Glycoprotein A34, a novel target for antibody-based cancer immunotherapy

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To identify novel, tissue-restricted cell surface proteins in cancer which can serve as targets for antibody-based diagnostics and therapeutics, a translated version of the expressed sequence tag database (tblastn) was mined for transcripts with similarity to the glycoprotein A33 (GPA33) colon cancer antigen. A novel human transcript, termed A34, was identified which encoded a putative cell surface protein, GPA34, which is approximately 30% identical to GPA33 and other members of the junctional adhesion molecule (JAM) family. Conventional end-point and quantitative real-time RT-PCR showed that A34 mRNA expression is highly tissue-restricted, as it is expressed predominantly in stomach and testis. A34 mRNA was also detected in 6/19 (31%) gastric cancers, 8/16 (50%) esophageal carcinomas, and 4/17 (23%) ovarian cancers, but not in lung, breast or colon carcinomas. A murine monoclonal antibody (mAb A34) was generated to the extracellular domain of the A34 protein and used to biochemically and immunohistochemically characterize the A34 antigenic system. The mAb A34 specifically recognized glycoproteins ranging in apparent size from 55-70 kDa, present in normal gastric mucosa and in COS-7 cells transfected with A34 cDNA. Of 31 different normal tissues examined by immunohistochemistry, GPA34 protein expression was detected primarily in normal stomach mucosa and testicular germ cells, and in the tumor cells of 5/17 (29%) gastric cancers, 7/11 (63%) esophageal cancers, and 2/21 (9%) ovarian cancers, in agreement with gene expression results. The A34 antigen and monoclonal antibody may be of considerable value for immunotherapy of different types of cancer.

Keywords: human, membrane glycoproteins, amino acid sequence homology, A34, mRNA, tissue distribution, immunohistochemistry

Introduction

Recent progress in the bioengineering of immunoglobulin molecules has led to a new generation of therapeutic monoclonal antibodies for the treatment of human cancer (1, 2). For example, chimerization, veneering and humanization of murine mAb, as well as the generation of human mAb in transgenic systems, have been used to reduce or eliminate the intrinsic immunogenicity of xenogenic antibodies and their subsequent neutralization in vivo, while phage display technologies allowed for further improvements in their binding affinities and specificities. Strategies for improving the efficacy of existing therapeutic monoclonal antibodies have also been introduced and include conjugating antibodies to novel cytotoxic agents or radionuclides, activation of antibody/toxin conjugates with pre-targeted pro-drugs, and the coupling of traditional chemotherapy with monoclonal antibody-based therapies. Several humanized/chimerized antibodies have received U. S. Food and Drug Administration (FDA) approval for the treatment of cancer, including anti-c-erbB-2/Her2neu (Trastuzumab) for breast cancer (3), anti-CD20 (Rituximab) for non-Hodgkin’s lymphoma (4), anti-CD52 (Alemtuzumab) for B cell chronic lymphocytic leukemia (5), and both anti-EGFR (Cetuximab) (6) and anti-VEGF (Bevacizumab) (7) for colon cancer.

Immunotherapeutic antibodies typically recognize and bind to antigens expressed on the surface of cancer cells. Despite major progress in antibody engineering, there has been little progress in identifying new targets for therapeutic antibodies on the cell surface of cancer cells. Considering the recent advances in gene discovery emanating from the human genome, transcriptome, and proteome projects, the prospect of identifying new cell surface molecules expressed primarily in cancer is quite promising. Thus, in the current study we searched the human expressed sequence tag (EST) database for novel transcripts encoding tissue-restricted cell surface proteins which could potentially serve as new targets for monoclonal antibody-based diagnostics and therapeutics. Glycoprotein A33, which is currently the focus of therapeutic antibody trials with humanized antibodies (8, 9, 10, 11, 12), was used as a model cell surface target. GPA33 antigen (43 kDa) is expressed predominately in normal human colonic mucosa and in 95% of primary and metastatic colorectal carcinomas (9). The A33 antigen belongs to the JAM family (13), which includes molecules such as the cossackie and adenovirus receptor (CXADR; 14), the cossackie and adenovirus receptor-like membrane protein (CLMP; 15), the cortical thymocyte receptor-like protein (CTXL; 16), the JAM-A/F11 receptor (17), JAM-B/VE-JAM (18), the JAM-C/Mac-1 receptor (19), the endothelial cell-selective adhesion molecule (ESAM; 20), and the brain/testis-IGSF protein (21). Members of the JAM family localize to tight and adhesion junctions of epithelial and...
endothelial cells, and are believed to mediate cell-cell adhesion (13).

