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ORIGINAL ARTICLE

Development of Novel Monoclonal Antibodies for Evaluation of Transmembrane Prostate Androgen-Induced Protein 1 (TMEPAI) Expression Patterns in Gastric Cancer

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Abstract Transmembrane prostate androgen-induced protein 1 (TMEPAI) is a single-span membrane protein, functionally involved in transforming growth factor beta signaling pathway. The particular protein presented in cells in three isoforms, which differs in the length of the soluble N-terminal extracellular domain, making it challenging for the immunochemical recognition. By using quantitative real-time polymerase chain reaction, we identified significant upregulation of *PMEPA1* gene expression in malignant tissues of patients with gastric adenocarcinoma. The main part of commercially available anti-TMEPAI antibodies are having polyclonal nature or not suitable for immunocytochemical localization of target protein in tissue specimens. Hence, we decide to generate a set of novel rat monoclonal antibodies (mAb) directed against conservative C-terminal cytoplasmic epitope. Immunoblotting analysis showed that monoclonal antibodies, 2E1, 6C6, and 10A7 were able to recognize specifically target protein in transiently transfected HEK293T and CHO-K1 cells. Especially established mAb, named 10A7, showed the excellent binding ability to target protein in immunohistochemistry. By using developed antibodies, we observed pronounced expression of TMEPAI in normal gastric epithelial cells while tumor cells from gastric adenomas, and adenocarcinoma samples were mostly negative for target protein expression. Also, we found that gastric epithelium cells lose the TMEPAI expression concurrently with severe dysplasia progression, which probably caused by a mechanism involving specific microRNA.

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Introduction

The transmembrane prostate androgen-induced protein 1 (TMEPAI) has attracted the significant attention nowadays [1] and was originally identified as a prostatic mRNA, the synthesis of which is induced by steroid hormones [2]. The certain polypeptide also named alternatively in literature as prostate transmembrane protein, androgen induced 1 (*PMEPA1*) or solid tumor-associated protein 1 (STAG1). Encoded by *PMEPA1* gene, located at chromosomal region 20q13, canonical protein product is a 31,6 kDa single-pass membrane protein, consisted of a soluble extracellular domain, α-helical transmembrane domain, and functional cytoplasmic domain (Fig. 1). Now it is known that TMEPAI exists in cells in three isoforms formed by alternative splicing mechanism, which differs in the length of the soluble extracellular domain.

This protein is functionally involved in the transforming growth factor beta (TGF-β) signaling pathway, a crucial regulator of malignant transformation, and metastatic tumor progression [3]. The TGF-β functions as a promoter of tumor progression by inducing epithelial-mesenchymal transition (EMT) during which tumor cells acquire the metastatic ability [4–6]. The major signaling pathway induced by TGF-β mediated by TGFβRII receptor complex and that indeed phosphorylation of SMAD2/3 resulting in activation of a transcriptional program [7, 8]. The activation of TGF-β induced directly *PMEPA1* gene expression by signaling pathway [9]. Moreover, it is known that TMEPAI and related proteins (e.g. C18 ORF1) interacting with TGF-β receptor signaling cascade mediators SMAD2/3, hence can block signal transduction [3, 10].

The TMEPAI isoform 3 apparently comprised of the only soluble intracellular domain and exceptionally not able to interact with SMADs and block TGF-β signaling [11]. In thecase of human cancers, inactivation of TGF-β signaling modules or mutations initiating in oncogenic signaling pathways is often observed and provide an essential background for further tumor progress [12].

TMEPAI protein was shown to regulate cell proliferation and differentiation of epithelial tissues, suggesting its role in the development of malignant tumors of various localizations [4, 11, 13]. The existing disparate data shows rather an ambiguous role of TMEPAI in tumorigenesis, which requires more detailed clarification. Increased gene expression of TMEPAI in the tumor tissue observed in patients with renal cell carcinoma, lung, colon, pancreatic, ovarian and breast cancer [4, 14]. Comparing full-length transcriptomes of invasive tumors to normal tissues (or pre-invasive tumors) revealed that TMEPAI is the most inducible gene in tumor tissues characterized by invasive growth. Accordingly, the increase of its expression is associated with poor prognosis of the clinical course of the disease [4, 15]. Earlier, other authors studied the role of *PMEPA1* in prostate cancer [16]. Their research shows disease progression in the case of decreased *PMEPA1* expression. It is possible that disorders in androgen receptors play a critical role in this case, since they, along with TGF-beta, regulate TMEPAI secretion into the prostate tissue. Recently, the potential role of PMEPA1 methylation in androgen receptor stability was postulated, and it becomes clearer how this process might effect on prostate tumorigenesis [17]. It was shown [6], that TGF-β decidedly upregulated the expression of TMEPAI during EMT in human lung adenocarcinoma via involvement of reactive oxygen species and insulin receptor substrate-1. Based on the fact that there are some organspecific features of target protein expression, it is necessary to interpret its role in carcinogenesis in different tissues and organs.

Earlier we had identified a panel of genes with upregulated expression of mRNA in gastric cancer (GC) tissues (e.g. *PMEPA1, GPNMB, SPARC, SULF1, CLDN4, GPRC5A, PI3, STK31, CEACAM6*) by applying a mathematical function characterizing frequency and increase of mRNA synthesis [18, 19]. Further expression levels of selected target genes were evaluated on the frozen paired tumor, and normal tissue samples from GC patients by using qRT-PCR. The expression level of *PMEPA1* in tumors has been revealed to be significantly

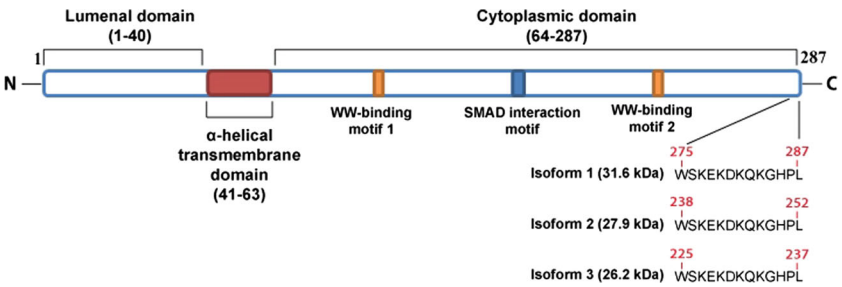


Fig. 1 A schematic representation of TMEPAI isoform 1 and selected epitope location. The topology of canonical target protein isoform illustrated, with structural domains and functional motifs. Localization and alignment of defined immunogenic epitope shown in relation to the certain type of protein isoform

altered compared to non-malignant tissues. Our data confirms the results observed by other researchers that demonstrate up-regulated *PMEPA1* gene expression in gastric tumors [20, 21]. The presented study was aimed to develop immunochemical instruments for TMEPAI expression patterns analysis in gastric tumors and adjacent non-malignant tissues.

Materials and Methods

Clinical Tumor Samples

The fresh and paraffin-embedded gastric adenocarcinoma samples and paired adjacent normal tissues were obtained from patients with gastric cancer (GC) diagnosis, who underwent surgical resection at the Department of Thoracic and Abdominal Surgery of Tomsk Cancer Research Institute in years 2004–2009. All procedures in this study were performed by the Helsinki Declaration (1964, amended in 1975 and 1983). This study approved by Ethical Committee of the Tomsk Cancer Research Institute and all patients signed an informed consent for voluntary participation. Clinical features of the cases were gained from hospital records, and clinicopathological variables were evaluated in a cohort of 55 patients (Table 1).

