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ORIGINAL ARTICLE

Expression of transglutaminase-2 isoforms in normal human tissues and cancer cell lines: dysregulation of alternative splicing in cancer

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Abstract The multiple enzymatic activities and functions of transglutaminase type 2 (TG2) may be attributed to alternative TG2 molecules produced by differential splicing of TG2 mRNA. Different RNA transcripts of the human TG2 gene (TGM2) have been identified, but the expression of TG2 multiple transcripts has never been systematically addressed. We have confirmed and rationalized the main TG2 variants and developed a screening assay for the detection of alternative splicing of TG2, based on real-time reverse-transcription PCR. We have quantified the multiple TG2 transcripts in a wide range of normal tissues and in cancer cell lines from four different sites of origin. Our data show a significant correlation in the expression of canonical and alternative TG2 isoforms in normal human tissue, but differences in alternative splicing of TG2 in cancer cell lines, suggesting that in cancer cells the alternative splicing of TG2 is a more active process.

Keywords Transglutaminase-2 · Alternative splicing · Splicing dysregulation · Normal tissue · Cancer · Cell lines

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Introduction

Transglutaminase type 2 (TG2) (EC 2.3.2.13) is a multifunctional ubiquitous protein primarily known for its role in protein transamidation, which is dependent on Ca^{2+} binding/GTP dissociation. Although TG2 has been considered a pro-apoptotic factor, recent studies have reported that TG2 is overexpressed in many cancers where it contributes to increased malignancy by exerting an anti-apoptotic and anti-anoikis pro-survival effect (Verderio et al. 2003; Verma and Mehta 2007). TG2 expression is induced by hypoxia in several tumour cell lines in a hypoxia-inducible factor 1 (HIF-1) dependent manner (Jang et al. 2010), thus contributing to the growth advantage of cells in solid tumours. The consequences of TG2 knockdown in cancer cell lines suggest that TG2 expression correlates with molecular and cellular events associated with tissue invasion such as matrix metalloproteinase-2 function (Satpathy et al. 2009), epidermal growth factor signalling/cell migration (Antonyak et al. 2009), and epithelial-to-mesenchymal transition of ovarian cancer and mammary epithelial cell lines (Shao et al. 2009; Kumar et al. 2010). Animal studies have linked TG2 to tumourigenecity of human cancer cell xenografts (Jang et al. 2010; Satpathy et al. 2007, 2009; Hwang et al. 2008). TG2 activation of nuclear factor-κB (NF-κB) has been implicated in several TG2-mediated tissue transformation events (Mann et al. 2006; Jang et al. 2010; Shao et al. 2009). However, whether this wide range of pro-survival functions is to be attributed to TG2-mediated Ca^{2+}-dependent transamidation or the other enzymatic and

structural functions of TG2 remains contradictory (Satpathy et al. 2009; Antonyak et al. 2009; Jang et al. 2010; Hwang et al. 2008). Since different RNA transcripts of TG2 have been identified in addition to the constitutively spliced full-length TG2 mRNA (Gentile et al. 1991), it remains a possibility that the function of TG2 in cancer may also depend on the pattern of expression of alternative TG2 molecules, which may be capable of exerting a diverse array of cellular roles in the cell (Tee et al. 2010).

The constitutively spliced full-length TG2 transcript, which was first reported by Gentile et al. (1991), encodes a polypeptide of 687 amino acid residues with an approximate molecular mass of 75 kDa. Two alternative transcripts encoding TG2 protein isoforms were subsequently cloned from retinoic acid-induced human erythroleukemia cells, revealing open reading frames coding for a polypeptide of 548 amino acid residues with a molecular mass of ~62 kDa (TGH, also known as Tgase S or TG2-S) (Fraij et al. 1992; Antonyak et al. 2006; Tee et al. 2010) and for a polypeptide of 349 amino acid residues with a predicted molecular mass of ~38 kDa (TGH2) (Fraij and Gonzales 1996). These alternative transcripts are generated through an intron retention mechanism and encode for truncated TG2 isoforms with a different C-terminus. Loss of the C-terminal GTP binding regulatory domain (Arg-580), which controls the response of TG2 to Ca^{2+} activation (Begg et al. 2006), is the main feature of this set of TG2 variants. Interestingly, expression of the short TG2 isoform (TGH) was found to be increased in Alzheimer's disease (AD) brains and it was suggested to be responsible for the marked increase in Ca^{2+}-dependent TG2 cross-linking measured in AD brains (Citron et al. 2001). The short form of TG2 has been attributed opposite cellular effects to full-length TG2. For example, in neuroblastoma cells it was found to induce cell differentiation unlike full-length TG2, which acted as a repressor of cell differentiation (Tee et al. 2010). When expressed as a Myc-tagged protein in NIH3T3 cells, the short form of TG2 was reported to be cytotoxic, whereas the full-length TG2 conferred a growth advantage of these cells (Antonyak et al. 2006). Two further alternatively spliced forms of TG2 have been more recently detected and characterized in human umbilical vein endothelial cells (HUVEC), vascular smooth muscle cells and leukocytes (tTGv1 and tTGv2) (Lai et al. 2007). These TG2 variants are generated through an atypical alternative splicing event (Lai et al. 2007) resulting in proteins of similar mass to full-length TG2, but once again with alternative C-terminus.

Alternative splicing provides cells with the opportunity to produce protein isoforms that confer growth and survival advantage and therefore these isoforms may be preferentially expressed by cancer cells. In other cases, splice variants are simply a consequence of the cellular alterations of cancer cells (e.g. aberrant cell splicing events), not necessarily the cause of the cancer phenotype, but their differential expression can be exploited in clinic for diagnostic and therapeutic purposes (Srebrow and Kornblihtt 2006; David and Manley 2010).

The expression of TG2 multiple transcripts has never been systematically addressed in normal tissue and cancer cells. We have developed a screening assay for the TG2 multiple variants based on real-time reverse-transcription PCR, and have systematically quantified the alternative splicing of TG2 in a wide range of normal tissue and cancer cell types from four different sites of origin. Our data suggest a significant correlation in expression of canonical and alternative TG2 transcripts in normal human tissue, but differences in alternative splicing of TG2 in human cancer cell lines.

Materials and methods

Human cancer cell lines and culture conditions

All culture media were supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM L-glutamine, unless otherwise stated. Cells were maintained in a 5% CO_2 humidified atmosphere at 37°C and utilised at passage <25, except for MDA231, OPCT1 and OPCT2 which were at a higher passage number. Reagents were obtained from Lonza, unless specified.

The human breast cell line MDA231 was generously donated by Dr. P. de Gremoux, Institut Curie, Paris, France. The human breast cancer cell lines MDA468, MCF7 and T47D were purchased from American Type Culture Collection (ATCC). The breast cancer-derived cell line BR293 was obtained from Dr. L. Li, The University of Nottingham Medical School, UK. The breast cancer cell lines MDA231, MDA468 and T47D were cultured in RPMI-1640; BR293 were cultured in EMEM supplemented with non-essential amino acids (NEAA); MCF7 were maintained in DMEM with NEAA.

