asp, which mimics deamidation, greatly reduced the binding activity to the antigen. In this study, we successfully avoided the risk of the deamidation of Asn28 by substituting the neighboring Gly with Arg. Site-directed mutagenesis of Asn28 is not always feasible because this method may cause significant reduction in mAb potency. However, hGC33VLa_Arg retained the binding activity and the ADCC activity (Fig. 2). The parental humanized GC33 (hGC33VHk/hGC33VLa) did not cause a reduction in the binding activity even at 60°C for 2 weeks, so we have yet to prove whether this alteration (Gly29 to Arg) can reduce the rate of deamidation.

Humanized anti-GPC3 antibodies are expected to be a potent immunotherapy for HCC patients. The antitumor activity and immunogenicity of this humanized antibody in human patients will be tested in clinical studies.

Acknowledgements

The authors thank Y. Nakata and M. Ohtaka for their technical assistance. They also thank F. Ford for proofreading the manuscript.

References

1 Filmus J. Glypicans in growth control and cancer. *Glycobiology* 2001; **11**:19R–23R.
2 Pilia G, Hughes-Benzie RM, MacKenzie A, Baybayan P, Chen EY, Huber R, *et al.* Mutations in GPC3, a glypican gene, cause the Simpson–Golabi–Behmel overgrowth syndrome. *Nat Genet* 1996; **12**:241–247.
3 Cano-Gauci DF, Song HH, Yang H, McKerlie C, Choo B, Shi W, *et al.* Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson–Golabi–Behmel syndrome. *J Cell Biol* 1999; **146**:255–264.
4 Capurro MI, Xiang YY, Lobe C, Filmus J. Glypican-3 promotes the growth of hepatocellular carcinoma by stimulating canonical Wnt signaling. *Cancer Res* 2005; **65**:6245–6254.
5 Hsu HC, Cheng W, Lai PL. Cloning and expression of a developmentally regulated transcript MXR7 in hepatocellular carcinoma: biological significance and temporospatial distribution. *Cancer Res* 1997; **57**:5179–5184.
6 Sung YK, Hwang SY, Park MK, Farooq M, Han IS, Bae HI, *et al.* Glypican-3 is overexpressed in human hepatocellular carcinoma. *Cancer Sci* 2003; **94**:259–262.
7 Midorikawa Y, Ishikawa S, Iwanari H, Imamura T, Sakamoto H, Miyazono K, *et al.* Glypican-3, overexpressed in hepatocellular carcinoma, modulates FGF2 and BMP-7 signaling. *Int J Cancer* 2003; **103**:455–465.
8 Yamauchi N, Watanabe A, Hishinuma M, Ohashi K, Midorikawa Y, Morishita Y, *et al.* The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. *Mod Pathol* 2005; **18**:1591–1598.
9 Takai H, Kato A, Kato C, Watanabe T, Matsubara K, Suzuki M, *et al.* The expression profile of glypican-3 and its relation to macrophage population in human hepatocellular carcinoma. *Liver Int* 2009; **29**:1056–1064.
10 Takai H, Kato A, Kinoshita Y, Ishiguro T, Takai Y, Ohtani Y, *et al.* Histopathological analyses of the antitumor activity of anti-glypican-3 antibody (GC33) in human liver cancer xenograft models: the contribution of macrophages. *Cancer Biol Ther* 2009; **8**:930–938.
11 Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, *et al.* Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. *Cancer Sci* 2009; **100**:1403–1407.
12 Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, *et al.* Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology* 2003; **125**:89–97.
13 Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, *et al.* Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. *Biochem Biophys Res Commun* 2003; **306**:16–25.
14 Hippo Y, Watanabe K, Watanabe A, Midorikawa Y, Yamamoto S, Ihara S, *et al.* Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. *Cancer Res* 2004; **64**:2418–2423.
15 Ikuta Y, Nakatsura T, Kageshita T, Fukushima S, Ito S, Wakamatsu K, *et al.* Highly sensitive detection of melanoma at an early stage based on the increased serum secreted protein acidic and rich in cysteine and glypican-3 levels. *Clin Cancer Res* 2005; **11**:8079–8088.
16 Nakatsura T, Kageshita T, Ito S, Wakamatsu K, Monji M, Ikuta Y, *et al.* Identification of glypican-3 as a novel tumor marker for melanoma. *Clin Cancer Res* 2004; **10**:6612–6621.
17 Ushiku T, Uozaki H, Shinozaki A, Ota S, Matsuzaka K, Nomura S, *et al.* Glypican 3-expressing gastric carcinoma: distinct subgroup unifying hepatoid, clear-cell, and alpha-fetoprotein-producing gastric carcinomas. *Cancer Sci* 2009; **100**:626–632.
18 Nakano K, Orita T, Nezu J, Yoshino T, Ohizumi I, Sugimoto M, *et al.* Anti-glypican 3 antibodies cause ADCC against human hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 2009; **378**:279–284.
19 Ishiguro T, Sugimoto M, Kinoshita Y, Miyazaki Y, Nakano K, Tsunoda H, *et al.* Anti-glypican 3 antibody as a potential antitumor agent for human liver cancer. *Cancer Res* 2008; **68**:9832–9838.
20 Kabat EA, Wu TT, Reid-Miller M, Perry HM, Gottesman KS. *Sequence of proteins of immunological interest.* Washington, DC: United States Department of Health and Human Services, United States Government Printing Offices; 1991.
21 Sato K, Tsuchiya M, Saldanha J, Koishihara Y, Ohsugi Y, Kishimoto T, *et al.* Humanization of a mouse anti-human interleukin-6 receptor antibody comparing two methods for selecting human framework regions. *Mol Immunol* 1994; **31**:371–381.
22 Ohtomo T, Tsuchiya M, Sato K, Shimizu K, Moriuchi S, Miyao Y, *et al.* Humanization of mouse ONS-M21 antibody with the aid of hybrid variable regions. *Mol Immunol* 1995; **32**:407–416.
23 Ono K, Ohtomo T, Yoshida K, Yoshimura Y, Kawai S, Koishihara Y, *et al.* The humanized anti-HM1.24 antibody effectively kills multiple myeloma cells by human effector cell-mediated cytotoxicity. *Mol Immunol* 1999; **36**:387–395.
24 Chothia C, Lesk AM, Gherardi E, Tomlinson IM, Walter G, Marks JD, *et al.* Structural repertoire of the human VH segments. *J Mol Biol* 1992; **227**:799–817.
25 Tomlinson IM, Cox JP, Gherardi E, Lesk AM, Chothia C. The structural repertoire of the human V kappa domain. *EMBO J* 1995; **14**:4628–4638.
26 Smithson SL, Srivastava N, Hutchins WA, Westerink MA. Molecular analysis of the heavy chain of antibodies that recognize the capsular polysaccharide of Neisseria meningitidis in hu-PBMC reconstituted SCID mice and in the immunized human donor. *Mol Immunol* 1999; **36**:113–124.
27 Kettleborough CA, Saldanha J, Heath VJ, Morrison CJ, Bendig MM. Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation. *Protein Eng* 1991; **4**:773–783.
28 Foote J, Winter G. Antibody framework residues affecting the conformation of the hypervariable loops. *J Mol Biol* 1992; **224**:487–499.
29 Ellis JH, Barber KA, Tutt A, Hale C, Lewis AP, Glennie MJ, *et al.* Engineered anti-CD38 monoclonal antibodies for immunotherapy of multiple myeloma. *J Immunol* 1995; **155**:925–937.
30 Chothia C, Novotny J, Bruccoleri R, Karplus M. Domain association in immunoglobulin molecules. The packing of variable domains. *J Mol Biol* 1985; **186**:651–663.

