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Secretion and Immunogenicity of the Meningioma-Associated Antigen TXNDC16

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In a previous study, we identified thioredoxin domain containing 16 (TXNDC16) as a meningioma-associated Ag by protein macroarray screening. Serological screening detected autoantibodies against TXNDC16 exclusively in meningioma patients’ sera and not in sera of healthy controls. TXNDC16 was previously found to be an endoplasmic reticulum (ER)–luminal glycoprotein. In this study, we show an additional ER-associated localization of TXNDC16 in the cytosol by in vitro synthesis, molecular mass shift assay, and flow cytometry. We were able to show TXNDC16 secretion in different human cell lines due to masked and therefore nonfunctional ER retrieval motif. A previously indicated exosomal TXNDC16 secretion could not be confirmed in HEK293 cells. The secreted serum protein TXNDC16 is bound in circulating immune complexes, which were found both in meningioma and healthy blood donor sera. Employing a customized array with 163 overlapping TXNDC16 peptides and measuring autoantibody reactivity, we achieved discrimination of meningioma sera from healthy controls with an accuracy of 87.2% using a set of only five immunogenic TXNDC16 epitopes. The Journal of Immunology, 2014, 193: 3146–3154.

With 35% of all primary brain tumors, meningiomas are the most common intracranial neoplasias. They occur with an average annual incidence of 7 in 100,000 and are generally benign (1, 2). We previously showed that meningiomas trigger a complex humoral immune response, suitable for a highly accurate minimally invasive detection by serological methods (3–5). Serological screening of recombinantly expressed clones revealed several meningioma-associated Ags reacting with patients’ sera. Tumor-associated Ags represent a class of biomarkers that are recognized by autoantibodies in tumor patients’ sera. Although in general, normal proteins are not antigenic, deregulated expression, altered posttranslational modifications, or subcellular localization often provoke loss of immune system’s self-tolerance (6–8).

Thioredoxin domain containing 16 (TXNDC16) emerged as one of the most important meningioma-associated Ags to discriminate meningioma patients from healthy controls. TXNDC16 was exclusively seroreactive with meningioma patients’ sera (3, 4). It has a molecular mass of ∼93 kDa (NP_065835.2), and the gene is ubiquitously expressed in normal and in many cancer tissues (9). Mitochondrial localization as suggested by Barbe et al. (10) was later disproved by Riemer et al. (11), who found TXNDC16 to be an endoplasmic reticulum (ER)–luminal glycoprotein. It contains a predicted N-terminal signal peptide of 27 aa that is supposed to be necessary for cotranslational translocation into the ER. The signal peptide of the polypeptide chain is bound by signal recognition particle (SRP) during translation. SRP subsequently binds to the SRP receptor at the ER membrane, and the ribosome–polypeptide–SRP complex is delivered to the Sec61 complex, where polypeptide elongation and translocation are sustained, and the signal peptide is cleaved by signal peptidase (12).

Riemer et al. (11) detected TXNDC16 in cell culture supernatant, which they explained by either the lack of a C-terminal ER retrieval motif or the artificial overexpression. In general, ER-luminal proteins possess C-terminal ER retrieval motifs, variants of the consensus sequence KDEL (13, 14). Human proteins containing this motif bind to one of the three KDEL receptors (ERD21, ERD22, and ERD23) in cis-Golgi and are retrotranslocated into the ER via coat protein complex (COP) I–mediated vesicular transport (13–18). TXNDC16 contains the C-terminal KDEL variant DKEI with an additional 6 aa encoded downstream.

TXNDC16 was previously detected in human parotid gland exosomes (19). Exosomes are small membrane vesicles with a diameter of 30–100 nm and show a characteristic cup-shaped morphology as observed by electron microscopy (20, 21). Exosomes derive from late endosomes/multivesicular bodies by invagination of the endosomal membrane and are released due to

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Abbreviations used in this article: CIC, circulating immune complex; COP, coat protein complex; DPBS, Dulbecco’s PBS; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HA, hemagglutinin; malPEG, maleimide–PEG; PBS-T, PBS-Tween; PEG, polyethylene glycol; PFF, paraformaldehyde; P/S, penicillin/streptomycin; PSS, DPBS containing 0.1% (w/v) saponin; RM, rough microsome; RT, room temperature; SRP, signal recognition particle; TXNDC16, thioredoxin domain containing 16.

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multivesicular bodies’ exocytic fusion with the plasma membrane. They represent an unconventional secretion pathway for cytosolic proteins (22–24).