Here we describe the identification and characterization of a new member of the JAM family, which is similar to GPA33, which we termed A34. This novel antigen was expressed, both at the mRNA and protein level, in gastric, esophageal, and ovarian cancers, while expression in normal tissues was restricted to testis and stomach. Our data indicate that the A34 antigenic system and the anti-A34 monoclonal antibody generated during the course of these studies may be of diagnostic and therapeutic value.

Results

Identification of the A34 mRNA transcript

In order to identify tissue-restricted, cell surface molecules with sequence similarity to the GPA33 colon cancer antigen, the amino acid sequence of GPA33 was compared with a translated, non-redundant nucleotide database (tblastn; 22). A novel transcript termed A34 was identified which, upon hypothetical translation, showed 32% amino acid identity with GPA33 (Figure 1), including limited conservation of a putative signal sequence, immunoglobulin (Ig)-like domains and a transmembrane domain. These features indicate that the A34 transcript encodes a cell surface protein. The A34 transcript is represented by Unigene cluster Hs.177164, which contains a full-length testis-derived cDNA clone, MGC:44287 (GenBank Acc. No. BC043216), as well as 19 other homologous ESTs, derived from normal testis (7 ESTs), normal stomach (3 ESTs), normal aorta (1 EST), normal nasopharynx (2 ESTs), and pooled normal tissues (2 ESTs), as well as uterine cancer (2 ESTs), pancreatic cancer (1 EST) and acute myelogenous leukemia (1 EST). This limited tissue distribution of homologous ESTs suggests that the A34 transcript is differentially expressed.

The full length A34 transcript, represented by testis-derived cDNA clone MGC:44287, consists of 3017 nucleotides, a length which is in agreement with the single hybridization signal of 3.1 kb detected on Northern blots of testis mRNA hybridized with an A34 cDNA probe (data not shown). The A34 transcript, as represented by MGC:44287, contains a 122 bp 5’ untranslated sequence, a 1731 bp 3’ untranslated sequence, and a 1164 bp protein coding sequence. The A34 nucleotide sequence was verified by sequencing an additional full length A34 EST clone, IMAGE:5266771 (GenBank Acc. No. BI459739), as well as four independently isolated cDNA clones generated by RT-PCR of human testis RNA and which encompass the entire protein coding region of A34. Both strategies yielded cDNA sequences identical to MGC:44287 in the protein coding regions, although IMAGE:5266771 contained a 712 bp deletion in the 3’ untranslated region corresponding to nucleotides 1702-2413 of MGC:44287.

Expression profile of A34 mRNA

Both conventional end-point RT-PCR and real-time quantitative RT-PCR were used to determine the mRNA expression profile of A34. End-point RT-PCR analysis detected A34 mRNA transcripts in normal testis and stomach, but not in 20 other normal adult tissues (Figure 2). In order to further investigate this tissue-restricted expression profile of A34 mRNA, real-time quantitative RT-PCR was performed using a normalized cDNA panel derived from 19 normal adult tissues. As shown in Table 1, A34 mRNA was expressed at high levels (at least 0.5 fg of cDNA template or a copy number of about 3000) in testis and stomach. Only trace levels of A34 mRNA (equivalent to 0.07 – 0.001 fg of cDNA template) were detected in 14 other normal tissues (pancreas, PBLs, spleen, esophagus, lung, brain, thymus, cervix, heart, liver, placenta, small intestine, breast, adrenal gland). A34 transcripts were not detected in ovary, prostate, colonic mucosa, kidney, and skeletal muscle.