The human prostate cancer cell lines LNCAP, DU145 and PC3 were obtained from ATCC. The prostate cell lines OPCT1 and OPCT2 were obtained from Onyvax Ltd. DU145 cells were cultured in DMEM supplemented with NEAA and 1 mM sodium pyruvate. LNCAP cells were cultured in RPMI-1640 growth medium supplemented with hydrocortizone (5 ng/ml) and testosterone (5 ng/ml). PC3 was grown in HAMS with NEAA. OPCT1 and OPCT2 were grown in KSFM supplemented with 2% (v/v) FCS and recombinant human epidermal growth factor (30 ng/ml).

The human melanoma cell lines FM3, Ma-Mel-11 (MM11), Ma-Mel-12 (MM12), Ma-Mel-26a (MM26a),

Ma-Mel-27 (MM27), Ma-Mel-28 (MM28), Ma-Mel-30 (MM30), Ma-Mel-45a (MM45a), and Ma-Mel-66a (MM66a) were obtained from Prof. Dirk Schadendorf, DKFZ, Heidelberg, Germany. All melanoma cell lines were cultured in RPMI-1640.

The human head and neck cancer cell lines CAL27 and A253 were obtained from Dr. Eric Tartour, Hopital Européen Georges Pompido, Paris, France. JHU11, JHU12, PCI-13 and PCI-30 were obtained from Prof. Robert Ferris, University of Pittsburgh, USA. The head and neck cancer cell lines were grown in RPMI-1640, except for Cal27 which was cultured in DMEM supplemented with NEAA.

Human normal tissues

Human tissue RNAs were obtained from Human Master panel (BD Clontech) consisting of total RNA from adrenal gland, colon, fetal brain, brain, cerebellum, fetal liver, liver, heart, kidney, lung, placenta, salivary gland, prostate, skeletal muscle, spleen, testis, thymus, thyroid gland, trachea, uterus, ovary, mammary gland and retina.

Prostate cancer biopsies

Prostate cancer biopsy tissues were collected and processed by Mr. Michael Bishop (Nottingham Urology Centre, Nottingham City Hospital, UK) as previously described (Walton et al. 2005), under the approved National Research Ethics.

RNA isolation and real-time reverse transcription (RT) PCR

Total RNA was isolated using RNA-STAT 60 reagents (AMS Biotechnology) according to the manufacturer's instructions. Residual traces of genomic DNA were eliminated by RNase free DNase (Qiagen) as per manufacturer's instructions. The quantity and quality of RNA was assessed spectrophotometrically by NanoDrop 8000 (Thermo Scientific) (typical range 500 ng/ul–3 µg/ul RNA). Total RNA (2 µg) was reverse transcribed with random primers (Promega) using M-MLV reverse transcriptase (Promega), at 37°C for 60 min according to manufacturer's instructions. The cDNA was amplified by real-time PCR employing iQSYBR green supermix (Bio-rad), with oligonucleotide-primers specific for the TG2 variants and house-keeping genes (Table 2). Real-time PCR reactions were performed in a Corbett Rotor-Gene™ 6000 rotary analyzer (initial denaturation: 95°C, 5 min; followed by 40 cycles: 95°C, 30 s; 58–63°C, 30 s; 72°C, 30 s). For all transcripts studied, a ramp temperature from 72 to 95°C was used to generate the melting curves which were used to check the homogeneity of the amplified transcripts. Further validation was carried out by end-point analysis of the amplified transcripts by agarose (1.5% w/v) gel electrophoresis and direct DNA sequencing (Eurofins MWG operon). Threshold cycle (Ct) values were the mean values of three separate PCRs, each run in duplicate. A no-template control was included to rule out contamination.

Quantifications and normalization to house-keeping genes

Quantifications were performed using standard curves of transcript-specific RT-PCR products, purified by QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions. The cDNA standards were quantified spectrophotometrically using NanoDrop 8000 and the copy number calculated from the concentration using the Avogadro number (6.02×10^{23} mol^{-1}). Serial dilutions of the transcript-specific cDNA standards with known amounts of input copy (10^2–10^8 molecules) were amplified by real-time PCR in duplicate and the corresponding Ct values plotted against the log copy number to generate standard curves. The reaction efficiency ($E = [10^{(-1/M)}]-1$), slope (M) and r squared (r^2) were determined by the Corbett Rotor-Gene™ 6000 series software, giving $R^2 > 0.94$; slope > -3.28 to < -2.94; efficiency approximately 1.0 (optimal values for reaction efficiencies, slope and r squared are equal to 1, -3.322 and 1, respectively). To normalize for RNA input and possible inefficiencies in cDNA synthesis, copy numbers obtained for the target genes were divided by the mean copy number of the corresponding internal control genes and multiplied by 100. Relative quantifications were conducted by the $2^{-\Delta\Delta Ct}$ relative quantification method (Livak and Schmittgen 2001). Optimal amplification efficiency for each primer set was verified by amplifying serial dilutions of cDNA (from OPCT1 prostate cancer line and from human placenta), and calculating the ΔCT ($Ct_{\text{avg reference genes}} - Ct_{\text{target gene}}$). The log cDNA dilutions versus corresponding ΔCT were plotted to evaluate the efficiencies of the target and internal genes, which need to be similar (i.e. slope close to zero) to satisfy the assumption of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The absolute values of the slope were 0.0579 (TGM2_v1), 0.0481 (TGM2_v2), 0.0806 (TGM2_v3), 0.133 (TGM2_v4a), and 0.0068 (TGM2_v4b).

Results and discussion

Classification of main TG2 variants and their detection by reverse transcription-PCR

In the attempt to rationalize the nomenclature of all the main isoforms of the TG2 gene (TGM2), we have designated the different TG2 variants by identical names

(TGM2_v), followed by a number reflecting the chronological order of their identification, according to the suggestion of the HUGO Gene Nomenclature Committee (HGNC) (Table 1). According to this nomenclature, we have named the classic long form as TGM2_v1 (Gentile et al. 1991), the short-form TGM2_v2 (Fraij et al. 1992), the third TG2 isoform TGM2_v3 (Fraij and Gonzales 1996) and the latest to be reported related isoforms TGM2_v4a and TGM2_v4b (Lai et al. 2007). Isoform-specific oligonucleotide primer pairs suitable for real-time PCR were designed to selectively amplify "diagnostic" portions of the TGM2 transcripts (Table 2). The forward primers typically anneal to 5′ regions shared by all the TG2 isoforms and the reverse primer is specific for the alternative terminal exons (exon 10b for TGM2_v2, exon 6b for TGM2_v3 exon 13b and 13c for TGM2_v4a and TGM2_v4b, respectively) (Fig. 1). Specificity of the PCR amplification was documented by validating each primer set through end-point analysis using agarose gel electrophoresis and melting curve test (Supplementary Fig. 1) and by DNA sequencing (not shown).

