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Does thyroid peroxidase provide an antigenic link between thyroid autoimmunity and breast cancer?

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Women with breast cancer (BC) and antithyroid peroxidase (TPO) autoantibodies (TPOAb) have a better prognosis than women lacking TPOAb. Sera from women with TPOAb displayed immunoreactivity to BC tissue by immunofluorescence that was not apparent in women without TPOAb. We hypothesize a BC/thyroid shared antigen that provides a target for humoral or cell-mediated immune activity; candidates include the sodium/iodide symporter (expressed in thyroid and BC), cross-reacting epitopes in TPO and lactoperoxidase (LPO) or TPO itself. As the association is with TPOAb, we investigated TPO expression in BC, breast peritumoral tissue (PT), other tissues (tumoral and not) and thyroid as positive control. Transcripts for known and novel TPO isoforms were detected in BC ($n = 8$) and PT ($n = 8$) but at approximately 10^4-fold lower than in thyroid while in non-BC tumors ($n = 5$) they were at the limit of detection. TPO was expressed also in adipose tissue ($n = 17$), 10^3-fold lower than in thyroid. Full length TPO (Mr 105–110 kDa) was detected in Western blots in the majority of examined tissues; preabsorption of the TPO antibody with recombinant TPO (but not LPO) reduced the signal, indicating specificity. The same occurred with some lower molecular weight bands, which could correspond to smaller TPO transcript isoforms, present in all samples. In conclusion, TPO is weakly expressed in BC and other tissues; this could partly explain the high frequency and protective role of TPOAb in BC patients. Further studies will investigate tissue specificity, function and immunogenicity of the novel TPO variants (some BC-specific) identified.

The association between benign thyroid diseases and breast cancer (BC) has long been known,[1,2] even if the topic has been a subject of debate for many years, with not all authors agreeing.[3–5] However, the majority of the studies support this association, and in particular they provide evidence for the relationship between BC and autoimmune thyroid disorders (ATD).[6–8] A high prevalence of antithyroid peroxidase (TPO) autoantibodies (TPOAb) has been found in both treated[9,10] and untreated[11] BC patients and the positive predictive value of serum TPOAb in BC patients with aggressive disease has been reported.[12,13] Only Jiskra *et al.*,[14] despite confirming a higher prevalence of TPOAb in BC patients, found no impact on relapse-free and overall survival; this discrepancy could be due to the relatively small and highly heterogeneous BC patients group. Recently Farahati *et al.* have found a significantly lower frequency of distant metastases in a large cohort of BC patients with serum TPOAb positivity.[15]

BC and follicular thyroid cells share similar functional properties such as the expression of the sodium/iodide symporter (NIS) and a peroxidase activity: TPO in thyroid cells and lactoperoxidase (LPO) in breast cells. Therefore, we hypothesize the presence of a shared antigen between BC

Key words: breast cancer, thyroid autoimmunity, thyroid peroxidase, antigen, alternative splicing

Abbreviations: AD: adipose tissue; ATD: autoimmune thyroid disorders; BC: breast cancer; BSA: bovine serum albumin; IF: immunofluorescence; IHC: immunohistochemistry; KC: kidney cancer; LPO: lactoperoxidase; MKC: hepatic metastasis of kidney carcinoma; MPO: myeloperoxidase; MW: molecular weight; NIS: sodium/iodide symporter; PC: pancreatic adenocarcinoma; PCR: polymerase chain reaction; PEG: polyethylene glycol; PKC: primary kidney carcinoma; PT: peritumoral breast tissue; QPCR: absolute real-time quantitative PCR; RT: reverse transcription; TgAb: autoantibodies to thyroglobulin; TPO: thyroid peroxidase; TPOAb: autoantibodies to TPO; TT: thyroid tissue; WB: Western blot

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What's new?
Women with breast cancer who have antibodies against the enzyme thyroid peroxidase (TPO) have a better prognosis than those without the antibodies. This study showed that TPO mRNAs and proteins can be found in breast cancer tissue. They found several novel isoforms of TPO in the breast cancer samples, suggesting that these tissue-specific isoforms could be useful in diagnosis.

and thyroid cells able to trigger a common immunoreactivity; this could explain firstly the association between BC and ATD and secondly the influence of ATD on BC prognosis. The shared antigen could be NIS, cross-reactive epitopes in TPO and LPO or, since serum TPOAb are frequent and abundant in BC patients, TPO itself.