Protein secretion offers a potential target for humoral immune response. Backes et al. (25) and Daniels et al. (26) showed an increased seroreactivity of prostate cancer patients against the secreted protein LEDGF/p75 in contrast to controls. Via in silico analyses, we recently calculated a significant enrichment of secreted and extracellular Ags in autoimmune diseases. In our previous study, TXNDC16 mRNA transcription was found upregulated by up to three times in meningiomas of TXNDC16 seroreactive patients (27).

In this study, we identify an additional ER-associated cytosolic localization of TXNDC16 and examined whether the potential TXNDC16 signal peptide is essential for its translocation into the ER as well as its ability to translocate GFP into the ER likewise. We show that also endogenous TXNDC16 is detectable at 100 kDa in supernatants of several human cell lines (e.g., benign and malignant meningioma cell lines). We addressed whether TXNDC16 is retained due to its C terminus or is secreted physiologically, maybe via an exosomal pathway in HEK293 cells.

In addition, we demonstrate that TXNDC16 occurs as processed serum protein with molecular masses of 55 and 70 kDa. The 55-kDa fragment of TXNDC16 was found in circulating immune complexes both in meningioma sera and healthy controls. We used customized peptide arrays of 163 TXNDC16 peptides to identify immunogenic TXNDC16 epitopes and asked whether discrimination of meningioma and control sera is possible with a specific subset selection of all immunogenic epitopes.

Materials and Methods
Primers and plasmids
Full-length TXNDC16 and GFP cDNAs were amplified from plasmids pCMV-Sport6-TXNDC16 (Open Biosystems) and pEGFP-N1 (Clontech), respectively. Vector pSG5 (Agilent Technologies) was used to express all variants of TXNDC16 and GFP in HEK293 and HeLa cells.

Cell lines and transient transfection
For transfection, human cervix carcinoma cell line HeLa (Deutsche Sammlung von Mikroorganismen und Zellkulturen), human embryonic kidney cell line HEK293 (Deutsche Sammlung von Mikroorganismen und Zellkulturen), the benign meningioma cell lines Ben-Men-1 and HBL-52, and two malignant meningioma cell line IOMM-Lee were used. Meningioma cell lines were kindly provided by Prof. Christian Mawrin (Otto-von-Guericke University of Magdeburg), we used. Meningioma cell lines were kindly provided by Prof. Christian Mawrin (Otto-von-Guericke University of Magdeburg). Cells were cultured in DMEM (Life Technologies) supplemented with 10% (v/v) heat-inactivated FBS (Biochrom) and 1% (v/v) penicillin/streptomycin (PS; Life Technologies) at 37°C and 5% CO₂. HeLa cells were transiently transfected with the nonliposomal transfection reagent FuGENE HD (Roche) according to the manufacturer’s instructions. HEK293 cells were transfected using the calcium phosphate method. In detail, 3 × 10⁵ cells were seeded per 145-cm² dish and cultured for 24 h. Cells were washed with Dulbecco’s PBS (DPBS) (Life Technologies) and covered with 40 ml freshly supplemented DMEM. A total of 50 μg plasmid DNA in a total volume of 4.7 ml sterile, deionized water was mixed with 600 μl CaCl₂ and 4.7 ml sterile filtered 2× HEPES-buffered saline (280 mM NaCl, 1.5 mM Na₂HPO₄, and 50 mM HEPES [pH 7.05]). Cells were incubated overnight with the transfection complex (CIC) precipitation was done at 4°C overnight. CICs were pelleted at 13,000 rpm for 10 min, washed twice with 3.5% PEG, and resuspended in a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Protein, CIC, and serum samples (10 μg protein/CIC for each sample or 0.5% serum, respectively) were denatured in 4× RotiLoad sample buffer (Roth) for 5 min at 95°C prior separation in a 10% SDS polyacrylamide gel at 125 V for 2 to 3 h. Separated proteins were transferred onto a polyvinylidene difluoride membrane (Amersham) by wet Western blotting at 25 V for 2 to 3 h.

Immunodetection
Polyvinylidene difluoride membranes were blocked with 5% (w/v) BSA (Sigma-Aldrich) in PBS-T (PBS-Tween; 10 mM NaH₂PO₄, 130 mM NaCl [pH 7], and 0.2% [v/v] Tween 20) or TBST (20 mM Tris, 137 mM NaCl [pH 7.6], and 0.1% [v/v] Tween 20), depending on primary Ab, for 1 h at RT or overnight at 4°C. The membranes were incubated with the following primary Abs for 1 h at RT or overnight at 4°C: anti–actin (HPA002543; rabbit polyclonal, 1:500 in PBS-T; Sigma-Aldrich), anti–CD63 (HPA010088; rabbit polyclonal, 1:500 in PBS-T; Sigma-Aldrich), anti–β-actin (A1978; mouse monoclonal, clone AC-15, 1:1000 in PBS-T; Sigma-Aldrich), anti–myc (ab9106; rabbit polyclonal, 1:500 in TBST; Abcam), anti–PDI (sc-20132; rabbit polyclonal, 1:400 in PBS-T; Santa Cruz Biotechnology), anti–GAPDH (sc-47724; mouse monoclonal, clone 0411, 1:1000 in PBS-T; Santa Cruz Biotechnology), or anti–GFP (rabbit polyclonal, 1:1000 in PBS-T; kindly provided by Prof. Richard Zimmermann, Medical School, Saarland University). The membranes were washed three times with PBS-T/TBST for 10 min at RT and subsequently incubated with initial volume of 0.01 M PBS.