RNA Extraction and qRT-PCR

Prior RNA isolation all tissue samples were homogenized by using Sartorius Mikro-Dismembrator U device (Eppendorf, Germany) at a velocity of 7300 rpm with liquid nitrogen cooling. Total RNA was extracted from tissue samples using an RNeasy mini kit plus DNAse I digestion (Qiagen, Germany) with standard protocol and reversely transcribed into cDNA by using a RevertAid kit with random hexanucleotide primers (Fermentas, Lithuania) following the manufacturer's instructions. The expression of *PMEPA1* mRNA was detected by using RT-PCR.It was performed in triplicate reactions in a volume of 15 μl containing 250 lM dNTPs Sibenzyme, Russia), 300 μM forward and reverse primers, 200 μM probe, 2.5 mM MgCl2, 1.5 μl 10× SE buffer (67 mM Tris–HCl pH 8.8, 16.6 mM (NH4)2SO4, 0.01% Tween-20), 2.5 U Hot Start Taq polymerase (Sibenzyme, Russia), and 50 ng of template cDNA.

The primer sequences for PMEPAI were:

5′- TGTTCCAGAGCATGGAGATCA -3′ (forward) and 5′-GTGCAGACAGCTTGTAGTGG-3′ (reverse), FAM 5′-CATCGTGGTGGTGATGATGGTGATG-3′ BHQ1 (probe), for ACTB: 5′-GAGAAGATGACCCAGATCATGTT-3′ (forward), 5′-ATAGCACAGCCTGGATAGCAA-3′(reverse), FAM 5′-AGACCTTCAACACCCCAGCCAT-3′ BHQ1 (probe). The Cq value of PMEPA1 mRNA was normalized to the reference gene ACTB, and the relative was performed according to ΔCq mathematical method.

Table 1 Baseline gastric cancer patient characteristics (n = 55)

Characteristics		Mean ± standard deviation (range)	n (%)
Age (years)	<50	46.7 ± 3.4	10 (18.18%)
	≥50	64.5 ± 7,5	45 (81.82%)
Gender	Male		30 (54.55%)
	Female		25 (45.45%)
Invasion of tumor depth	pT1 (mucosa/submucosa)		4 (7.27%)
	pT2 (muscle/subserosa)		18 (32.73%)
	pT3/4 (serosa/organ inv.)		33 (60%)
Lauren type	Intestinal		27 (49.09%)
	Diffuse		28 (50.91%)
Lymph node involvement	pN0		22 (40%)
	pN1		22 (40%)
	pN2		8 (14.55%)
	pN3		3 (5.45%)
Distant metastasis	Negative		51 (92.73%)
	Positive		4 (7.27%)
Differentiation	Well differentiated		2 (3, 64%)
	Moderately differentiated		18 (32.72%)
	Poorly differentiated		26 (47.28%)
	Undefined		9 (16.36%)

Cell Culture

Following types of mammalian cell lines were used for this study, Chinese hamster ovary – CHO-K1 (ATCC, USA), human embryonic kidney HEK293T (ATCC, USA), mouse myeloma P3X63-Ag8.653 (ATCC, USA). The CHO and HEK cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) containing 10% fetal bovine serum (FBS, Omega Scientific, USA) and 50 μg/ml gentamicin sulfate. The mouse myeloma cells were cultured in an RPMI-1640 medium, supplemented with 15% FBS and the same concentration of gentamicin sulfate. All the cell lines mentioned above were maintained at 37 °C under 5% CO2.

Plasmid Construction and Transfection

The original plasmid DNA (pcDNA3.1-V5-His-A/TMEPAI) for subcloning of a target gene was a kind gift from Prof. Mitsuyasu Kato (University of Tsukuba, Japan). The cDNA of TMEPAI cloned into *XhoI* and *EcoRI* sites into pIRES2-EGFP vector (BD Biosciences Clontech, USA). Transfection with vector pIRES2-EGFP/ TMEPAI was performed by using of FuGENE® HD Transfection Reagent (Promega, USA) according to the manufacturer instructions. Fixation of the cells and the microscopic study was carried out after 48 h of cultivation.

Production of Monoclonal Antibodies

The rat monoclonal antibodies against synthetic dodecapeptides, mimicked of the W275-L287 region from human TMEPAI coupled to ovalbumin (OVA) (PSL, Germany) were developed using the standard hybridoma technology, described earlier [22]. Antigen solution contained 50 μg peptide-OVA conjugate were injected intraperitoneally and subcutaneously to Lou/C rats using 5 nmol of CpG oligonucleotide (Tib Molbiol, Germany) as an adjuvant. An antigen boost without adjuvant was given to rats six weeks after the primary injection. The splenocytes were isolated and fused with mouse myeloma cells P3X63-Ag8.653 (ATCC, USA) at the ratio 3:1 with 50% polyethylene glycol-1500 solution (Roche, Germany). Following the fusion procedure, cells were cultured on 96-well plates, in RPMI-1640 medium, supplemented with 20% fetal calf serum, L-glutamine, non-essential amino acid solution (PAA, Germany), penicillin/streptomycin and aminopterin (Sigma, USA). The supernatants from hybridoma cells were tested by indirect enzyme-linked immunosorbent assay (ELISA) with the TMEPAI peptide-OVA conjugate and irrelevant peptides coupled to the same carrier for counterscreening. Bounded antibodies were detected by using a biotinylated mouse monoclonal antibodies (mAbs) against the rat IgG heavy chains, for avoiding the detection of IgM-producing cells. Supernatants selected by ELISA were used for additional screening of specific antigen-binding abilities in immunoblotting (IB). Hybridomas producing antibodies, applicable both for ELISA and IB were subcloned by limiting dilution method with following isotype determination using Rat immunoglobulin isotyping ELISA kit (BD Pharmingen, USA). The supernatants from following hybridoma clones 6C6, 2E1 and 10A7 were selected for mAbs purification by using affinity chromatography on Protein G Sepharose 4FF (GE Healthcare, USA). Eluted fractions were concentrated on Amicon-15 30 kDa cells (Millipore, USA) to 1.75–2.25 mg/ml (estimated by BCA assay), and purity of produced antibodies was controlled by electrophoresis (SDS-PAGE).

Protein Extraction and Immunoblotting

The total protein was extracted from HEK293T cells and tissue samples in a lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% Na-deoxycholate, 1% NP-40, 5 MUrea, 2 M Thiourea, 100 mM DTT, 1× cOmplete Lysis-M EDTA-free (Roche, USA). To determine protein concentration in the supernatants, BCA Protein Assay Kit (Pierce, USA) was used. Protein samples were quantified, loaded on 12.5% polyacrylamide gel (SDS-PAGE) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). PVDF membranes were incubated with primary rat monoclonal anti-TMEPAI antibodies diluted 1:1000 overnight at 4 °C; these antibodies were produced by different hybridomas mentioned above. Subsequently, washing with PBS, containing 0.5% Tween-20 (Sigma, USA) were performed, and membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (AffiniPure goat anti-rat IgG LC, Jackson ImmunoResearch Inc., USA) diluted 1:10,000 for 60 min at 30 °C. For the purpose of loading control primary HRP-labeled antibody anti-β-actin (ab197277, Abcam, UK) diluted 1:5000 wasused. Detection of the signal was performed by using an electrochemiluminescence (ECL) detection system (GE Healthcare, USA). All experiments were repeated in replicates, the intensity of protein bands was quantified using ImageLab densitometric software (Bio-Rad,USA).