Human TPO and LPO are heme-containing enzyme members of the family of mammalian peroxidases, which use hydrogen peroxide to oxidize a number of inorganic and organic substrates and they are active in a variety of anatomic sites[16]; other members of this group include myeloperoxidase (MPO) and eosinophil peroxidase. Human TPO is the only characterized component of the "microsomal" antigen involved in ATD and the measurement of serum TPOAb is generally considered a marker of thyroid autoimmunity.[17] The human TPO gene is about 150 kbp in size, located on chromosome 2, locus 2p25 and consists of 17 exons and 16 introns.[18] The full-length 3,048-bp transcript (TPO1) encodes a protein consisting of 933 amino acids, which has a short intracytoplasmatic tail, a transmembrane domain encoded by exon 15 and a large extracellular domain which contains the catalytic site. TPO gene undergoes alternative splicing to generate different TPO isoforms lacking one or more exons. To date eight isoforms have been described in thyroid cells, TPO 1, TPO 2,[19,20] TPO 3 or TPO Zanelli,[21] TPO 4, TPO 5, TPO 6, TPO 2/3 and TPO 2/4,[22] as illustrated in Table 1. Although TPO 3[23] and TPO 4[22] are able to reach the cell surface and show enzymatic activity, TPO 2 and TPO 5 lack enzymatic activity[21,22] but we are unaware of functional studies performed on the other TPO isoforms. In addition, TPO isoforms' sequences have been only partially characterized because of the difficulty in obtaining full length mRNA/cDNA.[22]

The apparent molecular weight (MW) of TPO in denaturing Western blot (WB) is a doublet at 105–110 kDa (Table 1), which may be due to variants of TPO[19,22] or differing degrees of glycosylation.[24,25]

The aim of this study is to explore the hypothesis that TPO is a thyroid/BC shared antigen by analyzing TPO gene expression (mRNA and protein) in relevant ex vivo samples.

Material and Methods

Tissues

Human surgical tissues (from scheduled operations and obtained with informed consent) were immediately frozen. Eight BC (seven infiltrating ductal adenocarcinoma and one mucinous carcinoma) and their eight corresponding peritumoral breast tissues (PT) were collected. Seventeen adipose tissues (AD) from various depots including abdominal ($n = 4$), subcutaneous ($n = 8$), knee ($n = 1$) and orbital fat ($n = 4$) were studied and also malignant tissues, including pancreatic adenocarcinoma (PC; $n = 3$), primary kidney carcinoma (PKC; $n = 1$) and hepatic metastasis of kidney carcinoma (MKC; $n = 1$) were obtained for controls. Human thyroid tissue (TT) from thyroidectomy for multinodular goiter was used as a positive control. Also collected were two

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Table 1. Summary of known TPO isoforms' characteristics

	mRNA analysis					Protein analysis			
TPO Isoform	N° exons	Exon lacked	bp	PCR F13R17[1]	PCR F2R17[2]	N° AA	MW (kDa) calculated	MW (kDa) on WB	Function
TPO 1	17	/	3,152	485	2,776	933	103	105–110	Yes
TPO 2	16	10	2,981	485	2,605	876	96	?	No
TPO 3	16	16	3,022	355	2,646	890	98	?	Yes
TPO 4	16	14	2,930	353	2,644	889	98	?	Yes
TPO 5	16	8	2,543	485	2,257	760	84	?	No
TPO 6	12	10, 12, 13, 14, 16	2,339	No product	1,963	662	73	?	?
TPO 2/3	15	10, 16	2,851	355	2,475	832	91	?	?
TPO 2/4	15	10, 14	2,849	353	2,473	832	91	?	?

bp = number of base pairs.
[1]Size of expected products (bp) with PCR using primer pair F13/R17.
[2]Size of expected products (bp) with LongRange RT-PCR using primer pair F2/R17. N° AA = number of amino acids; MW = molecular weight; kDa = kilo Dalton; WB = Western blot.

additional fresh frozen BC and nine paraffin-embedded BC sections (all infiltrating ductal adenocarcinoma), for a total of 19 different BC samples analyzed with various techniques (Supporting Information Table S1). Thyroid autoimmunity status was available only in 8/19 BC patients and 1/8 had serum TPOAb positivity (Supporting Information Table S1).