A34 mRNA expression was also examined in various tumor types by real-time quantitative RT-PCR. As shown in Table 1, moderate to high levels of A34 mRNA expression was detected in 6/19 (31%) gastric cancer specimens, 8/16 (50%) esophageal cancer specimens, and 4/17 (23%) ovarian cancer specimens. Only trace levels of A34 mRNA were detected in breast cancer (n = 13), colon cancer (n = 5), and lung cancers (n = 19). Thus, in the cDNA panels examined, A34 mRNA expression was...
Expression of A34 mRNA transcripts in various normal and malignant human tissues. A34 mRNA expression was analyzed by conventional end-point RT-PCR analysis in normal adult tissues using a commercially available source of cDNA templates (Rapid-Scan™ Gene Expression Panels, Origene, Rockville, MD). Four different cDNA template concentrations were analyzed ranging from 0.001 ng to 1.0 ng. At cDNA concentrations of 1.0 ng and 0.1 ng, A34 transcripts were detected only in normal testis and stomach, but not in 20 other normal tissues. A34 transcripts were not detected at lower cDNA concentrations. Lane assignments: 1, brain; 2, heart; 3, kidney; 4, spleen; 5, liver; 6, colon; 7, lung; 8, small intestine; 9, muscle; 10, stomach; 11, testis; 12, placenta; 13, salivary gland; 14, thyroid; 15, adrenal gland; 16, pancreas; 17, ovary; 18, uterus; 19, prostate; 20, skin; 21, PBLs; 22, bone marrow.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>A34 mRNA Expression (fg cDNA⁻¹)</th>
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<tbody>
<tr>
<td>Normal Tissues</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>0.000</td>
</tr>
<tr>
<td>PBLs</td>
<td>0.634</td>
</tr>
<tr>
<td>Prostate</td>
<td>0.000</td>
</tr>
<tr>
<td>Spleen</td>
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<tr>
<td>Testis</td>
<td>3.380</td>
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<tr>
<td>Thymus</td>
<td>0.008</td>
</tr>
<tr>
<td>Brain</td>
<td>0.009</td>
</tr>
<tr>
<td>Heart</td>
<td>0.001</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.000</td>
</tr>
<tr>
<td>Liver</td>
<td>0.001</td>
</tr>
<tr>
<td>Lung</td>
<td>0.011</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.001</td>
</tr>
<tr>
<td>Heart</td>
<td>0.001</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>0.000</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.070</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.003</td>
</tr>
<tr>
<td>Colon</td>
<td>0.000</td>
</tr>
<tr>
<td>Breast</td>
<td>0.003</td>
</tr>
<tr>
<td>Stomach</td>
<td>7.434</td>
</tr>
<tr>
<td>Esophagus</td>
<td>0.013</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.002</td>
</tr>
<tr>
<td>Cervix</td>
<td>0.006</td>
</tr>
<tr>
<td>Tumor Tissues</td>
<td></td>
</tr>
<tr>
<td>Gastric cancer (n = 19)</td>
<td>0.047, 0.004, 0.001, 0.003, 1.310, 0.570, 0.870, 0.003, 0.000, 0.270, 0.001, 3.170, 0.047, 0.060, 5.390, 0.360, 0.000, 1.230, 0.010</td>
</tr>
<tr>
<td>Esophageal cancer (n = 16)</td>
<td>2.210, 0.037, 0.345, 5.630, 0.651, 0.092, 1.159, 13.82, 0.064, 1.770, 0.706, 0.145, 0.010, 4.450, 8.148, 0.0577</td>
</tr>
<tr>
<td>Ovarian cancer (n = 17)</td>
<td>0.013, 0.001, 0.003, 2.090, 0.002, 0.024, 0.024, 0.003, 6.290, 0.530, 0.003, 0.010, 0.001, 0.003, 0.540, 0.003, 0.002</td>
</tr>
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</table>

The mRNA concentration is expressed as an equivalent value of A34 cDNA starting template in a 40 cycle real-time (TaqMan) PCR reaction.

The A34 protein

The predicted ATG start site, present at nucleotide 123 of clone MGC:44287, conforms to the Kozak consensus sequence for initiation of protein translation, and is followed by the longest possible open reading frame which is 1161 bp in length. The predicted A34 protein (Figure 3) consists of 387 amino acids (Mr 41,816), comprising three structural domains: an extracellular domain of 233 amino acids, a transmembrane domain of 23 amino acids, and an intracellular domain of 131 amino acids. The N-terminal 21 amino acids form a putative hydrophobic signal sequence with a possible cleavage site between residues 21 and 22. Amino acid residues 33-123 encompass an N-terminal, V-type immunoglobulin (Ig)-like domain containing two cysteine residues (C43, C116), which are predicted to form a disulfide bond. A segment of 31 amino acids separate the V-type Ig-like domain, from a second Ig-like domain of the C₂₄-type present at residues 154-218, which also contain two cysteine residues (C161, C211). A putative third disulfide loop is formed between cysteine residues C154 and C218 that span the C₂₃ loop. There are seven potential N-linked glycosylation sites in the extracellular domain of A34 (N32, N38, N98, N133, N190, N200, and N219). Given that the average size of an oligosaccharides chain is approximately 2.5-3.0 kDa, the carbohydrate portion of A34 could potentially contribute approximately 17.5-21 kDa. Thus the predicted size of the mature A34 protein (excluding the signal peptide of 2.3 kDa) is 57-60.5 kDa.