Table 1 Classification of main TG2 variants and new nomenclature

Isoform name	Alias	Accession number[b]	References (human form)
TGM2_v1	Isoform 1[a]	NM_004613.2	Gentile et al. 1991
TGM2_v2	TGH Tgase S TG2-S Isoform 2[a]	NM_198951.1	Fraij et al. 1992; Tee et al. 2010
TGM2_v3	TGH2 Isoform 3[a]	S81734.1	Fraij and Gonzales 1996
TGM2_v4a	tTGv1	None available	Lai et al. 2007
TGM2_v4b	tTGv2	None available	Lai et al. 2007

The different human TG2 variants designated by identical names (TGM2_v), followed by a number reflecting the chronological order of their identification (according to HUGO Gene Nomenclature Committee). Transcript aliases and accession numbers for cDNA sequences are listed where available

[a] Universal Protein Resource (Uniprot)

[b] National Center for Biotechnology Information (NCBI)

Expression analysis of the individual TG2 variants in human tissues and cancer cell lines by real-time PCR

The expression of the individual TG2 isoforms in a wide range of human tissues was evaluated using real-time reverse transcription PCR. Quantifications were performed using calibration curves of cDNA standards. Hypoxanthine ribosyltransferase (HPRT1) and TATA-box-binding protein (TBP) were the internal controls used as normalisers. HPRT1 was chosen as a previous study demonstrated that this house-keeping gene could replace the measurement of multiple frequently used house-keeping genes in a variety of normal and tumor tissues (de Kok et al. 2005). TBP was

Table 2 Oligonucleotides specific for human TG2 spliceforms

TG2 isoform name	Primer sequence (5′ → 3′)	Product size (bp)	Primer position
TGM2_v1	F CCTTACGGAGTCCAACCTCA	245	F-exon 11
	R CCGTCTTCTGCTCCTCAGTC		R-exon 12
TGM2_v2	F ACCGCTGAGGAGTACGTCTG	153	F-exon 10
	R TCAACAAATGCTCCAGGAA		R-exon 10b
TGM2_v3	F GGTGAGTGGCATGGTCAACT	226	F-exon 5
	R AGGGCTCATGACCCACATC		R-exon 6b
TGM2_v4a	F CCTTACGGAGTCCAACCTCA	214	F-exon 11
	R CTGGGATGTGGAGGTGCA		R-exon 13b
TGM2_v4b	F CCTTACGGAGTCCAACCTCA	214	F-exon 11
	R CACTGGTGTGGAGGTGCAGC		R-exon 13c
House-keeping genes			
HPRT1	F TGACACTGGCAAAACAATGCA	94	F-exon 6
	R GGTCCTTTTCACCAGCAAGCT		R-exon 7
TBP	F TGCACAGGAGCCAAGAGTGAA	132	F-exon 5/6
	R CACATCACAGCTCCCCACCA		R-exon 6

The primers are listed in the 5′ → 3′ direction. Each transcript variant was amplified using a sense primer annealing to a constitutive exon and a sequence-specific antisense primer. TGM2_v1, TGM2v_4a and TGM2v_4b share a common forward (F) primer annealing to exon 11 (Fig. 1); spliceoforms TGM2_v2 and TGM2_v3 have a forward primer annealing to exon 10 and exon 6, respectively. Transcript-specific reverse (R) primers are complementary to exon 10b (TGM2_v2), exon 6b (TGM2_v3), and exon 13b/c (TGM2_v4a/b). The amplicon size (bp) has been calculated. House-keeping genes were hypoxanthine ribosyltransferase (HPRT1) and TATA-box-binding protein (TBP)

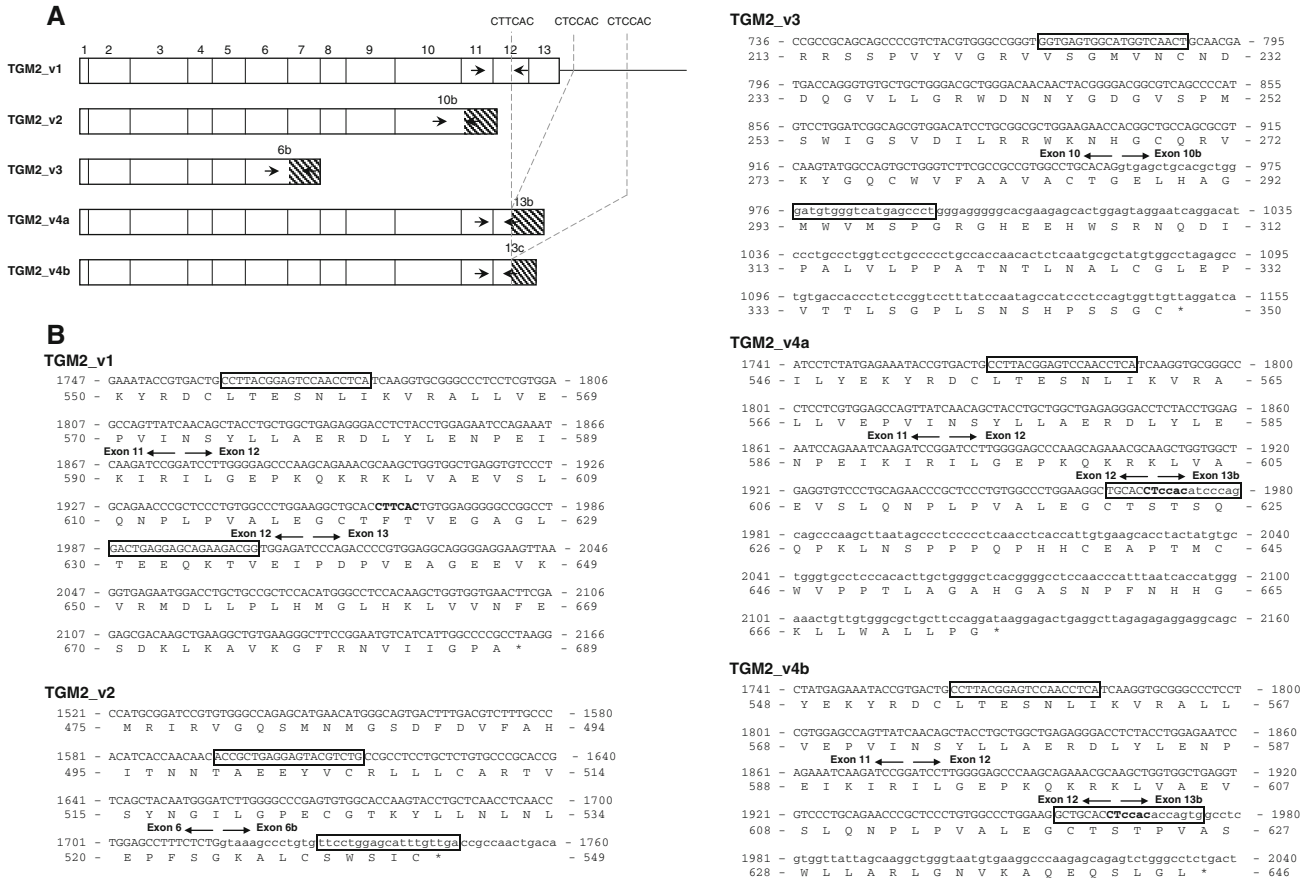
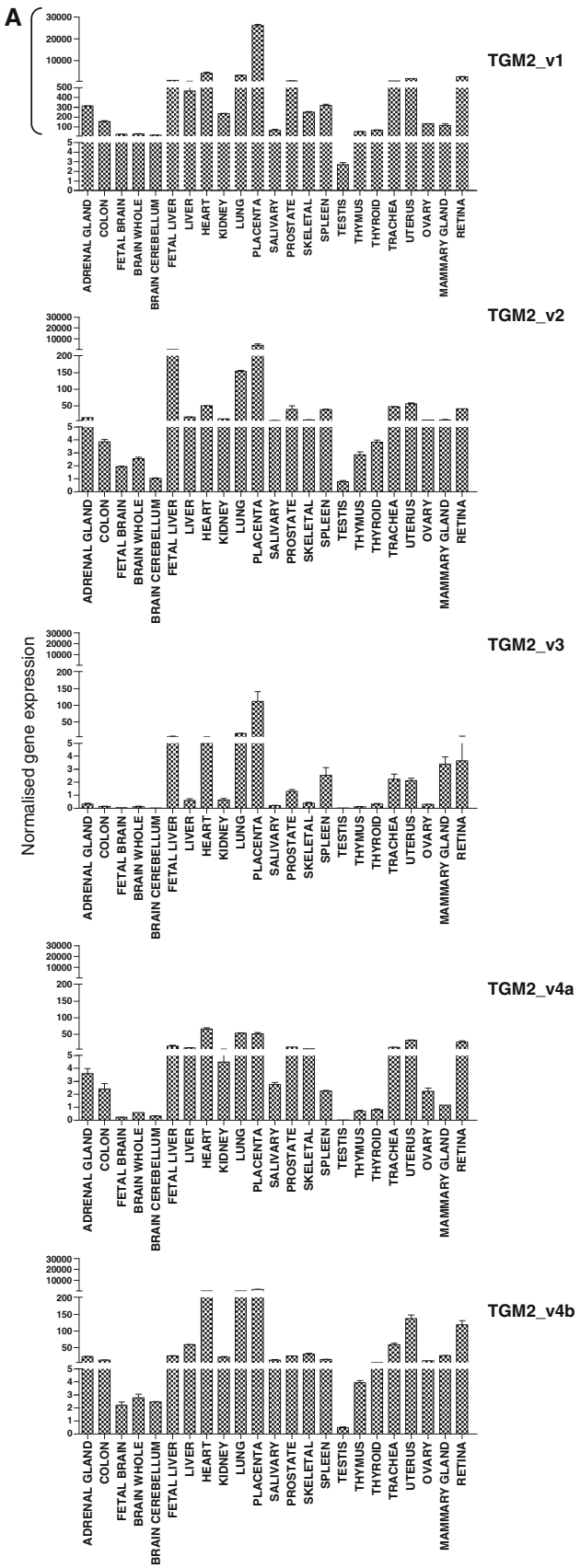