Indirect immunofluorescence

Indirect immunofluorescence (IF) was performed on four deparaffinized and rehydrated human BC tissue samples and also on human TT as positive control. The primary antibodies comprised pooled sera from women with high titers of TPOAb ($n = 4$) or free of TPOAb ($n = 3$); TPOAb determination was performed by immune enzymometric assay (Tosoh Bioscience, Tessenderlo, Belgium). Tissue sections were incubated in 1:10 dilution of the pooled sera at room temperature for 1 hr, followed by 1:70 dilution of polyclonal rabbit anti-human-IgG FITC-conjugated (Dako, DK-2600 Glostrup, Denmark) for 1 hr at room temperature. The same tissue sections were also incubated in 1:100 dilution of mouse monoclonal to TPO ab76935 (Abcam, Cambridge, UK) at room temperature for 1 hr followed by 1:50 dilution of Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Paisley, UK) at room temperature for 1 hr. A further five paraffin-embedded BC samples were analyzed in a modified protocol in which slides were initially blocked for 2 hr at room temperature with goat serum 10% and all primary and secondary antibodies were also diluted in goat serum 10% and incubated with tissue slides for 1 hr at room temperature. Primary antibodies were pooled human sera from women with high titers of TPOAb or free of TPOAb as above 1:10 dilution and mouse TPO monoclonal ab12500 (Abcam) 1:25 dilution. Secondary antibodies were, respectively, goat polyclonal to human IgG-H&L (FITC) ab97164 (Abcam) 1:500 dilution and Alexa Fluor 488 goat polyclonal anti-mouse IgG H + L (Life Technologies) 1:1,000 dilution. In all cases, images were analyzed using Olympus BX51 microscope, trinocular brightfield/fluorescence (Olympus America, Center Valley, PA).

Analysis of TPO mRNA expression

RNA isolation and reverse transcription-polymerase chain reaction. Total RNA was extracted from tissues and cells using TRIzol Reagent (Ambion, Life Technologies, Paisley, UK) as described in the manufacturer's protocol; reverse transcription used 1 µg total RNA, M-MLV reverse transcriptase (Promega, Madison, WI) and was primed with oligo dT for standard PCR or random hexamers for absolute real-time quantitative PCR (QPCR) in a standard protocol.

The resulting cDNA was amplified in standard PCR using Ampli Taq Gold DNA Polymerase (Applied Biosystems, Life Technologies, Paisley, UK) and primers designed using Primer 3 Plus software. The principal primer pairs used for PCR were forward in exon n° 13 (F13: 5′-ACGGGTATGAGCTCCAAGG-3′) and reverse in exon n° 17 (R17: 5′-TCTCGGCAGCCTGTGAGTAT-3′). PCR products obtained were electrophoresed on 2% agarose gel and stained with ethidium bromide. PCR single products were purified using Polyethylene Glycol (PEG) solution (26% PEG, 6.6 µM $MgCl_2$ and 0.6 M Na acetate), or extracted from cut gel fragments using the Qiaquick gel extraction kit (Qiagen, Manchester, UK) according to the manufacturer's instructions for sequence analysis performed using the BigDye Terminator Sequencing Kit (Applied Biosystems [ABI], Life Technologies) and an ABI Prism 377 DNA automatic sequencer (as previously described)[26].

QPCR. QPCR was conducted using SYBR Green incorporation measured on a Stratagene (La Jolla, CA) MX 3000. The TPO primers used for QPCR were forward in exon n° 2 (F2: 5′-GCTGTCTGTCACGCTGGTTA-3′) and reverse in the junction region between exons n° 3 and 4 (R3-4: 5′-TGAGGTTTCTCTGCATCGTG-3′). Standard curves, comprised of serial dilutions containing known copy number, were included in all experiments to allow calculation of absolute values for each sample (transcripts/µg input RNA). In addition, transcripts for a housekeeping gene, APRT, were measured so that values could be expressed relative to this (transcripts/1,000 APRT). In a single QPCR experiment, all measurements were made in triplicate; the standard curve was also run in at least duplicate in each reaction (as previously described)[27].

Whole TPO mRNA expression according to the different TPO isoforms (LongRange RT-PCR)

To characterize more fully previously reported TPO variants and those identified in this study by PCR, we generated cDNA using the Qiagen LongRange 2Step RT-PCR kit, according to the manufacturer's protocol, and TPO primers F2 and R17. Several tissues were analyzed including TT ($n = 1$), BC ($n = 2$) and subcutaneous AD ($n = 1$). The PCR amplification step was conducted in the presence and absence of Q solution; resulting products were electrophoresed on 1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer, stained, extracted, purified and sequenced as described above.