The amino acids in proximity to the carboxyl terminus of A34 form transmembrane and cytoplasmic domains. Hydrophobicity plots and transmembrane domain prediction software (23, 24) located a transmembrane domain at residues 234-256. This is followed by a C-terminal intracellular domain encompassing residues 257-387. Using functional site prediction software (25), the A34 intracellular domain was found to contain eight sites of potential serine/threonine...
Figure 3

Cartoon illustrating the similarities between the A34 and A33 protein structures.

Figure 4

Reactivity of monoclonal antibody A34 in SDS-PAGE immunoblots. (A) The mAb A34 reacts with proteins having apparent molecular weights (lane 1, MW standards) of 61-70 kDa under reducing conditions (lane 2), and 55-67 kDa under non-reducing conditions (lane 3) in detergent lysates from COS cells transfected with an A34 encoding plasmid, but not with detergent lysates from COS cells transfected with an A33 encoding plasmid (lane 4). (B) The mAb A34 reacts with proteins ranging in size from 45-70 kDa (lane 1, MW standards) in detergent lysates of human gastric mucosa (lane 2), but not in detergent lysates of human colonic mucosa (lane 3). Conversely, mAb A33 reacts with a protein having apparent molecular weights of 43 kDa in detergent lysates of human colonic mucosa (lane 4), but not in detergent lysates of human gastric mucosa (lane 3). (C) The mAb A34 reacts with proteins ranging in size from 45-70 kDa (lane 1, MW standards) in human gastric mucosa (lane 2), which upon treatment with N-glycanase PNGaseF, sialidase A, O-glycanase, β-(1-4) Galactosidase, and β-N-Acetylglucosaminidase (lane 3) are deglycosylated to proteins ranging in size from 33-50 kDa.

phosphorylation (casein kinase II phosphorylation sites) and three potential GSK3 phosphorylation sites. Two TRAF2-binding consensus motifs are present at amino acids 314-317 and 324-327. Furthermore, a unique pattern of glutamic acid/proline (‘EP’) repeats is found proximal to the carboxyl terminus of A34.

The domain organization and amino acid sequence of A34 places it in the junctional adhesion molecule family. Members of the JAM family are characterized by two extracellular Ig-like domains (V and C2 type), conserved cysteine residues in the extracellular domain, and a single transmembrane domain. An alignment of the amino acid sequences of A34, A33 and other members of the JAM-IgSF is provided in Supplementary Figure 1. The A34 amino acid sequence of the extracellular domain is 31%, 34%, 29%, 25%, 25%, 25%, 31% and 33% identical to A33 (75 amino acid overlap), CTXL (76 amino acid overlap), JAM-B (56 amino acid overlap), CXADR (58 amino acid overlap), JAM-A (59 amino acid overlap), JAM-C (58 amino acid overlap), ESAM (70 amino acid overlap), CLMP (62 amino acid overlap) and BT-IgSF (77 amino acid overlap) respectively, with conservation of at least 4 of the 6 cysteine residues in the extracellular domain. The intracellular domains of the JAM family, including A34, are poorly conserved.

In order to identify and characterize the A34 protein in vitro, a murine monoclonal antibody was generated to the extracellular domain of A34 (amino acids 35-231). In an immunoblot analysis, mAb A34 recognized two distinct protein bands in detergent lysates from COS-7 cells transfected with a plasmid encoding the full length A34 protein (Figure 4A). The proteins had apparent molecular sizes of 61 kDa and 70 kDa under reducing, and 55 kDa and 67 kDa under non-reducing, conditions in SDS-PAGE gels. This indicated that the proteins contain disulfide bonds. No mAb A34 reactivity was seen with COS-7 cells transfected with a plasmid encoding the full length A34 protein (Figure 4A). The proteins had apparent molecular sizes of 61 kDa and 70 kDa under reducing, and 55 kDa and 67 kDa under non-reducing, conditions in SDS-PAGE gels. This indicated that the proteins contain disulfide bonds. No mAb A34 reactivity was seen with COS-7 cells transfected with a plasmid encoding the closely related A33 antigen. Analysis of detergent lysates prepared from fresh human gastric mucosa, and colonic mucosa with mAb A34 (Figure 4B) detected at least four proteins of 45-70 kDa in the former, but not in the latter, samples. In contrast, mAb A33 reacted with a 43 kDa protein (A33 antigen) in human colonic mucosa, but not in human gastric mucosa.
To investigate if the A34 antigen is glycosylated, NP-40 (1%) lysates of human gastric mucosa were incubated with various exoglycosidases, and the digests subjected to immunoblot analysis using mAb A34. Enzymatic treatment with an exoglycosidase cocktail consisting of N-glycanase PNGaseF, sialidase A, O-glycanase, β(1-4)-Galactosidase, and β-N-Acetylglucosaminidase, resulted in the complete disappearance of the 55 kDa and 70 kDa mAb A34-reactive proteins and the appearance of five A34-reactive proteins with apparent molecular sizes of 50 kDa, 47 kDa, 44 kDa, 35 kDa, and 33 kDa (Figure 4C). A similar banding pattern was observed with N-glycanase PNGaseF digestion alone. Digestion of NP-40 lysates of gastric mucosa with sialidase or sialidase and O-glycanase did not result in any detectable downshift of the mAb A34-reactive bands. These results suggest that A34 protein is highly N-glycosylated and appears not to contain major amounts of O-linked carbohydrates or sialic acid in its sugar moieties.