Immunofluorescence

To visualize TMEPAI expression in transfected CHO-K1 cells, the method of indirect immunofluorescence was used. Cells were incubated with primary anti-TMEPAI 10A7 antibody in dilution 1:100 at 37 °C for 45 min with following PBS washing step. After that, cells were incubated with the TRITC-labeled secondary antibody (donkey anti-rat IgG H + L, Sigma, USA) in dilution 1:400, at 37oC for 40 min. To perform the nuclear DNA staining in fixed cells, the DAPI (VectorLabs, USA) was used. All immunofluorescence

experiments were done by using the Axio Imager Z1 Microscope (Carl Zeiss, Germany) with the appropriate filters set and camera AxioCam MRm (Carl Zeiss, Germany).

Immunohistochemistry

For detection of TMEPAI, 4 μm paraffin-embedded tissue sections were fixed on Superfrost Plus microscope slides (Thermo Scientific, USA). Then slides were deparaffinized and rehydrated by conventional method [23]. The endogenous peroxidase activity was blocked by incubation with Hydrogen Peroxidase Block (Thermo Scientific, USA) for 10 min at room temperature. After double washing in PBS for 30 min at room temperature the sections were incubated with primary rat anti-TMEPAI antibodies in dilution 1:100 at 25 °C for 60 min and stained with (HRP)-labeled secondary antibody (goat anti-rat IgG H&L, ab97057, Abcam, UK) diluted 1:200 for 60 min at 30 °C. The development of immunochemical reactions were performed by BioGenex Super Sensitive Non-Biotin HRP Detection System (BioGenex, USA) and visualized by diaminobenzidine tetrahydrochloride (DAB) for 10 min at room temperature; Meyer's hematoxylin was used for counterstaining. Negative controls were performed in the presence of secondary antibody only; rat IgG's isotype negative controls were carried out by appropriate replacement of primary anti-TMEPAI antibodies with the following MCA1124 (IgG2a), MCA1125 (IgG2b) and MCA2879 (IgG2c) (AbD Serotec, USA) under the same conditions. All immunohistochemical (IHC) experimentswere performed by using the Axio Scope A1 Microscope (Carl Zeiss, Germany); results were evaluated independently by two pathologists (EVK, MVZ). For each tissue section, ten representative fields with well-preserved tumor tissue were analyzed at magnification ×100 and × 400, and 100 carcinoma cells were counted.

MicroRNA Analysis

The study included 16 paired clinical tumor and normal gastric epithelium specimens from gastric cancer patients. Tissue samples were obtained during operation and immediately frozen in liquid nitrogen and then stored at −80 °C. These samples were homogenized with Sartorius Mikro Dismembrator U (Eppendorf, Germany) at 7300 rpm under cooling with liquid nitrogen. The RNA was extracted from the tissue samples using the mirVana™ kit (Ambion, USA) as per the manufacturer's instructions. Multiplex reverse transcription (RT) on miR templates was performed as described by Chen et al. Stem-loop RT primers are listed in Table 2.

Table 2 Primers and probes for reverse transcription and realtime PCR

miRNA	Sequence
Universal reverse primer	5′-GTGCAGGGTCCGAGGTAT-3'
miR-511–3p-RT:	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTGTC-3'
miR-511–3p-F:	5′- GCCGCAATGTGTAGCAAAA-3'
miR-511–3p-P:	5′-(FAM)-TCGCACTGGATACGACTCTGTC -(BHQ)-3'
miR-96-5p-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCAAA-3'
miR-96-5p-F	5′- CCGCTTTGGCACTAGCACAT-3'
miR-96-5p-P:	5′-(FAM)-CGCACTGGATACGACAGCAAA-(BHQ)-3'
miR-19a-3p-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTT-3'
miR-19a-3p-F	5′- CCGCTGTGCAAATCTATGCA -3'
miR-19a-3p-P	5′-(FAM)-TCGCACTGGATACGAC TCAGTTT-(BHQ)-3'
miR-194-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCACA-3'
miR-194-F	5′-GCCGCTGTAACAGCAACTCC-3'
miR-194-P	5′-(FAM)-CGCACTGGATACGACTCCACA-(BHQ)-3'
miR-301a-RT	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTTTGA-3'
miR-301a-F	5′-GCCGCCAGTGCAATAGTATT-3'
miR-301a-P	5′-(FAM)-CGCACTGGATACGACGCTTT-(BHQ)-3'
miR-191a -5p-RT:	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTGC-3'
miR-191a -5p-F:	5′- CGCCAACGGAATCCCAAAA-3'
miR-191a -5p-P:	5′-(FAM)-CGCACTGGATACGACCAGCTGC-(BHQ)-3'

RNA concentration and quality weremeasured with a NanoDrop-2000 spectrophotometer (Thermo Scientific, USA). The RNA integrity was estimated with capillary electrophoresis using TapeStation 2200 (Agilent Technologies, USA). Real-time PCR amplification was carried out on the RotorGene 6000 Real-Time PCR System (Corbett research, Australia). All reactions were carried out in duplicate in a total volume of 10 μl. Each reaction contained 1 μl RT product, 0.35 unit Taq DNA polymerase, 1× PCR buffer (pH = 8.3), 3.0 mM MgCl2, 200 μM each dNTP, 200 nM forward primer, 20 nM universal reverse primer, and 400 nM specific TaqMan probe. After initial activation of Taq polymerase at 95 °C for 10 min, the reactions were runfor 45 cycles at 95 °C for 15 s and 62 °C for 60 s. miR-191a was chosen for normalizing the data (Peltier HJ, Latham GJ., 2008). Micro-RNA expression value was scored as $\log_2$-transforemed fold change between relative quantity of target miR and miR-191a.

Statistical Analysis

The differences in *PMEPA1* mRNA and protein expression level were analyzed by The Mann-Whitney U-test. Median and range for the expression value were calculated by using software package Statistica 8.0 (StatSoft, Inc., USA). The TMEPA1 protein expression differences between the paraffin-embedded sections of cancer tissues and adjacent normal mucosa were compared using the Chi-square test.A one-sample t-test was used to assay the significance of the differences obtained during the microRNA expression analysis.

Results

Upregulation of *PMEPA1* mRNA Expression in Gastric Cancer

In this study, a total 55 pairs of clinical specimens from surgically treated patients were used. The characteristics of studied cohort were summarized in Table 1. To determine altered *PMEPA1* expression in a different types and grades of GC, the mRNA level of target protein were detected by real-time quantitative reverse transcription PCR (qRT-PCR) and normalized to ACTB. The rate of PMEPA1 gene expression in gastric tumor tissues was significantly higher compared to adjacent normal tissues. As a result increased mRNA level of PMEPA1 in the tumor compared with normal tissue was observed (2.63 (1.82; 3.84) versus 1.32 (0.79; 2.81), $p = 0.042$, median (range), Mann Whitney U test). On average, more than two-fold increase in expression of PMEPA1 was observed in 44% of patients (Fig. 2). When patients were stratified by histological type of tumor, the significantly increased expression level was observed only for the intestinal type of GC compare to

