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Humanization of JAA-F11, a Highly Specific Anti-Thomsen-Friedenreich Pancarcinoma Antibody and *In Vitro* Efficacy Analysis

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Abstract

JAA-F11 is a highly specific mouse monoclonal to the Thomsen-Friedenreich Antigen (TF-Ag) which is an alpha-O-linked disaccharide antigen on the surface of ~80% of human carcinomas, including breast, lung, colon, bladder, ovarian, and prostate cancers, and is cryptic on normal cells. JAA-F11 has potential, when humanized, for cancer immunotherapy for multiple cancer types. Humanization of JAA-F11, was performed utilizing complementarity determining regions grafting on a homology framework. The objective herein is to test the specificity, affinity and biology efficacy of the humanized JAA-F11 (hJAA-F11). Using a 609 target glycan array, 2 hJAA-F11 constructs were shown to have excellent chemical specificity, binding only to TF-Ag alpha-linked structures and not to TF-Ag beta-linked structures. The relative affinity of these hJAA-F11 constructs for TF-Ag was improved over the mouse antibody, while T20 scoring predicted low clinical immunogenicity. The hJAA-F11 constructs produced antibody-dependent cellular cytotoxicity in breast and lung tumor lines shown to express TF-Ag by flow cytometry. Internalization of hJAA-F11 into cancer cells was also shown using a surface binding ELISA and confirmed by immunofluorescence microscopy. Both the naked hJAA-F11 and a maytansine-conjugated antibody (hJAA-F11-DM1) suppressed *in vivo* tumor progression in a human breast cancer xenograft model

Abbreviations: Ab, Antibodies; ADCC, Antibody-dependent cellular cytotoxicity; Ag, Antigen; BLAST, Basic local alignment search tool; BSA, Bovine serum albumin; CDC, Complement-dependent cytotoxicity; CDRs, Complementarity determining regions; CHO, Chinese hamster ovary; CMV, Cytomegalovirus; DNA, Deoxyribonucleic acid; *E. coli*, *Escherichia coli*; EIA, Enzyme immunoassay; ELISA, Enzyme-linked immunosorbent assay; FCS, Fetal calf serum; FRs, Framework regions; Gal, Galactose; GalNAc, N-acetyl galactosamine; GlcNAc, N-acetyl glucosamine; HC, Heavy chain; Ig, Immunoglobulin; IgG, Immunoglobulin G; IgG1, Immunoglobulin G class 1; LC, Light chain; LDH, Lactate dehydrogenase; mAb, monoclonal antibody; NeuAc, Sialic acid; OD, Optical density; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; RNA, Ribonucleic acid; SD, Standard deviation; SEM, Standard error of the means; TF-Ag, Thomsen-Friedenreich antigen; VH, Variable region heavy chain; VL, Variable region light chain.

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in SCID mice. Together, our results support the conclusion that the humanized antibody to the TF-Ag has potential as an adjunct therapy, either directly or as part of an antibody drug conjugate, to treat breast cancer, including triple negative breast cancer which currently has no targeted therapy, as well as lung cancer.

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Background

Thomsen-Friedenreich antigen (TF-Ag) is a carbohydrate pancarcinoma antigen expressed on ~80% of human carcinomas including breast, lung, colon, bladder, ovary, prostate and stomach cancers, [1–10] but is cryptic on normal tissues. TF-Ag is also found on leukemia and lymphoma cells [11,12]. TF-Ag is expressed on tumor tissues due to changes in glycosylation in tumor cells which increases the expression of core saccharide structures devoid of further glycosylation, as opposed to normal cells where TF-Ag is hidden due to the addition of carbohydrate moieties [13–15]. TF-Ag is the disaccharide D-galactose-beta-(1–3)-N-acetyl galactosamine (Gal-β-(1–3)-GalNAc) which is alpha linked to a serine or a threonine residue on proteins [1]. The same disaccharide structure can appear beta-linked on glycolipids in normal tissues, so this linkage inclusion is important for tumor specificity [13]. Thus, the tumor restricted presence of this antigen makes this a promising target for cancer immunotherapy. In addition, TF-Ag plays a pivotal role in metastasis [16–22] where tumor cell surface TF-Ag binds and causes up-regulation of Galectin-3 (Gal-3) expression and mobilization on the surface of vascular endothelial cells. The TF-Ag and Gal-3 binding interaction causes the arrest of the tumor cell on the blood vessels followed by the endothelial integrin α3β1 stabilization/locking event, all steps required for establishing metastasis [23]. Further evidence supporting the role of TF-Ag in tumor metastasis stems from clinical studies showing that tumors that have greater metastatic activity were found to over-express TF-Ag [3–5,10]. Therefore, we hypothesized that therapy with an anti-TF antibody could create a survival advantage for patients with TF-Ag expressing tumors through direct killing, antibody-linked drug conjugate killing and/or by blocking tumor cell spread.