Immunohistochemical analysis of A34 protein expression

In a panel of 31 normal tissues (Table 2), A34 protein was detected by immunohistochemical analysis only in stomach mucosa (Figure 5, panels A and C), testis (Figure 5D), and to a much lesser degree in pancreas (Figure 5E). In stomach, the epithelial cells throughout the entire mucosa were stained (Figure 5A). No other tissue component was stained. The mucosal cells showed a typical membranous staining pattern (Figure 5, panels B and C). A similar staining pattern was observed in ductal epithelial cells of the pancreas; however in pancreas only focal cells were immunopositive (Figure 5E). In testis, only germ cells were stained with mAb A34. No staining was observed in the testicular interstitial tissue (Figure 5D).

A limited number of malignant tumors were analyzed. In gastric adenocarcinomas, 5/17 samples stained with mAb A34 and showed a mostly heterogeneous labeling, with only occasionally a homogeneous labeling of tumor cells (Figure 5, panels F and G). As in normal gastric mucosal epithelium, the staining pattern in tumor cells was membranous. In esophageal adenocarcinoma, 7/11 (63%) tumors were A34-positive, while only 2/21 (9%) ovarian cancer specimens showed A34 expression. No A34 expression was detected in eight colonic carcinomas.

The A34 gene

Analysis of the human genome database mapped the A34 gene to chromosome Xq22.1-22.3 and revealed no other sequences with a high sequence similarity, suggesting that A34 is a single
copy gene. The A34 gene is approximately 34 kb in length, equivalent to nucleotides 25298366 – 25332446 of the chromosome X genomic contiguous sequence, NT 011651.14. The A34 gene spans seven exons, whereby exon 1 encodes the 5’ untranslated region and a large portion of the signal sequence, exons 2 and 3 encode the V₁H-type Ig-like domain, exons 4 and 5 encode the C₂-like domain, exon 6 encodes the transmembrane domain and a portion of the cytoplasmic domain, and exon 7 encodes the remainder of the cytoplasmic domain and the 3’ untranslated region.

The murine A34 gene orthologue

A putative murine orthologue of A34 was identified on the basis of nucleotide similarities, tissue distribution of homologous ESTs, protein similarities, chromosomal location, and gene structure. Comparison of the human A34 nucleotide sequence with the mouse EST database showed more than 83% nucleotide identity with EST sequences belonging to murine Unigene cluster Mm.66893, which contains a full length murine EST clone, RIKEN cDNA 4930405J24 (GenBank Acc. No. NM_030181). There are currently 31 sequences in this Unigene cluster: 23 ESTs from normal testis, six ESTs from normal stomach and two ESTs from normal cecum. The full length mRNA transcript consists of 2182 nucleotides and encodes a protein of 407 amino acids, which is 73% identical (330 amino acids overlap) to human A34 and has a similar domain structure (233 amino acids, a transmembrane domain of 23 amino acids, and an intracellular region of 131 amino acids). There are 2 Ig-like domains (V₁H-type and C₂-like) and 7 sites of potential N-linked glycosylation in the extracellular region of the A34 protein. The protein has an apparent molecular weight of 45-70 kDa in both human gastric mucosa and COS-7 cells transfected with the A34 cDNA, consistent with the expected molecular weight of 54-58 kDa, predicted from nucleic acid translation and N-linked glycosylation motif analysis of the A34 transcript. Further analysis of the carbohydrate portion of A34 showed the presence of large amounts of N-linked glycosyl residues, with no obvious evidence for O-linked carbohydrates and sialic acid. The 70 kDa immunoreactive protein may represent a highly glycosylated form of the A34 protein. Thus, like GPA33 (9, 31, 32), naturally occurring A34 is a glycoprotein composed primarily of N-linked sugars. We propose terming the A34 protein, GPA34. On the basis of amino acid identities and protein domain similarities, GPA34 is a new member of the JAM family and the immunoglobulin superfamily. This is further substantiated by similarities in the organization of the GPA34 and other JAM family member genes, all of which encompass 7 exons encoding the same structural regions of their respective proteins. From a functional standpoint, members of the JAM family are believed to play a role in the assembly and stabilization of tight junctions via carboxyl terminal interactions with PDZ-domain containing scaffolding proteins (e.g. ZO-1), and by engaging in amino terminal homophilic interactions that link adjacent endothelial and epithelial cells (13).