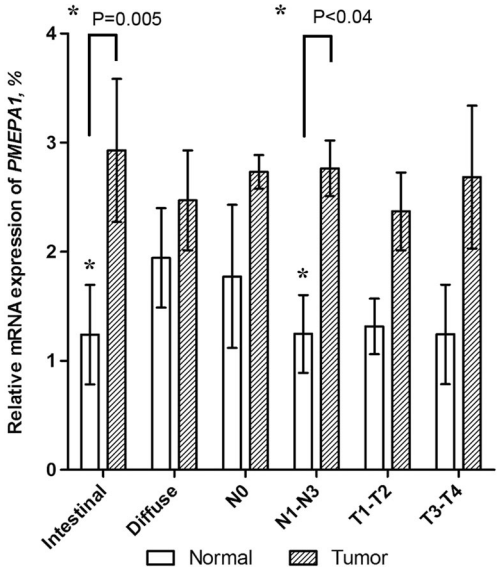


Fig. 2 Altered expression of PMEPA1 mRNA in gastric cancer. Analysis of quantitative real-time reverse transcription polymerase chain reaction showed that mean expression level of *PMEPA1* mRNA in tumor tissues was significantly higher compared to the value in adjacent normal tissues. Results were subdivided into groups according to Lauren classification (intestinal; diffuse); lymph node involvement (N0-N3) and depth of tumor invasion (T1-T4). The considerably increased level of expression was observed for the intestinal type of gastric cancer compare to normal tissue (Me (Q25-Q75), $N = 27$; $p = 0.0005$, Mann–Whitney U-test) and for patients with lymph node metastasis ($N = 33$, $p = 0.042$)

normal tissue (2.88 (2.35; 3.63) versus 1.1 (0.8; 1.7), $p = 0.000456$). More than two-fold increase of PMEPA1 expression was found in.

Fifty-eight percent of patients from this group. Notable the gene overexpression was detected only in case of patients with regional metastases (2.76 (2.12; 3.34) versus 1.22 (0.79; 1.51), $p = 0.042$).

Production and Characterization of Novel Rat Anti-TMEPAI Monoclonal Antibodies

Based on structural features and intracellular localization of target protein we had performed an in silico prediction of immunogenic epitopes, suitable for anti-TMEPAI monoclonal antibody generation. In thecase of transmembrane proteins, it is common to select the mimicking synthetic peptides based on N- or C-terminal amino acid sequences [24]. We had selected dodecapeptide segment, mimicking W275-L287 region of human TMEPAI, which is presented in all three isoforms of the target protein (Fig. 1).

The Lou/C rats were immunized intraperitoneally and subcutaneously with dodecapeptides conjugated to ovalbumin. In three weeks after immunization level of specific antibodies in rat serums were determined by ELISA. When antibody titers reached 1:16,000, immunoblotting was performed to evaluate the ability of candidate serums recognize recombinantly

expressed target protein in lysates of HEK293T cells. The rat splenocytes with preliminary defined high-titer antibody response were isolated for further fusion with mouse myeloma cells P3X63-Ag8.653. Hybridomas cells were screened for production of antibodies that able to bind immunogenic dodecapeptide and TMEPAI protein. Finally, we obtained three different clones (2E1, 6C6, and 10A7), which was successfully subcloned and stabilized. Three selected hybridoma clones were expanded to large-scale cultivation to get antibody-containing supernatants; IgG fraction was purified by using Protein G. Isotyping of rat immunoglobulins showed that antibodies from clones were as follows 6C6 - IgG2a, 2E1 - IgG2b and 10A7 - IgG2c. Selected monoclonal antibodies were tested for theability to recognize recombinantly overexpressed TMEPAI in HEK293T lysates (Fig. 3) andnative conditions inside the overexpressing CHO-K1 cells by immunofluorescence staining approach (Fig. 4). We have observed the nuclear-cytoplasmic localization of target protein in transfected cells while untransfected cells were characterized by subtle background expression of TMEPAI. Endosomal localization of target protein near the Golgi apparatus, visualized by nuclear-cytoplasmic staining, was also demonstrated earlier in cell cultures [3] and other types of tumors [2]. This kind of intracellular localization is typical for other proteins that have structural motifs interacting with the WW-domain of ubiquitin ligase Nedd4 [2, 25].

Analysis of TMEPAI Expression in the Clinical Samples Using Developed mAbs

Primary immunochemical assessment of TMEPAI protein expression were performed onpaired frozen and paraffin-embedded tissue samples from GC patients by using rat mAb C6C ontotal protein extracts. In this tissue extracts selected mAb reacted with canonical TMEPAIisoform 1 (32–33 kDa) and N-terminally shorter isoform 2 (27–28 kDa). Those isoforms were detected in paired tissue samples as visually intense bands (Fig. 5a). The abundance levels ofindividual TMEPAI isoforms were relatively quantified by the scoring of immunoblot signalintensities. We observed the significantly decreased expression levels of target protein isoform 1and isoform 2 (Fig. 5b) in a cohort of patients with T1-T2 and T3-T4 GC stages. Remarkably,TMEPAI mRNA expression had a significantly negative correlation with its protein expressionlevel.

Preliminary, we performed experiments to characterize specific binding of commerciallyavailable antibodies sc-85,829 (Santa Cruz Biotechnology Inc., USA) and ab128006 (Abcam,UK) to TMEPAI in paraffin-embedded tissue sections. Application of listed commercial anti-TMEPAI antibodies does not allow us to detect the presence of target protein in aclinical sample by IHC (data not shown). To evaluate the possibility of using developed monoclonal antibodiesto detect target protein in paraffin-embedded tissue, we used IHC assay of normal and humangastric carcinoma tissues sections. Here, we show that TMEPAI has a nuclear-cytoplasmic localization in gastric epithelium cells. Moreover, the positively stained target protein was detected only in normal cells, which additionally confirms our data of decreased protein expression in the gastric tumor tissue identified by immunoblot analysis (Fig. 4).

To elucidate the TMEPAI expression pattern during malignant transformation of the gastric epithelium, we performed analysis of protein abundance in the normal mucosa, adenoma with different types of dysplastic changes, and adenocarcinoma. As a result, we have found a phenomenon consisting of

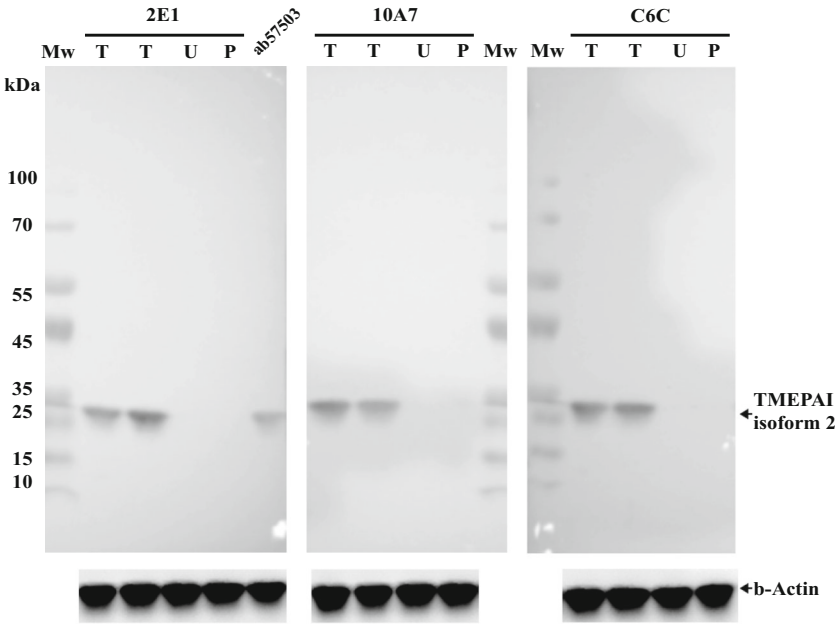


Fig. 3 Characterization of developed rat monoclonal antibodies by immunoblotting analysis. Cells HEK293T were transiently transfected with a plasmid pIRES2-EGFP/TMEPAI, encoding target protein (T). For the negative control propose the selected cells were kept untransfected (U) or transfected with control plasmid pIRES2-EGFP (P). After 48 h from transfection cells were lysed and total protein extracts were analyzed by immunodetection with purified mAbs 2E1, 10A7 or C6C respectively. Commercial anti-TMEPAI antibody (ab57503) was used for positive control; Loading control was performed by immunodetection of β-actin