Exosomes isolation
For depletion of bovine exosomes, FBS was ultra centrifuged overnight at 100,000 × g and 4°C. HEK293 cells were cultured for 24 h in DMEM with 10% (v/v) exosome-depleted FBS and P/S in three 175-cm² flasks. After calcium phosphate transfection, exosomes were released into FBS-free DMEM. Medium was collected, and detached cells, dead cells, and cell debris were removed by sequential centrifugation at 300 × g for 10 min, 2000 × g for 10 min, and 10,000 × g for 30 min, respectively, at 4°C each. Secre ted exosomes were subsequently pelleted at 100,000 × g for 1 h at 4°C. The supernatant was retained for further protein precipitation. The exosome-containing pellet was resuspended in an appropriate volume of DPBS (at least 100 μl), and the ultra centrifugation step was repeated. DPBS supernatant was discarded, and the exosomes were resuspended in 100 μl DPBS.

Circulating immune complex preparation
To remove any suspended particles, serum was first centrifuged at 10,000 rpm for 5 min. Serum supernatant was mixed with equal volume of 7% polyethylene glycol (PEG 8000) in borate buffer (0.1 M boric acid, 0.5 mM disodium tetraborate, and 75 mM NaCl [pH 8.4]). Circulating immune complexes (CIC) precipitation was done at 4°C overnight. CICs were pelleted at 13,000 rpm for 10 min, washed twice with 3.5% PEG, and resuspended with initial volume of 0.01 M PBS.

Cell lysis, protein precipitation, SDS-PAGE, and Western blotting
Cells were trypsinized, diluted in DMEM, pelleted, and washed once with DPBS (300 × g, 5 min each). Cells were resuspended in an appropriate volume (200 μl per 1 × 10⁵ cells) of cell lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.4, and 0.5% [v/v] Triton X-100) containing protease inhibitors (1 tablet of complete EDTA-free protease inhibitor mixture Roche) in 10 ml lysis buffer) and incubated 20 min on ice. Crude membrane fraction was pelleted at 20,000 × g and 4°C for 20 min. Supernatant was collected for subsequent protein precipitation. Cell lysates and exosomes harvested from cell culture supernatant were incubated on ice for 30 min in 10% (v/v) TCA, and precipitated proteins were pelleted at 15,000 × g for 15 min at 4°C. The protein pellet was resuspended in 300 μl ice-cold acetone and centrifuged at 20,000 × g for 5 min at 4°C. The air-dried protein pellet was dissolved in 100 μl DPBS with protease inhibitors (1 tablet of complete EDTA-free protease inhibitor mixture in 10 ml DPBS). Protein concentration was determined by measuring the absorbance at 280 nm in a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). Protein, CIC, and serum samples (10 μg protein/CIC for each sample or 0.5% serum, respectively) were denatured in 4× RotiLoad sample buffer (Roth) for 5 min at 95°C prior separation in a 10% SDS polyacrylamide gel at 125 V for 2 to 3 h. Separated proteins were transferred onto a polyvinylidene difluoride membrane (Amersham) by wet Western blotting at 25 V for 2 to 3 h.
with HRP-conjugated goat anti-rabbit IgG (H + L) (1:30,000; Dianova) or HRP-conjugated sheep-anti-mouse IgG (H + L) (1:50,000; Dianova), each diluted in 5% (v/v) nonfat dry milk/PBS-T or TBST for 1 h at RT. Proteins were detected using ECL Plus Western blotting Detection (GE Healthcare).

**Molecular weight shift**

HeLa cells were seeded at 2 x 10^5 cells per 55-cm² dish and transiently transfected with FuGENE HD (Roche) according to the manufacturer’s instructions. Molecular weight shift assay with maleimide-PEG (malPEG; 5 kDa) (Sigma-Aldrich) was performed as described previously (28). Proteins were separated by SDS-PAGE and detected by Western blotting, as described above.