In terms of cancer diagnosis and immunotherapy, the most intriguing aspect of A34 is its tissue-specific expression. Interestingly, although GPA33 and GPA34 are similar at a structural level, there are clear differences in their expression patterns. For example, GPA34 is expressed in normal stomach mucosa as well as in tumors of the upper gastrointestinal tract, while GPA33 is expressed in the lower gastrointestinal tract and associated malignancies (8). Furthermore, GPA34 differs from GPA33 in that it is mostly heterogeneously expressed, and is found in approximately 30% of gastric and 60% of esophageal cancers, while GPA33 is generally homogeneously expressed, and found in approximately 95% of colon cancers. GPA34 expression in normal tissues further distinguishes it from GPA33, but not other JAM family members such as JAM-B and BT-IgSF, which are also expressed in testicular germ cells (21, 33). The promoters for both GPA33 (34) and GPA34 contain conserved binding sites for the intestinal specific transcription factor, CDX1, which may account for their restricted expression in gastrointestinal tissues. In contrast to the GPA33 promoter, the GPA34 promoter also contains conserved binding sites for testis-related transcription factors, SRY and SOX-5, which may
account for its additional tissue-restricted expression in testis (unpublished data).

Several antigens are currently being evaluated for use as targets for antibody therapy of gastric cancer and/or adjuvants to cancer chemotherapy, such as Her2neu (35), EGFR (36), MUC1 (37), CEA (38), Lewis Y (39), EpCAM (40), ST4 (41), CC49 (42) and SC-1/CD55 (43). A number of difficulties have arisen with several antibodies directed against these targets, mainly the lack of tumor specificity, an inability to penetrate solid tumors, low binding affinities, the formation of immune complexes with shed antigen, and the neutralization of the antibody construct by host immune responses. Most of these shortcomings may be addressed through molecular reengineering of the antibodies into constructs that are more human (chimeric, veneered or humanized antibodies) or of different molecular size. Regarding gastric cancer, clinical trials have been initiated with a chimeric antibody to the Lewis Y antigen (39, 44) and a human antibody to SC-1 (43). Two other chimerized/humanized antibodies, Herceptin (anti-Her2neu; 35) and Ch F11-39 (anti-CEA; 38), have undergone pre-clinical evaluation in gastric cancer models. At present, the monoclonal antibody generated to GPA34 is being reengineered to become more human with the aim of initiating phase I imaging and therapeutic trials in patients with gastric and esophageal cancers.

Abbreviations
Ct, cycle threshold; GPA33, glycoprotein A33; GPA34, glycoprotein A34; JAM, junctional adhesion molecule

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were purchased from commercial sources (Rapid-Scan™ Gene Incorporated (Palo Alto, CA) and Ambion Incorporated the guanidinium thiocyanate method. Additional normal tissue
of the New York Branch of the Ludwig Institute for
samples, and standard archival formalin-fixed, paraffin-
Nordwest (Frankfurt, Germany) with patient consent and
Sloan-Kettering Cancer Center, New York Presbyterian
Tissue specimens and cells
Total RNA from tumor tissues and cell lines was prepared by
the guanidinium thiocyanate method. Additional normal tissue
RNA samples were purchased from Clontech Laboratories
Incorporated (Palo Alto, CA) and Ambion Incorporated (Austin, TX). The cDNA preparations derived from tumor
tissues and select normal tissues were prepared as previously
described (45). Additional normal tissue cDNA preparations
were purchased from commercial sources (Rapid-Scan™ Gene
Expression Panels, Origene, Rockville, MD; MTC™ panels I and
II, BD Biosciences, Palo Alto CA).