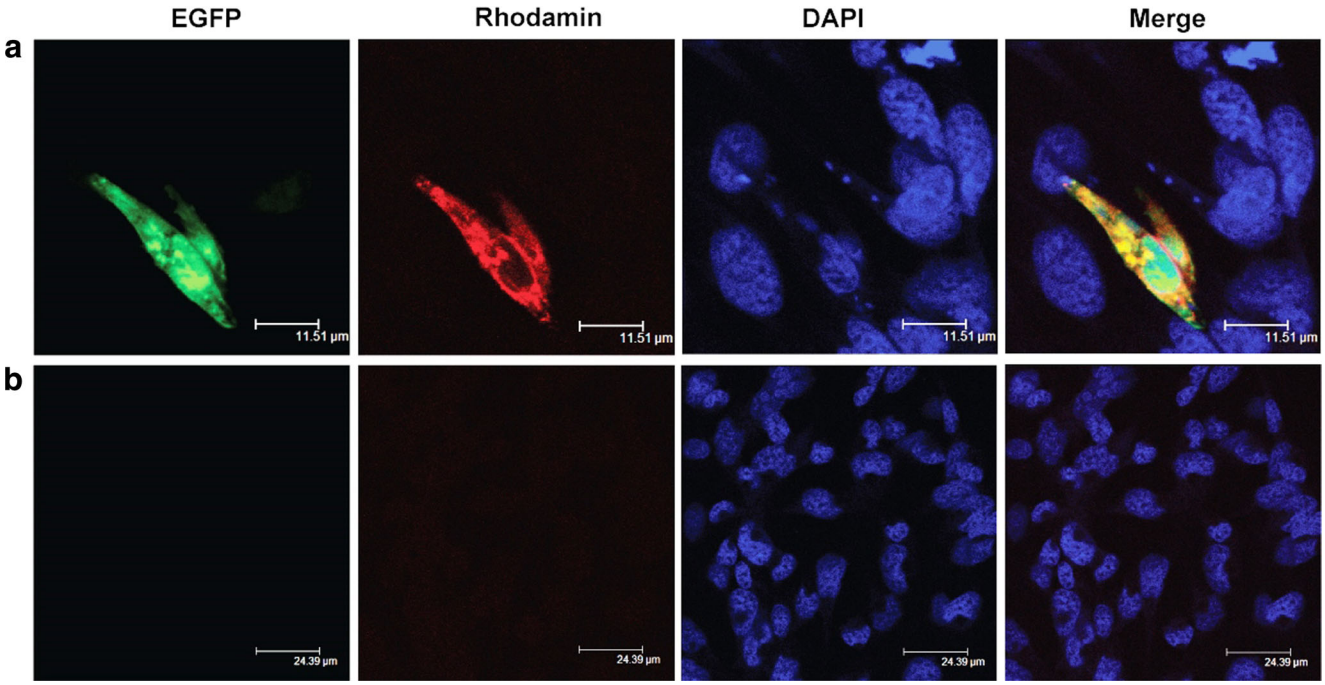


Fig. 4 Immunofluorescence staining of TMEPAI by rat monoclonal antibody 10A7. Cells CHO-K1 were transiently transfected with a plasmid pIRES2-EGFP/TMEPAI (**a**), encoding target protein and EGFP as transfection control; untransfected cells were used as negative control (**b**). To display cell nucleus DAPI (*blue fluorescence*) was used. Co-localization of fluorescence at three different wavelengths was shown

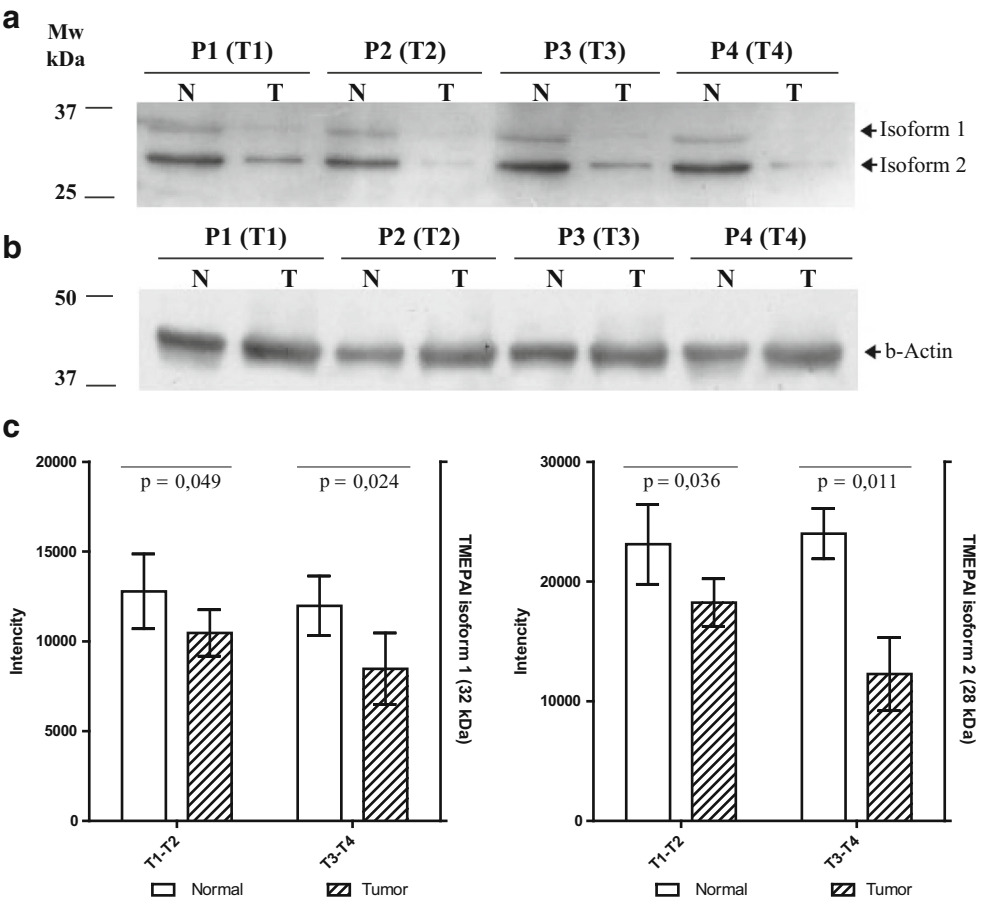


Fig. 5 Downregulation of TMEPAI expression levels in human intestinal gastric adenocarcinoma tissues ($N = 27$). The tissue samples were obtained from patients (P) with different stages of gastric cancer (T1-T4), the total protein extracts were separated on 12.5% SDS-PAGE under reducing conditions with subsequent immunodetection by rat monoclonal antibody 10A7. One representative immunoblotting from three experiments showing the expression of TMEPAI protein (**a**) in paired normal (N) and tumor (T) tissues. Target protein expression levels were determined using β-actin (**b**) as the endogenous control. The intensity of protein bands was measured using ImageLab densitometric software and divided according to the type of TMEPAI isoforms (**c**) and analyzed by Mann–Whitney U-test

increased expression of TMEPAI in normal gastric epithelial cells, while cells containing protein of interest in the adenomas and adenocarcinomas samples were practically absent (Fig. 6). An important fact is that the gastric epithelium cells lose the TMEPAI expression since the emergence of morphological signs of severe dysplastic changes also tissues sections of adenoma defined by high-grade dysplasia (Fig. 6). Analysis of adenoma sections with mild to moderate dysplasia has revealed same expression level of the target protein as normal epithelium (data not shown).