TPO protein expression analysis using WB

Protein extraction. Frozen tissue (200 mg) was diced using a razor blade and thawed in 600 µl of RIPA buffer (phosphate buffered saline PBS, 1% NP40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate [SDS]) containing 10 µl/ml proteases inhibitor (phenylmethanesulfonylfluoride [PMSF] in isopropanol) and homogenized with a Dounce homogenizer at 4°C. Samples were then centrifuged at 15,000g for 20 min at 4°C and the protein extract (supernatant) stored at −20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and WB. Extracted proteins (5–15 µg of TT and 50 µg for all other tissues) were boiled for 5 min in loading buffer (40% SDS 10%, 20% glycerol, 20% 0.5 M Tris pH 6.8, 16%

pure water, 2% pyronin Y 0.2%, 2% β-mercaptoethanol, 10 μl/ml PMSF 100 mM) and separated on 8% Tris-glycine SDS-acrylamide gels. After electrophoresis, proteins were transferred onto a polyvinylidine fluoride (PVDF) membrane Amersham Hybond-P (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and blocked with 5% non-fat dry milk powder (Marvel, Long Sutton, Spalding, UK) for 2 hr at room temperature followed by overnight incubation at 4°C with mouse monoclonal TPO ab76935 (Abcam) raised to TPO amino acids 672–780, diluted (1:1,000) with 5% bovine serum albumin (BSA [Sigma-Aldrich, Gillingham, Dorset, UK]). After three washes, membranes were incubated with peroxidase-labeled anti-mouse IgG (1:5,000) for 1 hr at room temperature and then developed with Amersham ECL Plus Western blotting detection system (GE Healthcare) according to manufacturer's instructions. Membranes were then stripped for 30 min at 60°C in buffer and reprobed with rabbit monoclonal anti-human â-actin IgG and peroxidase-labeled anti-rabbit IgG, following the same procedure as previously described.[28]

Primary antibody absorption test by antigen peptide. Primary TPO antibody ab76935 (8 μg) was preincubated overnight at 4°C with TPO recombinant fragments (8 μg) produced in bacteria either TPO 3 (Glu 471–Ser 720) or TPO 4 (Phe 709–Leu 993)[29] in 5% BSA. The same primary antibody absorption test was also performed using 10 μg bovine LPO (Sigma-Aldrich).

Indirect immunohistochemistry

Indirect immunohistochemistry (IHC) was performed on two frozen human BC tissue samples and frozen human TT as positive control, using Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA) with Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories), as described in the manufacturer's protocol. The primary antibody was the mouse monoclonal to TPO ab12500 (Abcam) diluted 1:25 in specific buffer and incubated with tissue sections at room temperature for 1 hr. Images were analyzed using Olympus BX51 microscope, trinocular brightfield/fluorescence (Olympus America).

Results

TPO antibodies display autoreactivity to a BC antigen

Figure 1 shows representative results obtained by indirect IF, in which pooled human sera from women with high levels of TPOAb were used as the primary antibody. In TT, used as a