**Flow cytometry**

Confluently grown and untransfected HeLa cells were harvested from three 75-cm² flasks, and cell aggregates were separated with 10% FBS in DPBS. Cells were pelleted for 5 min at 300 x g and RT, resuspended in 9 ml DPBS supplemented with 3% (v/v) BSA, and split into three aliquots. The first aliquot was left unpermeabilized, the other aliquots were permeabilized with 20 μM digitonin or 0.1% (v/v) Triton X-100, respectively, and pelleted at 300 x g. The permeabilized cells were washed twice with BSA/DPBS containing 2 μM digotonin or 0.01% Triton X-100, respectively, and pelleted at 300 x g for 5 min at RT. Three pelleted cell aliquots were resuspended in 1 ml primary Ab solution (anti-α-tubulin, T9026; mouse monoclonal, clone DM-1A; 1:1000; Sigma-Aldrich; anti-PDI, 1:500; and anti-TXNDC16, 1:500) and two pelleted cell aliquots in Ab-free BSA/DPBS. After incubation for 30 min at RT, cells were washed three times by adding 2 ml BSA/DPBS containing centrifuged at 300 x g for 5 min. Cells were fixed with 2% (v/v) PFA in DPBS for 10 min at 37°C, and the three washing steps were repeated. Cells were incubated for 30 min with secondary Abs Alexa Fluor 488 goat-anti-rabbit and Alexa Fluor 594 goat-anti-mouse (each 1:2000 in BSA/DPBS). As negative control, one aliquot previously not incubated with a primary Ab was incubated with both secondary Abs. Secondary Abs were removed, and cells were washed three times with BSA/DPBS. Cells were subsequently kept in fixative (1% [w/v] PFA/DPBS) until FACScan (BD Biosciences) measurement. A total of 2000 cells was analyzed per run.

**Protein transport and sequestration**

Cotranslational protein transport experiments were performed by synthesizing precursor polypeptides in reticulocyte lysate (Promega) in the presence of 35S-Methionine (1000 Ci/mmol). The in vitro synthesis reaction was prepared with 1 μg plasmid DNA according to manufacturer’s instructions and split into two aliquots (25 μl final volume), in one of which 6% (v/v) pancreatic ER-derived rough microsomes (RMs) were added (29). The samples were incubated for 60 min at 30°C. Each reaction was subsequently split into two aliquots and incubated with or without proteinase K (170 μg/ml) for 60 min at 0°C. The reaction was stopped by addition of PMSF (10 mM). Processed polypeptides were separated by SDS-PAGE and detected by phosphoimaging (Typhoon-Trico imaging system; GE Healthcare).

**Patients’ sera, peptide arrays, and bioinformatics**

Meningioma sera and sera from healthy controls were subsequently stored at −20°C after blood withdrawal. CellSpots peptide arrays were manufactured by Intavis with 163 TXNDC16 spanning peptides spotted as two replicates with overlapping 15-mer peptide sequences, see Supplemental Table I. Arrays were incubated according to the manufacturer’s instructions. Serum and secondary Ab (Cy5-conjugated sheep-anti-mouse IgG [H + L]; Dianova) were both diluted 1:1000 in 5% (v/v) nonfat dry milk/TBST (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], and 0.05% [v/v] Tween 20). Cy5 detection was performed with a Typhoon scanner (Typhoon Trio imaging system; GE Healthcare). Western blotting Proteins were detected using ECL Plus Western blotting Detection (GE Healthcare).

**Results**

**Cytosolic association of TXNDC16 with the ER**

Human TXNDC16 was previously found to be a soluble ER-luminal glycoprotein, cotranslationally translocated into the endoplasmic reticulum due to its predicted N-terminal 27-aa signal peptide (11). To confirm this subcellular localization, we performed immunostaining of HeLa cells transiently expressing TXNDC16 with a C-terminally added HA tag (TXNDC16-HA). By confocal microscopy, we detected a colocalization of TXNDC16-HA with the ER-luminal protein PDI (Fig. 1A). The N-terminal signal peptide plays an essential role in the translocation of TXNDC16 as a deletion variant of TXNDC16 lacking the first 27 aa (ΔSP-TXNDC16-HA), transiently expressed in HeLa cells caused an altered subcellular localization of the protein from ER-associated to cytosolic (Fig. 1A).

Next we examined the translocation of TXNDC16-HA into canine pancreatic ER-derived RMs using a cell-free 35S-methionine labeling in vitro transcription and translation system. As an ER-luminal protein, we expected TXNDC16 to be sequestered in the RMs after protease digestion as it could be shown for the κ L-chain of human IgGs (Fig. 1B). In this study, only the unprocessed κ L-chain precursor was accessible for proteinase K, but not the translocated and processed form. However, TXNDC16 did not yield similar results and was not sequestered despite RMs’ presence.