Comparison of miRNA Expression Using Real-Time PCR

To understand whether the expressionlevels of the selected miRNAs differs in normal and gastric cancertissue, a quantitative PCR was performed. The expression analysis of miR-511, miR-96, mir-19, mir-194 and mir-301 was held in the collection of frozen tumor and paired normal samples collected from 16 patients treated in Cancer Research Institute. We found asignificant increase of miR-19 in tumor tissue (fold changes – 7.9 ± 2.1) while miR-511expression was decreased (fold changes 3.7 ± 0.6). Furthermore, miR-96, mir-194, and mir-301 were tended to decrease, but these differences did not reach thelevel of significance ($p > 0.05$) (Fig. 7).

Discussion

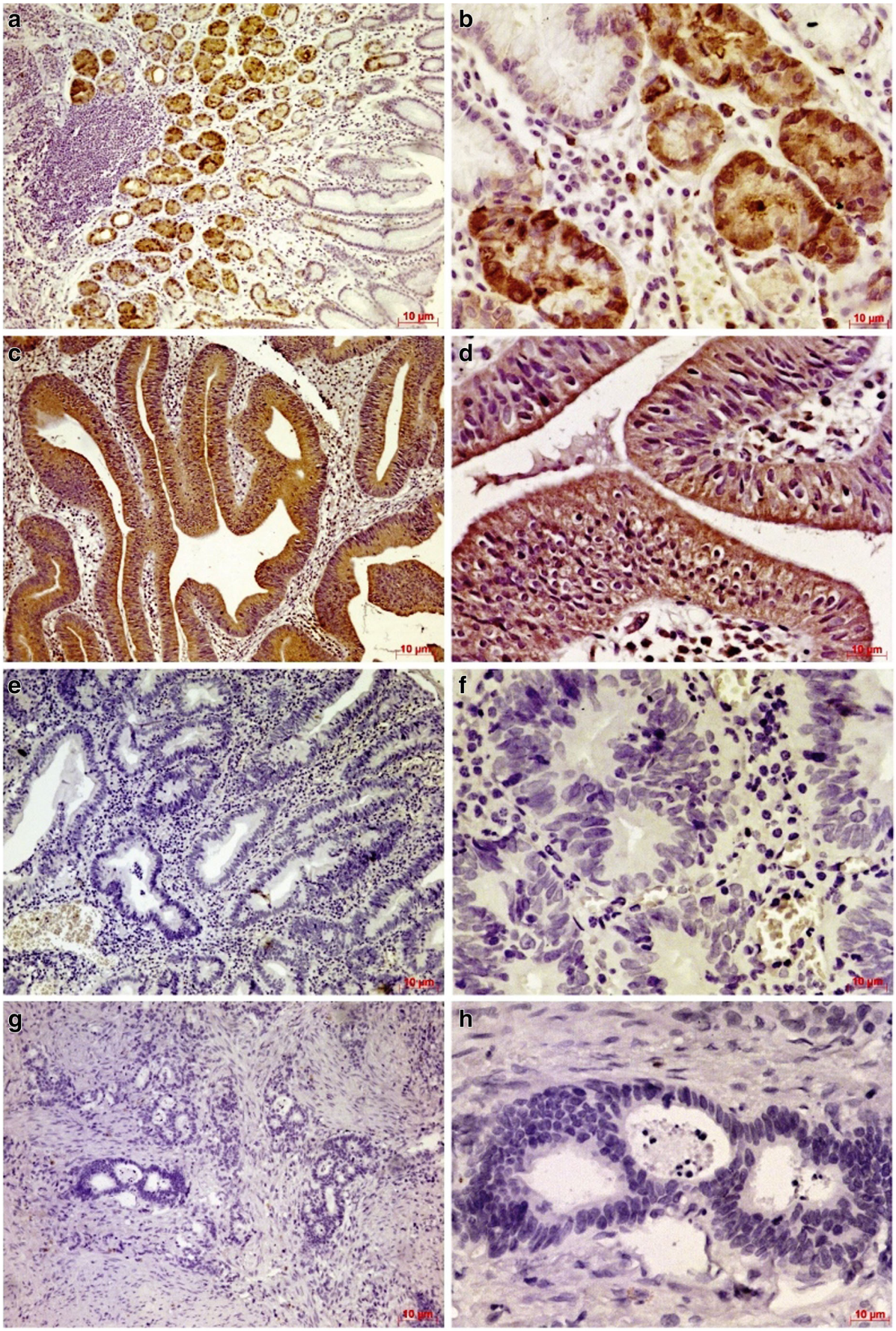
The gastric cancer is the fifth most common type of malignancy worldwide and the third main reason of cancer death [26]. Until now GC remains a serious diagnostic and therapeutic challenge. Recent improvements in prevention and treatment had appeared with thebetter long-time survival rate for patients with initial stages of GC, which is only about 30% of existing cases. Nevertheless, the efficacy of treatment for patients with progressed GC stages is imperfect, and 5-year survival is unsatisfied. The prediction of treatment results, based on clinical or histological characteristics are often unreliable.

Thus, understanding of molecular pathogenesis and identification of specific biomarkers for prediction of treatment outcome can be used for the development of personalized treatment approaches [27]. Interestingly GC cells express a broad spectrum of various growth factors, including TGF-β, which is predominantly up-regulated in poorly differentiated gastric carcinoma [28]. Besides main effects of TGF-β on metastatic ability and invasiveness of GC are driven by c-Jun N-terminal kinases (JNKs) and extracellular-signal-regulated kinases (ERKs) pathways excluding SMAD proteins [29]. However, decreased SMAD-related signaling also exists in GC and correlation between SMADs expression levels and outcome prognosis was discovered [30]. Also, TGF-β signaling is repealed by reduced expression of SMADs [31]. In this matter, the elucidation of TMEPAI involvement into TGF-β-inducible pathways become an actual task, due to demonstrated ability to regulate SMAD-signaling via its PY-motifs located at SIM domain [32]. It was also indicated, that reducing of TMEPAI expression by gene knockdown can suppress tumorigenic potential of lung cancer [4], breast cancer [14] and prostatic cancer [32].

The expression level of *PMEPA1* in tumors has been revealed to be significantly altered compared to non-malignant tissues. Our data confirms the results observed by other researchers that demonstrate up-regulated *PMEPA1* gene expression in gastric tumors [20, 21]. Until now in available literature, it faintly marked on TMEPAI immunochemical detection in the clinical samples. As well, there is no recommendation of Human Antibody Initiative database [33] for trustable commercially available mAbs that could provide researchers with the reliable detection of particular protein in human tissues. Addressing this problem is one of the most promising ways to the emerging needs of tissue-based proteomics [34–37]. We obtained three stable clones of hybridomas, which produced anti-TMEPAI antibodies. To evaluate the specificity we used immunoblot analysis and immunocytochemistry. It is known that the chain of events from normal gastric mucosa to adenocarcinoma includes chronic inflammation of the mucosa, atrophy, metaplasia and dysplasia of the epithelium. Dysplasia is characterized by significant alterations in the cellular elements, such as changing the size and shape of cells, nuclei pigmentation, anomalies of mitotic activity, which result in abnormal development of epithelial tissue [38].