RT-PCR analysis
Both conventional end-point RT-PCR and quantitative real-time
RT-PCR were performed. For end-point RT-PCR, oligonucleotide primers homologous to A34 were synthesized commercially
(A34 forward primer: 5’-ACTGTTGAGATCTAATGTCAC-3’, A34 reverse primer: 5’-AAGTTCTACACACAGCT-3’; Invitrogen Life
Technologies). The normal tissue cDNA templates used in end-point RT-PCR were derived from 24 human tissues (Rapid-Scan™ Gene Expression Panels, Origene), and were assayed at 4 different concentrations covering a 4-log range of approximately 0.001 ng - 1 ng cDNA. Thirty-five PCR amplification cycles
were performed at an annealing temperature of 60°C. The identity of selected PCR products was verified by DNA
sequencing (Cornell University DNA Services, Ithaca, NY).

For quantitative real-time RT-PCR, A34-specific TaqMan probes and PCR primers were designed using Primer Express
software (Applied Biosystems, Foster City, CA) and synthesized commercially (A34 tagman forward primer: 5’-GGAGGAGATGGGCCAATTCTATT-3’; A34 tagman reverse primer: 5’-CTCTGTATTAGATCTTTTAAATGCCC-3’; A34 tagman probe: 5-(and)-carboxylfluorescine (FAM) labeled 5’-CTTTTCTCAAGGTGGACAAGCTGTAGCCATT-3’; Invitrogen Life Technologies). Multiplex PCR reactions were prepared using 2.5 µl of cDNA diluted in TaqMan PCR Master Mix supplemented with VIC labeled human β-glucuronidase (GUS) endogenous control probe/primer mix (Applied Biosystems), 200 nM 6-FAM labeled gene-specific TaqMan probe, and 900 nM A34 forward and reverse primers. Forty PCR amplification cycles were performed at an annealing temperature of 60°C. Thermal cycling and fluorescent monitoring were performed using an ABI 7700 sequence analyzer (Applied Biosystems). The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct), was determined for each sample.

The relative level of A34 mRNA expression was measured by real-time RT-PCR using commercially-prepared, normalized
cDNA samples from 16 non-malignant tissues (MTC™ panels I and
II, BD Biosciences), as well as from laboratory-prepared cDNA preparations derived from various normal and tumor
tissues. Triplicate PCR reactions were prepared for each cDNA
sample, and the Ct determined for each sample. Resultant Ct
values were further normalized by subtracting the Ct value
obtained from the GUS endogenous control (ΔCt = Ct FAM –
Ct VIC). The concentration of A34 specific mRNA in experimental samples relative to normal tests was calculated by
subtracting the normalized Ct values obtained with normal
tests from those obtained with similarly prepared experimental
samples (e.g. ΔΔCt = ΔCt of tumor - ΔCt of normal tests), and
the relative concentration was determined (relative concentration = 2-ΔΔCt). The A34 transcript copy number was calculated by multiplying the relative concentration by the A34 transcript copy number present in a calibrator source of testis-
derived cDNA (MTC™ panel II, BD Biosciences).

Northern blot analysis
A Northern blot containing polyA+ RNA (2 µg/lane) from various normal tissues was obtained commercially (BD
Biosciences). An A34 cDNA probe (nucleotides 222-543 of
MG44287) was labeled using the Bright star Psoralen-Biotin
Kit (Ambion Inc., Austin, TX) and hybridized to the membrane

Materials and methods
Tissue specimens and cells
Tumor and normal tissues were obtained from Memorial Sloan-Kettering Cancer Center, New York Presbyterian Hospital, Roswell Park Cancer Institute, and Krankenhaus Nordwest (Frankfurt, Germany) with patient consent and Institutional Review Board approval. The specimens consisted of various normal tissues was obtained commercially (BD Biosciences). An

RNA and cDNA preparations
Total RNA from tumor tissues and cell lines was prepared by

Northern blot analysis
A Northern blot containing polyA+ RNA (2 µg/lane) from various normal tissues was obtained commercially (BD
Biosciences). An A34 cDNA probe (nucleotides 222-543 of
MG44287) was labeled using the Bright star Psoralen-Biotin
Kit (Ambion Inc., Austin, TX) and hybridized to the membrane
for 15 hours at 68°C. After washing, the hybridization signal was developed using the Bright Star Bio-Detect Kit, according to the manufacturer’s instructions (Ambion).

**Generation of anti-A34 monoclonal antibodies**

A PCR fragment corresponding to nucleotides 223-817 of MGC44287 encoding amino acid residues 35-231 of the extracellular domain was subcloned into the pET23d expression plasmid (Novagen/EMD Biosciences, Madison, WI) and the protein expressed in the *E. coli* strain BL21. The protein was purified as per the manufacturer’s protocol.