To exclude the possibility of insufficient translocation efficiency into the RMs, we investigated the subcellular localization of TXNDC16 by Western blotting. We used HeLa cells transiently expressing myc-TXNDC16-DKEL, an N-terminally myc-tagged version of the protein with a 6-aa truncation at its C terminus. These amino acids acidic flanks a KDEL-like ER retrieval motif (DKEEL), subsequently proven in this study to enrich the intracellular protein yield of TXNDC16. The myc-tag was inserted downstream of the C terminus of TXNDC16. After immunoblotting with myc Abs, the membrane-impermeable cysteine modification reagent caused a molecular mass shift of 5 kDa per accessible endogenous cysteine. ER-luminal proteins are protected from malPEG binding due to the nonpermeabilized ER membrane. No molecular shift was expected for myc-TXNDC16-DKEL after digitonin and malPEG treatment (Fig. 1C).

**Validation**

Validation was used as described previously (5). Unprocessed raw data can be downloaded from National Center for Biotechnology Information Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/index.cgi). accession number GSE58949. The study was approved by the local ethics committee (approval numbers 44/05, 67/06, 68/06, and 42/07).

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marker PDI yielded a fluorescence signal, >80% of these cells fluoresced after incubation with the TXNDC16 Ab similarly to cells incubated with Abs against α-tubulin that served as a cytosolic marker (Fig. 1D). As control, due to ER-membrane permeabilization with Triton X-100, cells showed increased fluorescence after incubation with PDI Ab. Nevertheless, it remains unclear how TXNDC16 is retrotranslocated into the cytosol. Despite its retrotranslocation into the cytosol, confocal microscopy did not show TXNDC16 as soluble cytosolic protein. Therefore, we propose that TXNDC16 is linked to the cytosolic ER membrane surface through a currently unknown mechanism.

Nonexosomal secretion of TXNDC16

Riemer et al. (11) previously detected TXNDC16 in cell culture medium of various nontransfected cell lines including HeLa, HEK293, as well as the benign meningioma cell lines Ben-Men-1 and HBL-52 and the malignant meningioma cell line IOMM-Lee. After TCA precipitation of the supernatant and SDS-PAGE, we performed immunoblotting with a polyclonal Ab recognizing a C-terminal epitope of TXNDC16. TXNDC16 was detected in all supernatant fractions at different expression levels (Fig. 2A). We did not detect GAPDH in supernatant fractions for which we confirmed the presence of TXNDC16. These data indicate that the TXNDC16 that was found in supernatant fractions does not stem from the cytosol of dead cells. Notably, in comparison with HeLa and HEK293, virtually no TXNDC16 was detectable in all meningioma cell lysates, thus supporting a physiological secretion of TXNDC16 in meningioma cells.

Nonexosomal secretion of TXNDC16
we examined the potential function of the masked DKEL retrieval sequence by transient expression of different TXNDC16 variants in HeLa cells. We generated an N-terminally myc-tagged and C-terminally truncated TXNDC16 form without the six flanking amino acids (myc-TXNDC16-DKEL) as well as an N-terminally myc-tagged cytosolic form with wild-type C terminus (DSP-myc-TXNDC16). As control, we used N-terminally myc-tagged TXNDC16 with wild-type C terminus (myc-TXNDC16). After TCA precipitation of cell culture supernatant and immunoblotting with primary myc-Ab, we observed marginal intracellular myc-TXNDC16 in contrast to very high protein levels in the supernatant (Fig. 2B). Myc-TXNDC16-DKEL was not detected in the supernatant but showed an increased intracellular yield, which supports the missing ER retrieval capacity of the masked DKEL sequence. Interestingly, in addition to its cytosolic localization, a small amount of DSP-myc-TXNDC16 was present in the supernatant likely due to unconventional secretion.