Epithelial dysplasia is a morphological marker of an increased risk of cancer, but this risk has not yet been exactly evaluated in each patient. Apparently, in most cases mild and moderate dysplasia can regress or remain stable, though doubtless that there is a possibility of progression of these changes to adenocarcinoma. Severe dysplasia may also be subjected to regression, but the probability of transition to malignancy varies over a broad range from 8 to 75%. It is rather important to clarify the definition of the adenocarcinoma risk during pathological changes of gastric mucosa.

The observed decrease of target protein expression in tumor samples combined with increased gene expression can be explained by microRNAs-related posttranscriptional regulation mechanisms. For the period of the last decade, a considerable amount of information concerning the role of microRNA in carcinogenesis as oncogenes and tumor suppressors was accumulated. The participation of these nucleic acids in the regulation of apoptosis, cell cycle, and metastasis are well-known [39]. There are many miRNAs which expression is reduced (miR −24, miR −129-2, miR-451, miR-9) and increased (miR-130b, miR-106b, miR-93, miR-222, miR-221) in gastric cancer [40]. Recently, Zhou and colleagues identified a panel of microRNAs with significantly altered expression in gastric cancer cells after TGF-β treatment [41].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◀ **Fig. 6** Representative immunohistochemical staining for TMEPAI expression during the progress of GC with antibody 10A7. **a**, **b** high expression of TMEPAI in normal gastric tissues; **c**, **d** ahigh expression of TMEPAI in gastric adenomatous polyps; **s**, **f** weak expression of TMEPAI in gastric adenomatous polyps with high-grade dysplasia; **g**, **h** absence of TMEPAI in poorly differentiated gastric adenocarcinoma. Antibody 10A7 was used in dilution 1:100. The digital images analyzed with the AxioVision Software software (**a**, **c**, **e**, **g** with ×100 and **b**, **d**, **f**, **h** with ×400-fold magnification). The micrograph indicates the amplified morphology of tissue, *red scale bars* - 10 μm

We carried out an in silico identification of microRNAs which can potentially bindTPMEPAI. An initial set of miRNAs were obtained from five databases: TargetScan, miRWalk, miRDB, miRBase, miRAD. After that we have selected several miRNAs, namely – miR-511, miR-96, miR-19, miR-194 and miR-301,which potentially binds TMEPAI transcript with higher probability takes into consideration secondary structure and free energy of miRNA-target duplex formation. We evaluated expression level of selected microRNAs in normal and tumor tissues of gastric cancer patients. It turns out that increased expression in tumor tissue compared to normal specimens has only one miRNA - mir-19.

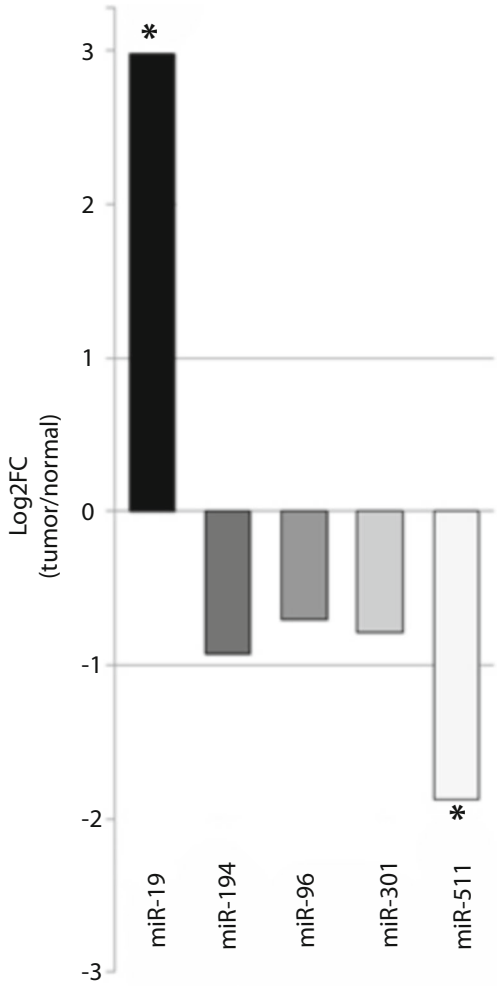

Fig. 7 Expression ratio (log2) of investigated microRNA obtained by qRT-PCR. The figure shows mean normalized expression ratio (Log2 fold change) for miR-511, miR-96, mir-19, mir-194 and mir-301. A bar value >1 represents >2-fold increases, whereas a bar value <−1, represents a > 2-fold decrease. Only microRNA mir-19 was found to be significantly up-regulated in thegastric tumor. Mir-194, mir-96 and mir-301 were insignificantly dawn-regulated in gastric cancer while the expression level of mir-511 was observed to be greatly decreased in thetumor. One-sample t-test was used to assay the significance of the differences obtained during the analysis

The miR-19 is amember of miR-17-92 clusterwhich encodes 6 single mature miRNAs. miR-19 has been identified as the key responsible for this oncogenic activity [42]. Li et al. (2015) has shown that miR-19 overexpression activated some EMTand mobility-related genes in lung cancer cells, which is likely responsible for the switch on of the epithelial-mesenchymal transition, as strongly supported by mesenchymal-like morphological conversion, decreased cell adhesion, and increased cell motility and invasion [43]. The same data was obtained during astudy on colorectal cancer cells [44]. Furthermore, overexpression of mir-19 was also shown in gastric cancer cells, wherein the increase correlates with tumor metastasis [45] and proliferative activity of tumor cells [46]. In the systematic review of microRNA expression profiling studies in human gastric cancer held by Shrestha S. (2014) was shown miR-19 overexpression in tumor tissue in five independent studies [47]. Median fold change of miR-19 overexpression in gastric cancer was 3.99, which corresponds to data obtained in our study.

To confirm miR-19 ability to block TMEPAI expression, it is necessary to carry out the additional studies on stablegastric cancer cell lines. However, even now it can be assumed due to its potential ability to silence TMEPAI gene expression, miR-19 has been hailed as apotential therapeutic agent since the decrease of TMEPAI production is a poor prognostic factor.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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References

1. Stone L (2015) Prostate cancer: TGF-beta signalling regulator PMEPA1 halts metastases tobone. Nat Rev Urol 12(7):362
2. Xu LL et al (2000) A novel androgen-regulated gene, PMEPA1, located on chromosome20q13 exhibits high level expression in prostate. Genomics 66(3):257–263