Fig. 1 Schematic representation of the spliceforms of the TG2 gene. **a** Exons are *numbered*, primer positions depicted by *arrows* and novel exons are *shaded*. TGM2_v1 is the full-length TG2 form, consisting of 13 exons and 12 intervening sequences (IVS). TGM2_v2 results from the read-through of a donor splice site into the consecutive IVS10, generating a novel terminal exon (exon 10b). TGM2_v3 results from the reading-through of a donor splice site into the consecutive IVS6, generating a novel terminal exon (exon 6b). TGM2_v4a and TGM2_v4b are generated via an atypical splicing event at a CTT/CCAC motif positioned in exon 12, with the formation of a novel terminal exon in the 3′-UTR (exon 13b and exon 13c) and an altered reading frame. **b** The *boxed* nucleotides represent the oligonucleotide primer annealing sites used for specific amplification of the spliceoforms by quantitative RT-PCR. Novel exonic regions are in *lowercase letters* and the exon/exon boundaries shown by *arrows*. The CTT/CCAC motif is in *bold font* and the termination codon shown as an *asterisk*

found to be the most stable reference gene from a set of candidates house-keeping genes in a panel of tissues and cell lines similar to those used in this study (analysis by geNorm http://www.multid.se/genex/hs400.htm) (data not shown).

The TG2 variants were all found to be expressed in the analysed human tissues although at variable levels (Fig. 2a). TGM2_v1 was the highest expressed TG2 isoform (mean expression value ± SD was 1,858 ± 1,129) followed by TGM2_v2 (209 ± 171), TGM2_v4b (127 ± 75.1), TGM2_v4a (13.2 ± 2.6) and TGM2_v3 (6.7 ± 4.0). Placenta, lung and heart displayed the highest levels of expression for most of the TG2 isoforms. Liver, prostate, trachea, uterus and retina also exhibited considerable expression levels of TG2 variants. All brain tissues and testis had generally the lowest expression of TG2 variants and testis was the only tissue of the panel where the level of one TG2 variant (TGM2_v4a) was undetectable (Fig. 2a). Notably, TGM2_v1, TGM2_v2 and TGM2_v3 were found to be much higher (by ∼twofold, ninefold and twenty fold, respectively) in fetal liver compared to adult liver. The relative proportion of full-length TG2 transcript TGM2_v1 to the other isoforms was constant, independent of the level of transcription, as shown by linear correlation (Fig. 2b). On average, TGM2_v1 transcript contributed to 84% (±6%, SD) of the total expression of the TG2 gene (TGM2) in normal tissues (Fig. 2c).

Real-time PCR was also utilized to quantitate the expression of the individual TG2 isoforms in a wide range of well-established cancer cell lines, normalizing gene expression by the reference genes HPRT1 and TBP. Details of the site of origin and important characteristics of the tumor cell lines used in this study are indicated in Table 3. Other factors such as duplication time, laboratory



◀**Fig. 2** Quantification of TG2 variants expression in a panel of 23 human tissue RNAs by real-time RT-PCR. **a** TG2 RNA transcripts were quantified by real-time RT-PCR, using isoform-specific oligonucleotide-primers (Table 2) and standard curves of cDNA standards as described in the "Methods". For each gene analysed, the number of transcript molecules was determined by comparing the Ct of the sample with the corresponding standard curve. The values were then normalised to the mean expression of reference genes HPRT1 and TBP. **b** Correlation between the expression of TGM2_v1 and the alternative splicing variants was analysed with GraphPad Prism software. Values relative to all the tissue considered in the study are plotted. R^2 indicates the fitting with the regression line. r Pearson's coefficient of linear correlation; $p < 0.05$ indicates correlation. **c** Percentage expression of TGM2_v1 compared to the expression of all the TGM2 isoforms in each normal tissue. The average expression of TGM2_v1 is shown by the *dashed line*

adaptation and possible hypoxic niches in the cell monolayers were not monitored, although they could influence the pattern of TG2 gene expression. Most cancer lines expressed the full-length transcript TGM2_v1, which was particularly high in prostate cancer cell lines DU145 and OPCT1, the breast cancer cell line MDA231 and melanoma cell lines (Fig. 3a). Head and neck cancer cells exhibited a lower, in some cases undetectable, level of TGM2_v1 (Fig. 3a). Published reports suggest that the expression of TG2 is variable amongst cancer cell lines in culture. Our findings for the canonical transcript TGM2_v1 are in general agreement with previous reports showing variable expression levels of TG2 activity or immune labeling in prostate cancer cells PC3, DU145 and LNCaP, the latter displaying no enzyme activity or immune-reactivity (Friedrichs et al. 1995). Concerning TG2 expression in breast cancer cell lines, previous work based on western blotting and enzymatic assays showed high levels of full-length TG2 in the metastatic cell line MDA-231 and low or undetectable levels in MCF-7, MDA-468 and T4D cells (Ai et al. 2008; Mehta et al. 2004). These findings are consistent with our measurements of TGM2_v1 transcript in these cell lines, except for the higher level than expected in MCF7 cells compared to previous work, which reported transcription silencing of TG2 expression by hypermethylaton in this cell line (Ai et al. 2008; Mehta et al. 2004; Herman et al. 2006). As TGM2_v1 is the predominant isoform in MCF-7 cells, we do not know the reason for this discrepancy, although culturing cells in D-glucose rich (25 mM)-DMEM compared to the low D-glucose (5.5 mM)-Eagles' MEM, which is routinely used for MCF-7 cell culture (Ai et al. 2008), may be responsible for the induction of TG2 transcription, as reported for other cell types (Skill et al. 2004). Induction of TG2 gene by doxorubicin was also reported in MCF-7, suggesting variability depending on culture conditions (Ai et al. 2008; Mehta et al. 2004; Herman et al. 2006).