The nonfunctional ER retrieval motif was further examined using several GFP fusion proteins. All GFP variants were fused with the TXNDC16 signal peptide at their N termini but differed at their C termini. We examined wild-type GFP C terminus (SP-TX-GFP), C-terminal fusion with DKEL (SP-TX-GFP-DKEL), and C-terminal fusion with the C-terminal 10 TXNDC16 aa (SP-TX-GFP-TX-Cterm). We first investigated if TXNDC16 signal peptide also facilitates GFP translocation into the ER. Confocal microscopy of transiently transfected HeLa cells shows that SP-TX-GFP is colocalized with the ER-luminal protein PDI in contrast to ubiquitous localization of overspressed wild-type GFP. In HEK293 cells nonfunctional ER retention of TXNDC16 C terminus is shown by different secreted GFP variants. Secretd GFP with wild-type C terminus (SP-TX-GFP) as well as secreted GFP with TXNDC16 C terminus (SP-TX-GFP-TX-Cterm) are not retained intracellularly, in contrast to secreted GFP with C-terminal ER retrieval motif (SP-TX-GFP-DKEL). N-terminally myc-tagged TXNDC16 is not secreted via an exosomal secretory pathway in HEK293 cells. Exosomal fraction was determined by presence of tetraspanins CD63 (B, D, E). HEK293 cells were grown in 145-cm² dishes. Cells were transiently transfected with calcium phosphate method 24 h after seeding and cultured in FBS-free medium for additional 48 h. Total of 5 µg of each protein sample was loaded into separate wells of a 10% SDS-PAGE gel. Western blotting was performed with same samples but different membranes.

**FIGURE 2.** Nonexosomal secretion of TXNDC16. (A) TXNDC16 is secreted by different human cell lines, including benign (Ben-Men-1, HBL-52) and malignant (IOMM-Lee) meningioma cell lines. All cells were grown in 75-cm² flasks with FBS-free DMEM for 72 h. In meningioma cell lines, endogenous TXNDC16 could not be detected in cell lysates, in contrast to HEK293 and HeLa. (B) In HEK293 cells, TXNDC16 is not retained intracellularly by a masked KDEL-like ER retrieval motif (DKEL). N-terminally myc-tagged TXNDC16 with wild-type C terminus (myc-TXNDC16) is found in supernatant but marginally detected in cell pellet, whereas the C-terminally truncated TXNDC16 variant with C-terminal DKEL (myc-TXNDC16-DKEL) is retained and not secreted. Cytosolic TXNDC16 (DSP-myc-TXNDC16) is also found in supernatant likely due to unconventional secretion. (C) Immunostaining of HeLa cells transiently overexpressing GFP fusion protein with N-terminal TXNDC16 signal peptide (SP-TX-GFP) or wild-type GFP, respectively. Cells were grown on coverslips in 35-mm dishes for 48 h after FuGENE HD transfection and fixed with 4% PFA. Overexpressed GFP fusion protein with N-terminal TXNDC16 signal peptide (SP-TX-GFP) is directed to secretory pathway and enriched in Golgi apparatus (see arrow) in contrast to ubiquitous localization of overexpressed wild-type GFP. (D) in HEK293 cells nonfunctional ER retention of TXNDC16 C terminus is shown by different secreted GFP variants. Secretd GFP with wild-type C terminus (SP-TX-GFP) as well as secreted GFP with TXNDC16 C terminus (SP-TX-GFP-TX-Cterm) are not retained intracellularly, in contrast to secreted GFP with C-terminal ER retrieval motif (SP-TX-GFP-DKEL). (E) N-terminally myc-tagged TXNDC16 is not secreted via an exosomal secretory pathway in HEK293 cells. Exosomal fraction was determined by presence of tetraspanins CD63 (B, D, E). HEK293 cells were grown in 145-cm² dishes. Cells were transiently transfected with calcium phosphate method 24 h after seeding and cultured in FBS-free medium for additional 48 h. Total of 5 µg of each protein sample was loaded into separate wells of a 10% SDS-PAGE gel. Western blotting was performed with same samples but different membranes.
exosome-free supernatant, and proteins from cell lysate were separated by SDS-PAGE. We used a polyclonal Ab against the exosomal marker CD63 (31, 32). Although the exosomal fraction was verified by presence of CD63, we did not detect TXNDC16 in the exosomal fraction of HEK293 cells (Fig. 2E). Still, these findings do not exclude an exosomal secretion of TXNDC16 in other cell lines or cell types. Nevertheless, TXNDC16 is a secreted protein and therefore offers itself as an accessible target for immune response.

**TXNDC16 in circulating immune complexes in the serum**

Because TXNDC16 was found in the supernatant of meningioma cells and because autoantibodies against TXNDC16 were detected in sera of patients with meningioma, we set out to confirm the presence of TXNDC16 in patients’ sera. In detail, we isolated sera from patients diagnosed with meningioma and performed Western blotting. The above-mentioned polyclonal Ab that recognized a C-terminal epitope of the endogenous TXNDC16 detected two distinct bands at ~55 and 70 kDa (Fig. 3A), which likely result from proteolysis of the 100-kDa protein found in the cell lysates (Fig. 2A, 2B). Because TXNDC16 was also found in the supernatant of HEK293 cells and because TXNDC16 was ubiquitously expressed (33), we also tested for the presence of TXNDC16 in sera of healthy controls and found the same signals as for the meningioma samples.