3. Watanabe Y et al (2010) TMEPAI, a transmembrane TGF-beta-inducible protein, sequestersSmad proteins from active participation in TGF-beta signaling. Mol Cell 37(1):123–134
4. Vo Nguyen TT et al (2014) TMEPAI/PMEPA1 enhances tumorigenic activities in lungcancer cells. Cancer Sci 105(3):334–341
5. Azami S et al (2015) Cooperative induction of transmembrane prostate androgen inducedprotein TMEPAI/PMEPA1 by transforming growth factor-beta and epidermal growth factorsignaling. Biochem Biophys Res Commun 456(2):580–585
6. Hu Yet al. (2013) TMEPAI regulates EMT in lung cancer cells by modulating the ROS and IRS-1 signaling pathways. Carcinogenesis 34(8):1764–1772
7. Ellenrieder V (2008) TGFbeta regulated gene expression by Smads and Sp1/KLF-liketranscription factors in cancer. Anticancer Res 28(3A):1531–1539
8. Gratchev A et al (2008) Activation of a TGF-beta-specific multistep gene expression programin mature macrophages requires glucocorticoid-mediated surface expression of TGF-betareceptor II. J Immunol 180(10):6553–6565
9. Brunschwig EB et al (2003) PMEPA1, a transforming growth factor-beta-induced marker ofterminal colonocyte differentiation whose expression is maintained in primary and metastaticcolon cancer. Cancer Res 63(7):1568–1575
10. Nakano N et al (2014) C18 ORF1, a novel negative regulator of transforming growth factorbeta signaling. J Biol Chem 289(18): 12680–12692
11. Fournier PG et al (2015) The TGF-beta signaling regulator PMEPA1 suppresses prostate cancer metastases to bone. Cancer Cell 27(6):809–821
12. Nagaraj NS, Datta PK (2010) Targeting the transforming growth factor-beta signalingpathway in human cancer. Expert Opin Investig Drugs 19(1):77–91
13. Li H et al (2015) Silencing of PMEPA1 accelerates the growth of prostate cancer cellsthrough AR, NEDD4 and PTEN. Oncotarget 6(17):15137–15149
14. Singha PK et al (2014) TGF-beta induced TMEPAI/PMEPA1 inhibits canonical Smadsignaling through R-Smad sequestration and promotes non-canonical PI3K/Akt signaling byreducing PTEN in triple negative breast cancer. Genes Cancer 5(9–10):320–336
15. Saadi A et al (2010) Stromal genes discriminate preinvasive from invasive disease, predictoutcome, and highlight inflammatory pathways in digestive cancers. Proc Natl Acad Sci U S A 107(5):2177–2182
16. Hirokawa YS et al (2007) High level expression of STAG1/PMEPA1 in an androgenindependentprostate cancer PC3 subclone. Cell Mol Biol Lett 12(3):370–377
17. Sharad S et al (2014) Methylation of the PMEPA1 gene, a negative regulator of the androgenreceptor in prostate cancer. Epigenetics 2014.9(6):918–927
18. Volkomorov V et al (2013) Search for potential gastric cancer markers using miRNAdatabases and gene expression analysis. Exp Oncol 35(1):2–7
19. Grigoryeva ES et al (2013) Expression of Cyclophilin a in gastric adenocarcinoma Patientsand its inverse association with local relapses and distant metastasis. Pathol Oncol Res 20(2):467–473
20. Rae FK et al (2001) Characterization of a novel gene, STAG1/PMEPA1, upregulated inrenal cell carcinoma and other solid tumors. Mol Carcinog 32(1):44–53
21. Rajkumar T et al (2010) Identification and validation of genes involved in gastrictumorigenesis. Cancer Cell Int 10:45
22. Rottach A et al (2008) Generation and characterization of a rat monoclonal antibody specificfor PCNA. Hybridoma (Larchmt) 27(2):91–98
23. Shi SR, Liu C, Taylor CR (2007) Standardization of immunohistochemistry forformalin-fixed, paraffin-embedded tissue sections based on the antigen-retrieval technique: fromexperiments to hypothesis. J Histochem Cytochem 55(2):105–109
24. Huang M et al (2014) Generation of a monoclonal antibody specific to a new candidate tumorsuppressor, cell adhesion molecule 2. Tumour Biol 35(8):7415–7422
25. Harvey KF et al (2002) N4WBP5, a potential target for ubiquitination by the Nedd4 familyof proteins, is a novel Golgi-associated protein. J Biol Chem 277(11):9307–9317
26. Fock KM (2014) Review article: the epidemiology and prevention of gastric cancer. Aliment Pharmacol Ther 40(3):250–260
27. Duraes C et al (2014) Biomarkers for gastric cancer: prognostic, predictive or targets oftherapy? Virchows Arch 464(3):367–378
28. Hofler H, Becker KF (2003) Molecular mechanisms of carcinogenesis in gastric cancer. Recent Results Cancer Res 162:65–72
29. Fu H et al (2009) TGF-beta promotes invasion and metastasis of gastric cancer cells byincreasing fascin 1 expression via ERK and JNK signal pathways. Acta Biochim Biophys Sin Shanghai 41(8): 648–656
30. Shinto O et al (2010) Phosphorylated smad 2 in advanced stage gastric carcinoma. BMC Cancer 10:652
31. Kubiczkova L et al (2012) TGF-beta - an excellent servant but a bad master. J Transl Med 10:183
32. Liu R et al (2011) PMEPA1 promotes androgen receptor-negative prostate cell proliferationthrough suppressing the Smad 3/4-c-Myc-p 21 Cip1 signaling pathway. J Pathol 223(5):683–694
33. Bjorling E, Uhlen M (2008) Antibodypedia, a portal for sharing antibody and antigenvalidation data. Mol Cell Proteomics 7(10): 2028–2037
34. Bordeaux J et al (2010) Antibody validation. BioTechniques 48(3): 197–209
35. Mischak H et al. (2010) Recommendations for biomarker identification and qualification inclinical proteomics. Sci Transl Med 2(46):46ps42
36. Uhlen M et al (2015) Proteomics. Tissue-based map of the human proteome. Science 347(6220):1260419
37. Huttenhain R et al (2012) Reproducible quantification of cancer-associated proteins in bodyfluids using targeted proteomics. Sci Transl Med 4(142):142ra94
38. Flejou JF (2005) Barrett's oesophagus: from metaplasia to dysplasia and cancer. Gut 54(Suppl 1):i6–12
39. Jansson MD, Lund AH (2012) MicroRNA and cancer. Mol Oncol 6(6):590–610
40. Wu WK et al (2010) MicroRNA dysregulation in gastric cancer: a new player enters the game. Oncogene 29(43):5761–5771
41. Zhou H et al (2013) TGF-beta 1 alters microRNA profile in human gastric cancer cells. Chin J Cancer Res 25(1):102–111
42. Grillari J, Hackl M, Grillari-Voglauer R (2010) miR-17-92 cluster: ups and downs in cancer and aging. Biogerontology 11(4):501–506
43. Li J, Yang S, Yan W et al (2015) MicroRNA-19 triggers epithelial-mesenchymal transition of lung cancer cells accompanied by growth inhibition. Lab Investig 95(9):1056–1070
44. Cellura D, Pickard K, Quaratino S et al (2015) miR-19-mediated inhibition of transglutaminase-2 leads to enhanced invasion and metastasis in colorectal cancer. Mol Cancer Res 13(7):1095–1105
45. Tsai MM, Wang CS, Tsai CY et al (2014) MicroRNA-196a/-196b promote cell metastasis via negative regulation of radixin in human gastric cancer. Cancer Lett 351(2):222–231
46. Wu Q, Yang Z, An Y et al (2014) MiR-19a/b modulate the metastasis of gastric cancer cells by targeting the tumour suppressor MXD1. Cell Death Dis 27(5):e1144
47. Shrestha S, Hsu SD, Huang W et al (2014) A systematic review of microRNA expression profiling studies in human gastric cancer. Cancer Med 3(4):878–888