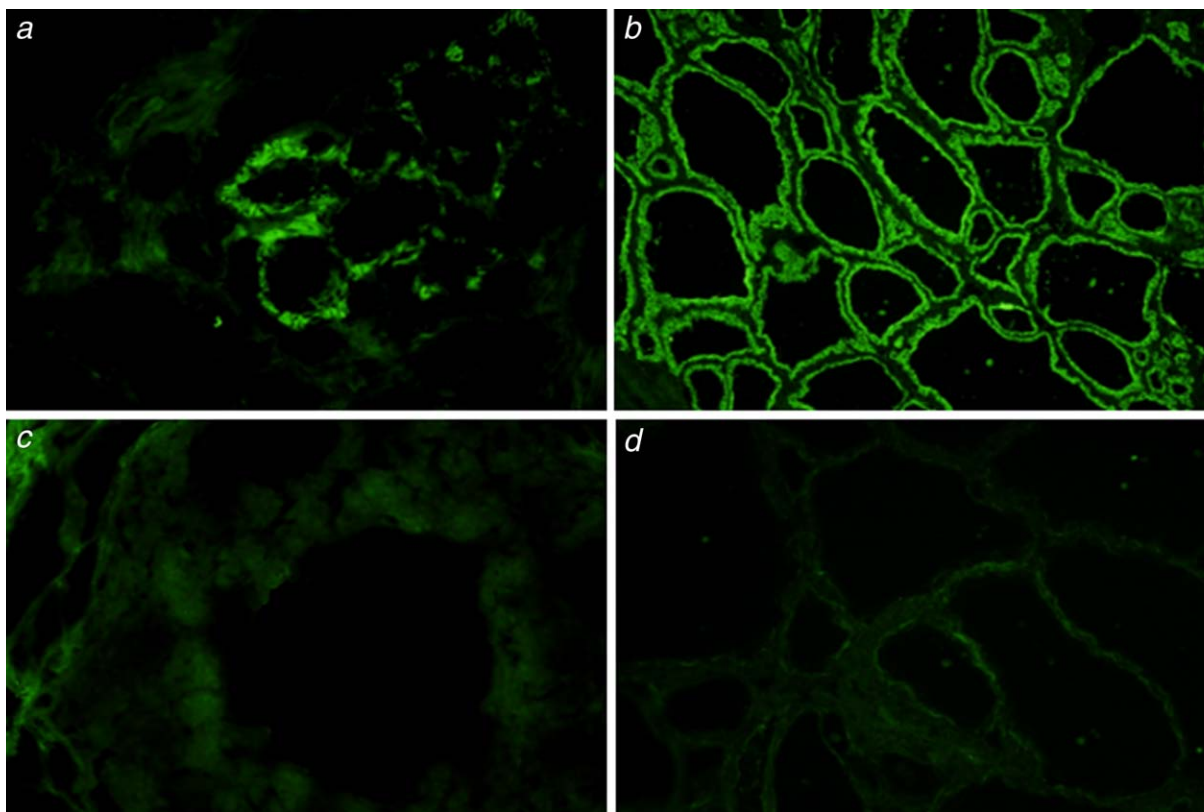


Figure 1. Indirect immunofluorescence results using pooled human sera (phs) as primary antibody. Panel (*a*): breast cancer (BC) tissue incubated with high levels TPOAb-positive phs. Panel (*b*): thyroid tissue (TT) incubated with high levels TPOAb-positive phs (positive control). Panel (*c*): BC incubated with TPOAb-free phs. Panel (*d*): TT incubated with TPOAb-free phs (negative control). In panels (*a*) and (*b*), a positive signal (green stain) is present, which is absent in panels (*c*) and (*d*), where only a weak aspecific staining (negative) is visible. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

positive control, the IF signal was strongest on the apical surface of the thyroid cells and most likely reflects binding to TPO. In BC tissues, immunoreactivity was apparent in 9/9 tested, while the signals were absent from TT and BC when using pooled TPOAb-free human sera. Similar results were obtained using the mouse monoclonal to TPO antibody (Supporting Information Fig. S1). The results indicate immunoreactivity to BC in women with TPO antibodies, thus we conducted experiments to investigate TPO expression in BC.

TPO transcripts expression in ex vivo tissues

Figure 2*a* shows results obtained in standard reverse transcription-polymerase chain reactions (RT-PCR) in representative TT, BC and PT samples after 50 PCR cycles. After 40 PCR cycles, the expected amplicons for F13 and R17 (485 bp and 353/355 bp, as shown in Table 1) were obtained only in TT, 8/8 BC, 5/8 PT and 9/9 AD (Fig. 2*b*). These four tissue types also expressed unexpected products of 220 bp and 130 bp; sequencing of the 220 bp amplicon revealed that it is a novel TPO isoform lacking exons 14 and 16 (most abundant in BC and AD) but we were unable to identify the 130 bp product. Sequencing of additional bands from 300 to 500 bp revealed they were all nicked products of the 485 bp or 353/355 bp bands. In other tumors such as PC, PKC or MKC, TPO transcripts were detected only after 50 cycles and in particular only the 485 bp product in 2/5 tumors; no other amplicons were obtained.