Murine hybridomas were generated as follows. BALB/c mice were immunized three times intraperitoneally, at 3-week intervals, with 10 µg purified A34 recombinant protein in adjuvant (Monophosphoryl Lipid A/Trehalose Dicorynomycolate, Sigma, St. Louis, MO). A booster injection consisting of 10 µg purified A34 recombinant protein alone was given three days prior to fusion. Spleen cells from immunized mice were fused with the mouse myeloma cell line SP2/0, and supernatants from sequentially cloned populations were screened against the immunizing protein by solid phase ELISA. In the case of positive supernatants, this was followed by immunohistochemistry and immunoblotting (see below).

**Immunohistochemistry**

Various dilutions of hybridoma supernatants were screened for immunoreactivity on frozen tissues with known A34 mRNA expression. Cuts (5 µm) of snap-frozen, O.C.T.™-embedded specimens were fixed in cold acetone for 10 minutes. Endogenous peroxidase activity was blocked with 1% H₂O₂ for 10 min. Primary antibody was incubated overnight at 5°C, and subsequently detected with a biotinylated horse-anti-mouse Ig (Vector, Labs, Burlingame, CA), followed by an avidin-biotin complex system (ABC-Elite, Vector Labs). The chromogen 3,3’-diaminobenzidine (Liquid DAB, BioGenex, San Ramon, CA) was used, and hematoxylin was used as a counterstain. Monoclonal antibodies from three IgG1 producing hybridomas, #342, #564, and #970, were shown to have identical recognition patterns and specificities. The antibody produced by hybridoma #342, termed monoclonal antibody A34 (mAb A34), was purified by protein G affinity chromatography and used in subsequent experiments. In order to analyze the expression profile of A34 protein by immunohistochemistry, formalin-fixed paraffin embedded tissues were probed with purified A34 antibody after antigen retrieval as previously described (45).

**COS-7 cell transfectants**

A cDNA fragment corresponding to nucleotides 96-1289 of MGC44287, and containing the complete open reading frame of A34 and its translation initiation sequence, was isolated from normal testis by RT-PCR (primers A34ClonF2, 5’-CTGCTGTCCTCAACCTAACTC-3’, and A34ClonR2, 5’-AGGCCATGCTTACAACCTACAC-3’) and subcloned into the pCDNA3.1 mammalian expression plasmid (Invitrogen Technologies). Preparation of the full length pCDNA3.1 mammalian expression plasmid (Invitrogen Life Technologies). The expression plasmid A34/pCDNA3 construct was previously reported (9). COS-7 cells were transfected with the A33 and A34 cDNA constructs using Lipofectamine™ Reagent (Invitrogen) as per manufacturer’s instructions.

**Immunoblotting and glycosylation analysis**

Gastric mucosa and colonic mucosa were mechanically dissected from human tissue specimens. Tissue lysates were prepared with ice-cold PBS containing 1% NP40 and Halt Protease Inhibitor Cocktail Kit (Pierce, Rockford, IL). COS-7 transfectants were lysed in RIPA (radioimmunoprecipitation assay) buffer. Solubilized proteins were separated from tissue/cell debris by centrifugation (1,000 x g for 10 min followed by 16,000 x g for 30 min at 4°C), and the resultant supernatants filtered through a 0.45 µm filter. Total protein concentration was determined using the Pierce BCA Protein Assay Reagent Kit.

For immunoblot analysis, approximately 50 µl aliquots of total protein were separated by electrophoresis on 10% Bis-Tris SDS-PAGE gels in MOPS buffer (Invitrogen). Proteins were transferred to PVDF (polyvinylidene difluoride) membranes and probed with mAb A34 (1 µg/ml), mAb A33 (1 µg/ml) and an isotype-matched monoclonal antibody (1 µg/ml), followed by horseradish peroxidase-conjugated rabbit anti-mouse Ig (1:20,000; Jackson ImmunoResearch laboratories Inc., West Grove PA). Reactivity was visualized with NEN Chemiluminescence Plus Reagent (Perkin Elmer, Boston, MA). Detergent lysates from human gastric and colonic mucosa (50 µg of total protein) were subjected to treatment with various glycosidases: N-Glycanase PNGaseF (5 mU) Sialidase A (5 mU), O-Glycanase (1.25 mU), β-(1-4)-Galactosidase (3 mU), and β-N-Acetylgalactosaminidase (40 mU) using the Prozyme Enzymatic Deglycosylation Kit (Glyko, San Leandro, CA) according to manufacturer’s instruction and deglycosylation was monitored by immunoblot analysis.

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**Supplemental data**

**Supplementary Figure 1.** Amino acid sequence alignment of A34 and other JAM family members.