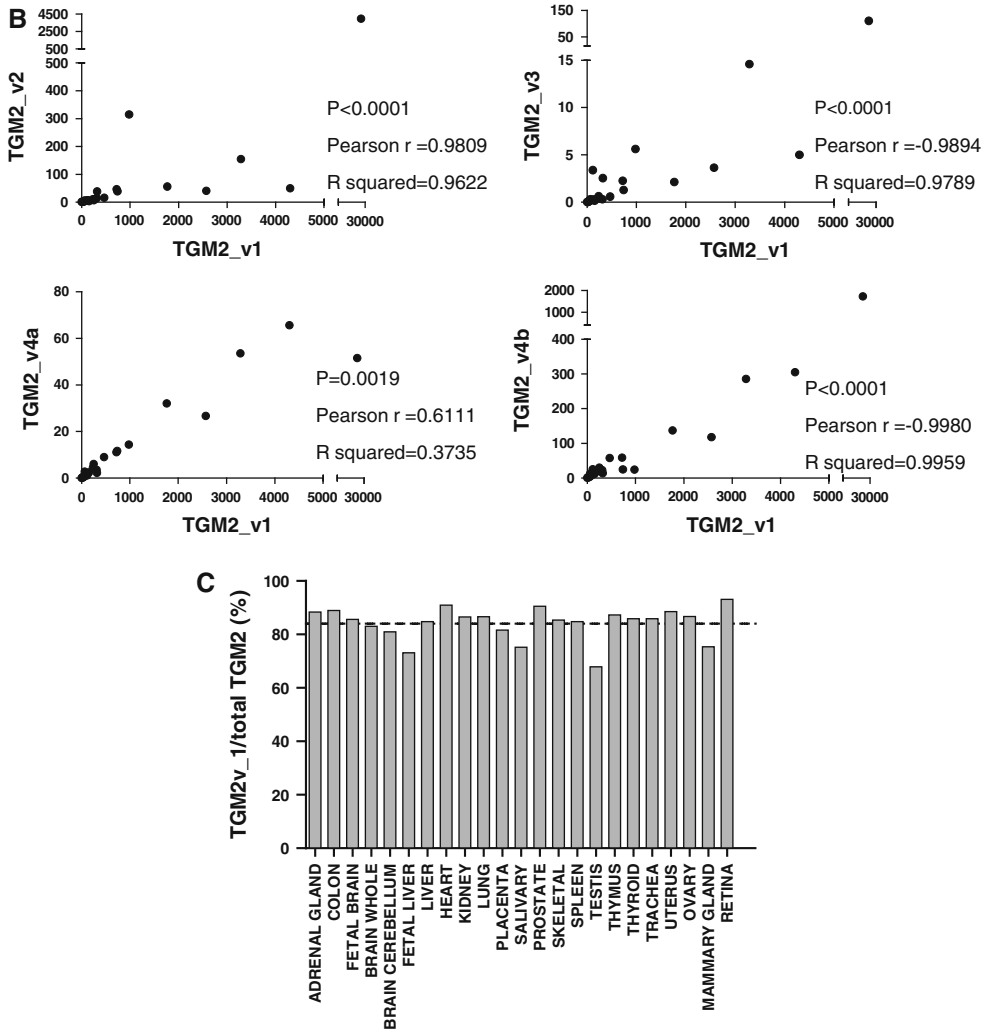


Fig. 2 continued

Expression of the alternative transcript TGM2_v2 followed a similar trend to that of full-length transcript TGM2_v1 although the proportion of TGM2_v2 expression was always lower than TGM2_v1 (Fig. 3a, b). Instead, expression of TGM2_v3 did not correlate with TGM2_v1, as low levels of TGM2_v3 could exclusively be detected in two prostate cell lines, PC33 and OPCT1 (Fig. 3a, b). Expression of TGM2_v4a and TGM2_v4b was more widespread throughout the cell lines, but with some differential expression depending on the site of origin. Prostate cancer lines preferentially expressed TGM2_v4a and not TGM2_v4b; on the contrary breast cancer cell lines MDA468 and MCF7 preferentially expressed TGM2_v4b and not TGM2_v4a. The full-length TG2 transcript TGM2_v1 correlated with TGM2_v4a but not with TGM2_v4b (Fig. 3b). A significantly higher level of TGM2_v4b than constitutively spliced TGM2_v1 was measured in melanoma cell lines FM3, MM30 and MM45a [$p < 0.02$ (paired T test)], and a similar level of TGM2_v1 and TGM2_v4b in MM11. These findings suggest dysregulation of alternative splicing of TGM2 in cancer cell lines compared to normal tissue. Notably, the breast cancer cell line MDA468 did only express TGM2_v4a among all the TG2 isoforms examined. In cancer cell lines a significant correlation was found between TGM2_v1 and TGM2_v2 (Fig. 3b), while no correlation could be drawn between TGM2_v1 and either TGM2_v3, which was detectable in two cancer cell lines, or TGM2_v4a-b. On average, TGM2_v1 transcript contributed to 57% (± 33%, SD) of the total expression of TGM2 in the cancer cell lines, confirming variability of TGM2 splicing amongst the cell lines (Fig. 3c).

Expression analysis of the individual TG2 variants in samples of human prostate cancer

Since our findings suggested a high expression of TG2 variants in prostate cell lines and tissue, we next analyzed a

Table 3 Site of origin and characteristics for the cancer cell lines used in this study

Name	Source of tissue	Pathology	Androgen receptor	PSA
Prostate cancer cell lines				
LNCAP	Derived from left supraclavicular lymph node	Prostatic adenocarcinoma	+	+
DU145	Derived from brain	Prostatic adenocarcinoma	+	–
PC3	Lumbar vertebral metastasis	Prostatic adenocarcinoma	+	–
OPCT1	Prostatic epithelial cells from prostatectomy tissue	Prostatic adenocarcinoma	+	+ (weakly)
OPCT2	Prostatic epithelial cells from prostatectomy tissue	Prostatic adenocarcinoma	+	–

Name	Source of tissue	Pathology	TNM stage
Head and neck cancer cell lines			
PCI-13	Oral cavity	Squamous cell carcinoma	T4N1M0
PCI-30	Oral cavity	Squamous cell carcinoma	T3N1M0
A253	Submaxillary salivary gland	Epidermoid carcinoma	–
Cal27	Tongue	Squamous cell carcinoma	–
JHU011	Larynx	Squamous cell carcinoma	T3N0
JHU012	Oral cavity	Squamous cell carcinoma	T1N2b