To analyze whether TXNDC16 immune complexes are present in patient’s serum, we isolated CICs from patients’ sera using PEG and performed Western blotting using the same polyclonal Ab for TXNDC16. As shown in Fig. 3B, the TXNDC16 Ab only detected the signal of 55 kDa in the CICs of meningioma sera. In addition, we isolated CICs from healthy donors and found the same signal as for the meningioma samples.

**TXNDC16 epitopes achieve high classification accuracy of meningioma sera**

Because TXNDC16 was discovered as a meningioma-associated Ag including upregulated mRNA expression in meningioma tissues of seroreactive patients, we aimed at identifying specific linear TXNDC16 epitopes exclusively recognized by meningioma patients’ autoantibodies (3, 4, 27). We used customized TXNDC16 peptide arrays with 163 peptides (15-mers) as duplicates overlapping in sequence and analyzed 24 meningioma sera and 19 sera of healthy controls. We could not determine any TXNDC16-specific epitopes neither recognized by all analyzed meningiomas nor with control sera (Fig. 4A). Yet we computed a set of five immunogenic epitopes out of the 163 peptides (feature selection) with which we could successfully discriminate both meningioma and control serum groups with an accuracy of 87.2%, a specificity of 83.7%, and a sensitivity of 90% compared with an accuracy of 53.8%, a specificity of 24.2%, and a sensitivity of 77.3% when all peptides were used (Table I). When examining the set of five epitopes, only peptide G06 was exclusively seroreactive with 8 of 24 meningioma sera, with 6 sera showing weak seroreactivity and 2 sera showing moderate seroreactivity (Fig. 4B). Peptide B12 was nearly exclusively seroreactive with meningioma sera, showing strong seroreactivity in three cases and moderate and weak seroreactivity in one case each, but also showed a weak seroreactivity with one control serum. Peptide sequences of the five best epitopes are summarized in Table II.

**Discussion**

In this study, we demonstrated that in addition to its previously reported occurrence as a soluble ER-luminal glycoprotein, TXNDC16 shows an ER-associated subcellular localization in the cytosol, as confirmed by confocal microscopy, molecular mass shift, and flow cytometry. Although Riemer et al. (11) conducted alkali extraction of crude membranes to postulate soluble ER-luminal localization of TXNDC16, these findings are not in conflict with our results. Yamazaki et al. (34) recently identified MIZ1 (Mizu-Kussei 1) of TXNDC16, these findings are not in conflict with our results. Because TXNDC16 was found in the supernatant of meningioma cells and because autoantibodies against TXNDC16 were detected in meningioma sera, we aimed at identifying specific linear TXNDC16 epitopes exclusively recognized by meningioma patients’ autoantibodies (3, 4, 27). We used customized TXNDC16 peptide arrays with 163 peptides (15-mers) as duplicates overlapping in sequence and analyzed 24 meningioma sera and 19 sera of healthy controls. We could not determine any TXNDC16-specific epitopes neither recognized by all analyzed meningiomas nor with control sera (Fig. 4A). Yet we computed a set of five immunogenic epitopes out of the 163 peptides (feature selection) with which we could successfully discriminate both meningioma and control serum groups with an accuracy of 87.2%, a specificity of 83.7%, and a sensitivity of 90% compared with an accuracy of 53.8%, a specificity of 24.2%, and a sensitivity of 77.3% when all peptides were used (Table I). When examining the set of five epitopes, only peptide G06 was exclusively seroreactive with 8 of 24 meningioma sera, with 6 sera showing weak seroreactivity and 2 sera showing moderate seroreactivity (Fig. 4B). Peptide B12 was nearly exclusively seroreactive with meningioma sera, showing strong seroreactivity in three cases and moderate and weak seroreactivity in one case each, but also showed a weak seroreactivity with one control serum. Peptide sequences of the five best epitopes are summarized in Table II.