The murine monoclonal IgG_3 antibody JAA-F11 [24] is highly specific in that it binds to the alpha-linked tumor-associated TF-Ag and not the beta-linked structure known to be localized on the surface of normal tissues such as the kidney and natural killer (NK) cells [25–27]. The *in vivo* specificity of JAA-F11 was observed using iodine 124-labeled JAA-F11 in the mouse 4T1 breast cancer model, the nude-xenograft human breast cancer model (MDA-MB-231(triple negative)) and the SCID-xenograft human breast cancer model (with DU4475 triple negative tumors) [27–29]. Notably, JAA-F11 did not significantly bind to the kidneys or other organs in the mice, which is a promising indicator of safety and specificity for future therapy and diagnostic imaging in humans. Conversely, the less specific anti-TF-Ag murine antibody, Tru-Scint MAb 170H.82, which appears to bind to both the alpha- and beta-linked TF-Ag, was withdrawn after Phase III clinical trials, possibly because despite the promising tumor imaging sensitivity observed, this antibody also bound normal kidney tissue [29]. Studies have shown that JAA-F11 blocks metastasis in an established *in vivo* 4T1 metastatic breast cancer model, as well as in *in vitro* and *ex vivo* models [16]. Previous *in vitro* whole cell binding assays revealed that JAA-F11 targets approximately 80% of 41 breast cancer cell lines tested, regardless of estrogen receptor (ER), progesterone receptor (PR) or Her2 status, targeting ability that included 82% of triple negative breast cancers tested (TNBC) [28]. Furthermore, we recently demonstrated that JAA-F11 is rapidly internalized by tumor cells within 1 hour [28]. This property can be exploited to bring drugs or toxins into the cancer cells in a more targeted way to reduce normal cell toxicity and increase tumor cell killing [30,31]. Altogether, these pre-clinical data obtained with JAA-F11 suggest this antibody as a potential therapeutic agent for the treatment of cancer.

The fact that TF-Ag is associated with many human cancers, including triple negative breast cancer, warrants its exploitation for therapeutic uses. Murine antibodies, however, have limited therapeutic applications due to their immunogenicity, short serum half-lives, and weak human effector functions [32–34]. Human immune response to mouse JAA-F11 prevents its direct clinical use, so humanization is necessary to decrease the immunogenicity of the mouse antibody and to allow it to remain in circulation for a longer time. As humanization can affect specificity and affinity because the framework regions can have an effect on conformation, understanding and conserving the initial antibody structure and testing the humanized constructs is important. This specificity is particularly important in our anti-carbohydrate antibody response since TF-Ag alpha is tumor associated while TF-Ag beta is on normal tissues.

Here we report the construction, production, and characterization of two humanized variants of the mouse mAb JAA-F11. Humanization of JAA-F11 was achieved by using a complementarity determining region (CDR) grafting process for the mouse VL and VH regions and subsequent construction into a full length human IgG1 antibody. The antibody was produced in CHO-K1 cells after stable transfection with two recombinant expression vectors. The purified hJAA-F11 antibodies were compared to the mouse [13,16,18,23,24,27,28,37] and chimeric JAA-F11 in terms of specificity, relative affinity, and effector functions. The overall goal of this study is to generate a humanized monoclonal antibody to the tumor antigen TF-Ag, which has high specificity, low immunogenicity and high biological efficacy.

Materials and Methods

Human use Ethics Statement

Blood for cytotoxicity assays was obtained voluntarily from healthy donors with approval (421397–4) granted by University at Buffalo Health Science Institutional Review Board.

Cells, Cell Lines, and Mammalian Expression Vectors

Adherent Chinese hamster ovary cells (CHO-K1; ATCC #CCL-61™, Manassas, VA) were maintained in Ham's F12 medium (Corning Cellgro, Manassas, VA) supplemented with 10% fetal calf serum (FCS; Hyclone) at 37°C and 5% carbon dioxide (CO_2) in humidified air. The tumorigenic human breast cancer cell line MDA-MB-231 was kindly provided by Dr. Julian Gomez-Cambronero (Wright State University,