Name	Source of tissue	Classification	Details of metastasis	Stage of diagnosis
Melanoma cell lines				
Ma-Mel-11	Skin	Not classified (Unknown)	Abdomen-right	IV
Ma-Mel-12	Skin	Not classified (Unknown)	Lower leg-right	IV
Ma-Mel-19	Squamous cell-skin	Superficial spreading melanoma	Thigh-right	I
Ma-Mel-26a	Lymph node	Not classified	Abdomen-left	IIB
Ma-Mel-27	Acral	Acral lentiginous melanoma	Palmar/Plantar-right	III
Ma-Mel-28	Occult	Not classified (unknown)	Unknown	IV
Ma-Mel-30	Squamous cell-skin	Acral lentiginous melanoma	Finger/toe-left	II
Ma-Mel-45a	Occult	Not classified (unknown)	Unknown	IV
Ma-Mel-66a	Squamous cell-skin	Not classified (unknown)	Unknown	Unknown
FM3	Metastatic lymph node	Unknown	Metastatic-lymph nodes	Unknown

Name	Source of tissue	Pathology	Estrogen/progesterone receptor	Luminal type/basal	Her2	TP53 gene mutations
Breast cancer cell lines						
MDA231	Pleural effusion	Adenocarcinoma	−/−	Basal B	+	R280 K
MDA468	Pleural effusion	Adenocarcinoma	−/−	Basal A	−	R273H
T47D	Pleural effusion	Invasive ductal carcinoma	+/+	Luminal A	−	L194F
MCF7	Pleural effusion	Invasive ductal carcinoma	+/+	Luminal A	+	Wild type
BR293	Not characterised					

Prostate cancer cells lines LNCAP, DU145 and PC3 were sourced from American Type Culture Collection (ATCC), OPCT1 and OPCT2 from the provider Onyvax; PSA (prostate specific antigen) levels from Sobel and Sadar 2005; head and neck cancer cell lines from Lin et al. 2006, Melanoma cell lines from Prof. Dirk Schadendorf (DKFZ, Heidelberg), breast cancer cell lines from Neve et al. 2006, TNM (Classification of Malignant Tumors)

small group of prostate clinical samples. Patients ($n = 4$) were all aged 71–78 and presented variable prostate specific antigen (PSA) level (1.2–709 ng/ml) [Gleanson score, 10 (5 + 5)]. TGM2 expression in prostate cancers was compared to gene expression in normal prostate by comparative real-time PCR using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and HPRT1 and TBP as internal controls. The validity of the calculation was confirmed by examining the efficiency of amplification of each target gene and the internal controls, which was similar as described in the "Methods". Starting from the ΔCt values ($Ct_{Internal\ controls} - Ct_{Target}$), the fold change in gene expression of the individual TG2 isoforms in prostate cancer relative to the corresponding prostate tissue gene

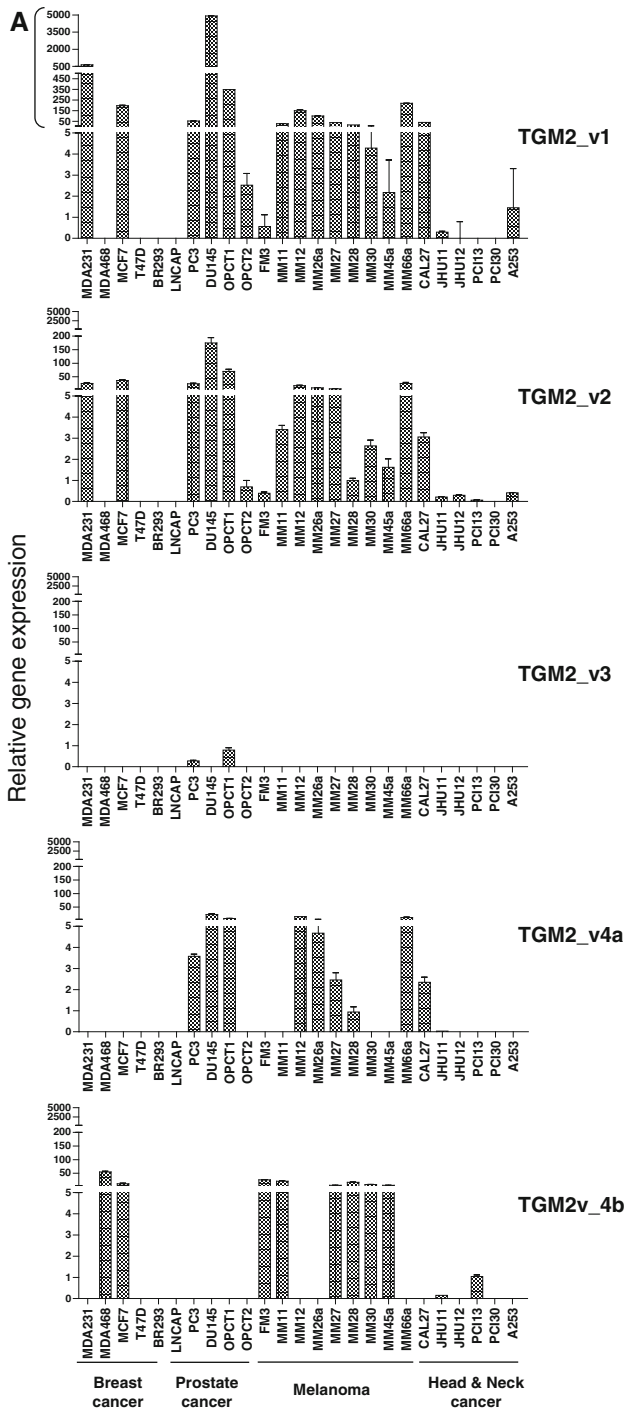


Fig. 3 Quantification of TG2 variants in cancer cell lines by real-time RT-PCR. **a** Quantifications of gene expression and normalization were performed as described in legend to Fig. 2a. **b** Correlation between the expression of TGM2_v1 and the alternative splicing variants was analysed as described in legend to Fig. 2b. Values relative to all the cancer cell lines considered in the study are plotted. R^2 indicates the fitting with the regression line. r, Pearson's coefficient of linear correlation; $p < 0.05$ indicates correlation. **c** Percentage expression of TGM2_v1 compared to the expression of all the TGM2 isoforms in each individual cancer cell line. The average expression of TGM2_v1 is shown by the *dashed line*

value is reported in Fig. 4. TGM2_v1, TGM2_v2, TGM2_v3 and TGM2_v4b were all found to be expressed in prostate cancer and normal tissue whilst TGM2_v4a was not found in any specimen. The average expression of TGM2_v2 and TGM2_v3 tended to be higher than TGM2_v1 and TGM2_v4b in prostate cancer tissues compared to normal tissue (Fig. 4). Although a larger number of samples would be required for statistical considerations, these data are supportive of the idea of differential TGM2 splicing in prostate cancer.