31 Jung S, Spinelli S, Schimmele B, Honegger A, Pugliese L, Cambillau C, *et al.* The importance of framework residues H6, H7 and H10 in antibody heavy chains: experimental evidence for a new structural subclassification of antibody V(H) domains. *J Mol Biol* 2001; **309**:701–716.
32 Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. *J Biol Chem* 1987; **262**:785–794.
33 Huang L, Lu J, Wroblewski VJ, Beals JM, Riggin RM. *In vivo* deamidation characterization of monoclonal antibody by LC/MS/MS. *Anal Chem* 2005; **77**:1432–1439.
34 Robinson NE, Robinson AB. Molecular clocks. *Proc Natl Acad Sci U S A* 2001; **98**:944–949.
35 Chothia C, Lesk AM. Canonical structures for the hypervariable regions of immunoglobulins. *J Mol Biol* 1987; **196**:901–917.
36 Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, *et al.* Conformations of immunoglobulin hypervariable regions. *Nature* 1989; **342**:877–883.
37 Singer II, Kawka DW, DeMartino JA, Daugherty BL, Elliston KO, Alves K, *et al.* Optimal humanization of 1B4, an anti-CD18 murine monoclonal antibody, is achieved by correct choice of human V-region framework sequences. *J Immunol* 1993; **150**:2844–2857.