FIGURE 3. TXNDC16 serum and in CICs. Serum samples from patients diagnosed with meningioma and sera of healthy blood donors were diluted 1:200 with PBS and separated by SDS-PAGE. Following Western blotting, a polyclonal Ab that recognized a C-terminal epitope of the endogenous TXNDC16 identifies two distinct fragments of TXNDC16 (55 and 70 kDa) in meningioma and in control sera. The same Ab identifies a signal at ~100 kDa for the myc-TXNDC16-DKEL in protein lysate of transfected HeLa cells. CICs were isolated from patients’ sera and sera of healthy blood donors using PEG. Total of 10 μg of PEG precipitated CICs was separated by SDS-PAGE. Following Western blotting, the Ab against TXNDC16 identifies a signal at 55 kDa in CICs of meningioma patients’ sera and in the sera of healthy controls.
demonstrated that TXNDC16 is not retained in the ER and thus secreted into cell culture medium. The absence of GAPDH in supernatant fractions that contained TXNDC16 indicates that TXNDC16 in cell supernatant is not due to cell death. In addition, we tested for b-actin as a structural protein, which was also absent in the supernatant. Furthermore, we found comparably less TXNDC16 in the meningioma cell lysates than in the supernatant, providing further evidence that the presence of TXNDC16 in the supernatant is not simply the result of lysed cells. However, we are aware that GAPDH in the supernatant might have been actively secreted, maybe via exosomes, and that b-actin as a structural protein is less likely to be found in the supernatant (46, 47). Nevertheless, the present evidence points toward secretion as cause of the TXNDC16 presence in the cell supernatant. Although TXNDC16 was discovered in human parotic gland exosomes in a proteomic study of Gonzales-Begne et al. (19), we could not confirm TXNDC16 detection in HEK293 exosomes.

It has to be elucidated whether TXNDC16 is secreted by the classical ER/Golgi dependent or an unconventional secretory pathway. The comparison of the subcellular localization of TXNDC16 (Fig. 1A) and the secreted GFP variant SP-TX-GFP (Fig. 2C) argues for the unconventional pathway. In contrast to TXNDC16, a prominent Golgi localization of SP-TX-GFP was observed as expected for secreted proteins. There may be multiple potential secretory mechanisms for TXNDC16 as previously described for proprotein convertase 7, which was reported to be secreted via the COP II-mediated vesicular transport as well as via Golgi-independent vesicular transport lacking COP II (48). The detection of TXNDC16 protein in patients’ serum is in agreement with the presence of autoantibodies against TXNDC16. The detection of TXNDC16 protein in normal sera is in agreement with the finding of this protein in the supernatant of non-meningioma cells (HEK293 cells) and an ubiquitous expression pattern (33). Apparently, the cytosolic 100-kDa TXNDC16 is processed into two C-terminal proteins of 55 and 70 kDa during the release into the serum. Notably, the Ab does not detect additional signals next to the 55- and the 70-kDa signal in the sera and next to the 100-kDa signal in the cell lysate. Only the 55-kDa signal detected in the serum is found in CICs, again both in patients and in healthy donors. There are previous reports on immunogenic proteins in CICs not only in tumor patients but also in normal controls (49, 50). Our data indicate that the potential proteolysis especially into proteins of 55 kDa may be a necessary prerequisite to elicit an autoantibody response against TXNDC16. However, the presence of TXNDC16 in the serum and specifically in the CICs of healthy donors also shows that other mechanisms must be present to explain our finding of frequent autoantibodies.

Table I. Accuracy, specificity, and sensitivity for discrimination of meningioma sera from healthy control sera using all 163 TXNDC16 peptides or using a subset of 5 immunogenic epitopes after feature selection, respectively

<table>
<thead>
<tr>
<th>Features</th>
<th>Accuracy</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>53.8%</td>
<td>24.2%</td>
<td>77.3%</td>
</tr>
<tr>
<td>A12, B12, C08, D19, and G06</td>
<td>87.2%</td>
<td>83.7%</td>
<td>90%</td>
</tr>
</tbody>
</table>

Serum classification was performed by linear support vector machines with 20 repetitions of standard 10-fold cross-validation.
against recombinantly expressed TXNDC16 in serum of meningioma patients. To understand these mechanisms that explain the autoantibody response against this TXNDC16, it is necessary to have a more profound understanding of the meningioma development. As stated >10 y ago, “a theory that explains the production of autoantibodies needs also to be a theory of etiology and pathogenesis of the diseases in which they occur” (51).

A potential upregulated but not yet confirmed TXNDC16 secretion in meningioma, caused by previously described TXNDC16 mRNA upregulation, could favor a patient’s humoral immune response. Therefore, it is necessary to investigate potential immunomucogenic epitopes for tumor diagnosis, as demonstrated for cancer/testis Ag NY-ESO-1. Zeng et al. (52) identified a 40-aa protein fragment of NY-ESO-1 recognized by autoantibodies in a wide range of patients’ sera suffering from different tumor entities. In this study, we determined one TXNDC16 epitope exclusively recognized by autoantibodies in one-third of all tested meningioma sera and one epitope recognized in approximately one-fifth of meningioma sera but also in one control serum. In addition, we achieved highly accurate differentiation of meningioma sera from controls by peptide array with just a small number of peptides of the meningioma-associated Ag TXNDC16. Still these findings need to be validated with an independent set of sera.

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Disclosures

The authors have no financial conflicts of interest.

References