Conclusions

Alternative splicing is a major mechanism for generating diversity in the transcriptome and hence the proteome, and underlies the apparent disparity between the estimate of the number of genes in the human genome (24,000) and the 100,000 proteins that are synthesized (Keren et al. 2010). Cancer cells may take advantage of this flexibility to produce isoforms that promote their own growth and survival (David and Manley 2010).

The multiple enzymatic activities and functions of TG2 suggest that alternative TG2 molecules, capable of exerting a diverse array of functions, may be produced by splicing of TG2 gene product. Of the different TG2 transcripts that we have described, TGM2_v4a and TGM2_v4b arise from splice site selection, whereas TGM2_v2 and TGM2_v3 are generated through the use of alternative polyadenylation sites in intron 10 and intron 6, respectively, with the result that the splice donor site at the end of the preceding exon is not used, and intronic sequence is thus included in the transcript. This is a recognised mechanism for generating proteins with differing C-termini (Millevoi and Vagner 2010). All of the alternative splice forms encode truncated TG2 isoforms with different C-terminal amino acid sequences to the full-length TG2 form (Gentile et al. 1991). The alternative splicing of TG2 appears to provide cells with TG2 protein isoforms with intrinsically higher protein transamidation power, as they escape the normal GTP-regulation (Tee et al. 2010). Here, for the first time all the TG2 variants reported by the literature have been rationalized and their expression systematically searched and quantified in a wide panel of human tissues and cancer cell lines.

We have developed a rapid diagnostic method for the detection of TG2 variants, which is based on real-time RT-PCR and isoform-specific primers. We have shown that all the TG2 variants are expressed in the human tissues analyzed and that the expression of the alternative TG2 transcripts is generally lower but highly correlated to that of full-length TG2 transcript. Testis was found to express a low level of TG2 variants and was the only tissue with

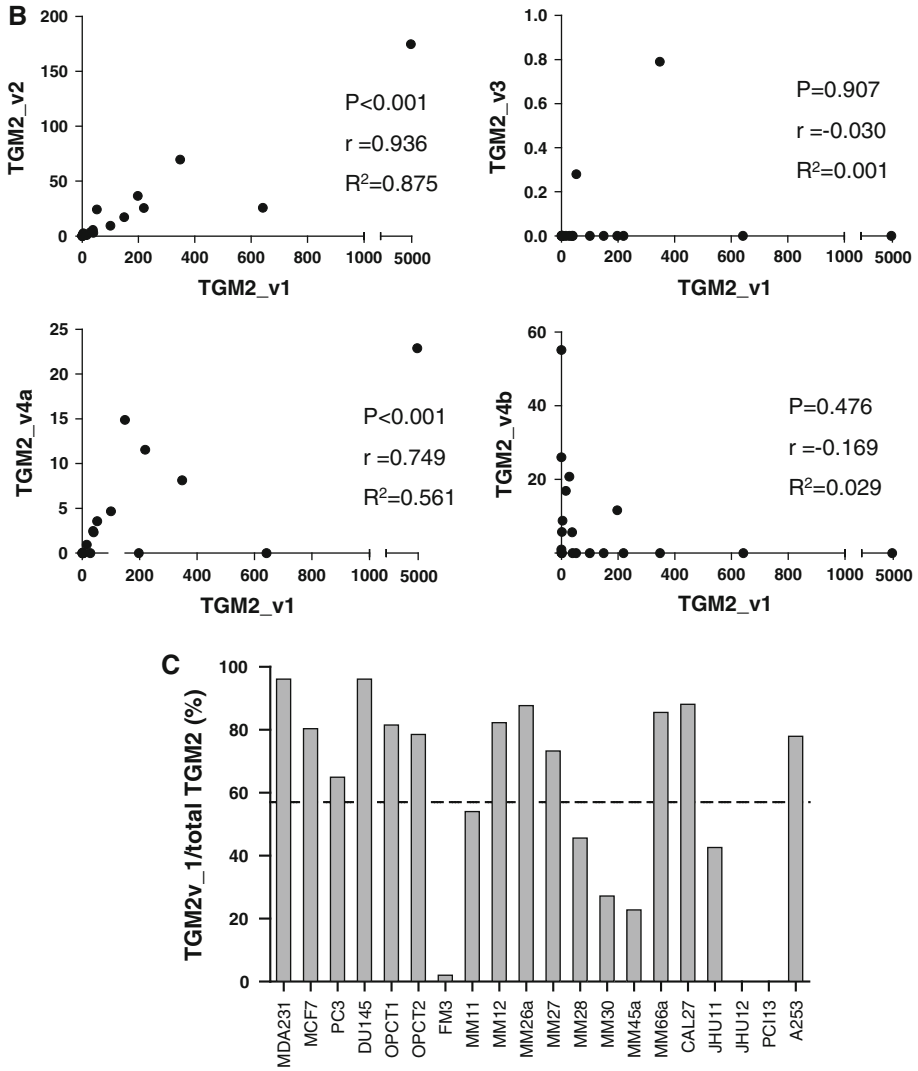


Fig. 3 continued

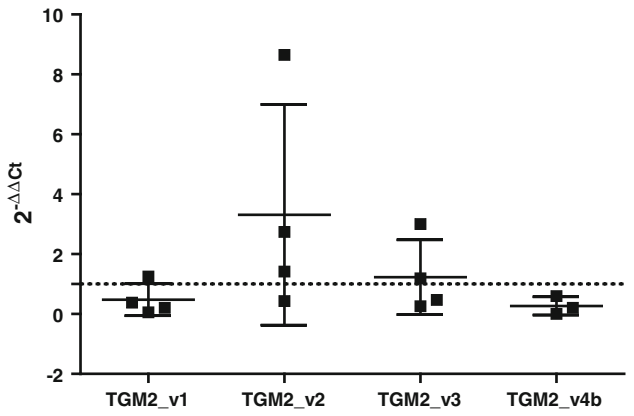


Fig. 4 Relative expression of TG2 variants in prostate cancer. TG2 transcripts were amplified by real-time RT-PCR, using isoform-specific oligonucleotide-primers and quantified by the $2^{-\Delta\Delta Ct}$ relative method (Livak and Schmittgen 2001). Normal prostate ($2^{-\Delta\Delta Ct} = 1$) was the reference tissue (*dashed line*). The values were normalised to the mean expression of reference genes HPRT1 and TBP

undetectable levels of one TG2 isoform. This conservation of the relative levels of all the TG2 isoforms would suggest that the alternative splicing pattern is constitutive in normal cells, probably through some stochastic mechanism. In contrast, in cancer cell lines, there is more variability in the relative levels of the expression of the different isoforms, in that the expression of the full-length TG2 transcript did not correlate well with that of the alternative TG2 variants.

Initial data with melanoma cell lines and human prostate cancer biopsies and cell lines suggest a preferential expression of specific TG2 variants. Thus, it is likely that in cancer cells the alternative splicing of TG2 [e.g. in the case for TGM2_v2 and TGM2_v3, modification of the interplay between splicing and 3′ end processing (Millevoi and Vagner 2010)] is a more active process, consistent with the reported large scale alterations in alternative splicing in cancer cells (David and Manley 2010).

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References

Ai L, Kim WJ, Demircan B, Dyer LM, Bray KJ, Skehan RR, Massoll NA, Brown KD (2008) The transglutaminase 2 gene (TGM2), a potential molecular marker for chemotherapeutic drug sensitivity, is epigenetically silenced in breast cancer. Carcinogenesis 29(3):510–518

Antonyak MA, Jansen JM, Miller AM, Ly TK, Endo M, Cerione RA (2006) Two isoforms of tissue transglutaminase mediate opposing cellular fates. Proc Natl Acad Sci USA 103(49):18609–18614

Antonyak MA, Li B, Regan AD, Feng Q, Dusaban SS, Cerione RA (2009) Tissue transglutaminase is an essential participant in the epidermal growth factor-stimulated signaling pathway leading to cancer cell migration and invasion. J Biol Chem 284(27): 17914–17925

Begg GE, Carrington L, Stokes PH, Matthews JM, Wouters MA, Husain A, Lorand L, Iismaa SE, Graham RM (2006) Mechanism of allosteric regulation of transglutaminase 2 by GTP. Proc Natl Acad Sci USA 103(52):19683–19688

Citron BA, SantaCruz KS, Davies PJ, Festoff BW (2001) Intron-exon swapping of transglutaminase mRNA and neuronal Tau aggregation in Alzheimer's disease. J Biol Chem 276(5):3295–3301

David CJ, Manley JL (2010) Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. Genes Dev 24(21):2343–2364

de Kok JB, Roelofs RW, Giesendorf BA, Pennings JL, Waas ET, Feuth T, Swinkels DW, Span PN (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. Lab Investig; J Tech Methods Pathol 85(1):154–159

Fraij BM, Gonzales RA (1996) A third human tissue transglutaminase homologue as a result of alternative gene transcripts. Biochim Biophys Acta 1306(1):63–74

Fraij BM, Birckbichler PJ, Patterson MK Jr, Lee KN, Gonzales RA (1992) A retinoic acid-inducible mRNA from human erythroleukemia cells encodes a novel tissue transglutaminase homologue. J Biol Chem 267(31):22616–22623

Friedrichs B, Riedmiller H, Goebel HW, Rausch U, Aumuller G (1995) Immunological characterization and activity of transglutaminases in human normal and malignant prostate and in prostate cancer cell lines. Urol Res 23(5):301–310

Gentile V, Saydak M, Chiocca EA, Akande O, Birckbichler PJ, Lee KN, Stein JP, Davies PJ (1991) Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases. J Biol Chem 266(1):478–483

Herman JF, Mangala LS, Mehta K (2006) Implications of increased tissue transglutaminase (TG2) expression in drug-resistant breast cancer (MCF-7) cells. Oncogene 25(21):3049–3058

Hwang JY, Mangala LS, Fok JY, Lin YG, Merritt WM, Spannuth WA, Nick AM, Fiterman DJ, Vivas-Mejia PE, Deavers MT, Coleman RL, Lopez-Berestein G, Mehta K, Sood AK (2008) Clinical and biological significance of tissue transglutaminase in ovarian carcinoma. Cancer Res 68(14):5849–5858

Jang GY, Jeon JH, Cho SY, Shin DM, Kim CW, Jeong EM, Bae HC, Kim TW, Lee SH, Choi Y, Lee DS, Park SC, Kim IG (2010) Transglutaminase 2 suppresses apoptosis by modulating caspase 3 and NF-kappaB activity in hypoxic tumor cells. Oncogene 29(3):356–367

Keren H, Lev-Maor G, Ast G (2010) Alternative splicing and evolution: diversification, exon definition and function. Nat Rev Genet 11(5):345–355

Kumar A, Xu J, Brady S, Gao H, Yu D, Reuben J, Mehta K (2010) Tissue transglutaminase promotes drug resistance and invasion by inducing mesenchymal transition in mammary epithelial cells. PLoS One 5(10):e13390

Lai TS, Liu Y, Li W, Greenberg CS (2007) Identification of two GTP-independent alternatively spliced forms of tissue transglutaminase in human leukocytes, vascular smooth muscle, and endothelial cells. Faseb J 21(14):4131–4143

Lin CJ, JR Grandis, TE Craey, SM Gollin, TL Whiteside, WM Koch, RL Ferris, SY Lai (2006) Head and neck squamous cell carcinoma cell lines: established models and rationale for selection. Wiley InterScience. http://www.intersceince wiley.com

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. Methods 25(4):402–408

Mann AP, Verma A, Sethi G, Manavathi B, Wang H, Fok JY, Kunnumakkara AB, Kumar R, Aggarwal BB, Mehta K (2006) Overexpression of tissue transglutaminase leads to constitutive activation of nuclear factor-kappaB in cancer cells: delineation of a novel pathway. Cancer Res 66(17):8788–8795

Mehta K, Fok J, Miller FR, Koul D, Sahin AA (2004) Prognostic significance of tissue transglutaminase in drug resistant and metastatic breast cancer. Clin Cancer Res 10(23):8068–8076

Millevoi S, Vagner S (2010) Molecular mechanisms of eukaryotic pre-mRNA 3′ end processing regulation. Nucleic Acids Res 38(9):2757–2774

Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, Gray JW (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10(6):515–527

Satpathy M, Cao L, Pincheira R, Emerson R, Bigsby R, Nakshatri H, Matei D (2007) Enhanced peritoneal ovarian tumor dissemination by tissue transglutaminase. Cancer Res 67(15):7194–7202

Satpathy M, Shao M, Emerson R, Donner DB, Matei D (2009) Tissue transglutaminase regulates matrix metalloproteinase-2 in ovarian cancer by modulating cAMP-response element-binding protein activity. J Biol Chem 284(23):15390–15399

Shao M, Cao L, Shen C, Satpathy M, Chelladurai B, Bigsby RM, Nakshatri H, Matei D (2009) Epithelial-to-mesenchymal transition and ovarian tumor progression induced by tissue transglutaminase. Cancer Res 69(24):9192–9201

Skill NJ, Johnson TS, Coutts IG, Saint RE, Fisher M, Huang L, El Nahas AM, Collighan RJ, Griffin M (2004) Inhibition of transglutaminase activity reduces extracellular matrix accumulation induced by high glucose levels in proximal tubular epithelial cells. J Biol Chem 279(46):47754–47762

Sobel RE, Sadar MD (2005) Cell lines used in prostate cancer research: a compendium of old and new lines–part 1. J Urol 173(2):342–359

Srebrow A, Kornblihtt AR (2006) The connection between splicing and cancer. J Cell Sci 119(Pt 13):2635–2641

Tee AE, Marshall GM, Liu PY, Xu N, Haber M, Norris MD, Iismaa SE, Liu T (2010) Opposing effects of two tissue transglutaminase protein isoforms in neuroblastoma cell differentiation. J Biol Chem 285(6):3561–3567

Verderio EA, Telci D, Okoye A, Melino G, Griffin M (2003) A novel RGD-independent cel adhesion pathway mediated by

fibronectin-bound tissue transglutaminase rescues cells from anoikis. J Biol Chem 278(43):42604–42614

Verma A, Mehta K (2007) Tissue transglutaminase-mediated chemoresistance in cancer cells. Drug Resist Updat 10(4–5): 144–151

Walton TJ, McCulloch TA, Rees RC, Bishop MC (2005) Obtaining fresh prostate cancer tissue for research: a novel biopsy needle and sampling technique for radical prostatectomy specimens. Prostate 64(4):382–386