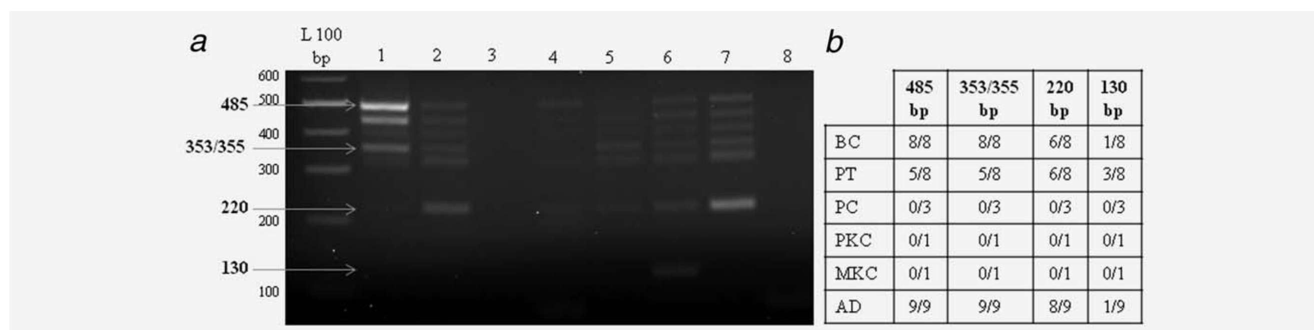
Primer pair F2/R3-4, which should detect all known TPO isoforms and hence provide a measure of total TPO transcripts, was used in QPCR. In TT ($n = 2$), we obtained an average 6×10^6 transcripts/μg input RNA. In BC ($n = 8$) the mean was 8.9×10^2 ($\pm$sem) transcripts/μg input RNA, which was not significantly different from the level in PT ($n = 8$) but 10^4-fold less than in TT. As breast tissue contains fat, we measured TPO transcripts in AD from various depots ($n = 17$); the mean was 9.2×10^3 ($\pm$sem) transcripts/μg input RNA with no depot-specific differences noted but 10^3-fold less than in the TT (and tenfold higher than in BC). In the other tumors (PC, PKC and MKC), TPO transcripts were at the limit of detection.

In an attempt to characterize the various TPO isoforms detected by standard RT-PCR, we used primers in exons 2 (location of start codon) and 17 (location of stop codon) in LongRange RT-PCR; Table 1 and Figure 3 summarize the expected products and our results, respectively. We used TT as a positive control, in which we obtained a faint amplicon at 2,700 bp only in the presence of Q solution (Fig. 3*a*); sequencing confirmed that it was the full length TPO 1 isoform (Fig. 3*b*). The most abundant thyroid transcript was the previously described TPO 5, which lacks exon 8, and hence enzymatic activity. Additional smaller transcripts were also present in TT but these did not correspond to other TPO isoforms, either previously reported or described in this study.

In nonthyroid samples, many different bands were detected; a 2,000 bp variant was present in BC n°1 (with Q solution) lacking exons 8–9 or in BC n°2 lacking exon 8 (TPO 5); a 1,500–1,700 bp variant lacking exons 8–10 was present in BC n°1 (with Q solution) and AD; while a 1,000 bp transcript in which exon 7 then skipped to exon 15 was abundant in the two BC (without Q); a short transcript comprising exons 2 linked to 15–17 was present in AD, BC n°2 and TT. As in the case of TT, we did not find other known TPO isoforms or the new variant lacking exons 14 and 16; Figure 3*b* summarizes all TPO isoforms found with LongRange RT-PCR using primer pair F2/R17.

TPO protein expression in ex vivo tissues

In WB using extracts from human TT probed with the TPO monoclonal antibody ab76935, we observed proteins of apparent molecular mass of 105–110 kDa, corresponding to TPO 1 (Table 1). When exposing the blots for extended times (up to 40 min) combined with loading ten times more protein extract than for TT, we were able to detect similar bands in



	485 bp	353/355 bp	220 bp	130 bp
BC	8/8	8/8	6/8	1/8
PT	5/8	5/8	6/8	3/8
PC	0/3	0/3	0/3	0/3
PKC	0/1	0/1	0/1	0/1
MKC	0/1	0/1	0/1	0/1
AD	9/9	9/9	8/9	1/9

Figure 2. PCR products obtained using primer pair F13/R17. Panel (*a*): Results after 50 cycles (representative samples) in 1 = thyroid tissues (positive control); 2, 4, 7 = breast cancer (BC); 3, 5, 6 = peritumoral breast tissues (PT); 8 = negative control (pure water). L 100 bp = ladder 100 base pairs. The expected 485 and 353/355 bp bands and other intermediate bands (nicked products) are expressed by all samples, except for 3, 8. The 220 bp unexpected band is clearly expressed in 2, 4, 5, 6, 7, faint in 1 and absent in 3, 8. The 130 bp unexpected band is expressed only in 6. Panel (*b*): Summary of PCR products after 40 cycles obtained in all samples. BC = breast cancer, PT = peritumoral breast tissues; PC = pancreatic adenocarcinoma; PKC = primary kidney carcinoma; MKC = hepatic metastasis of kidney carcinoma; AD = adipose tissue.

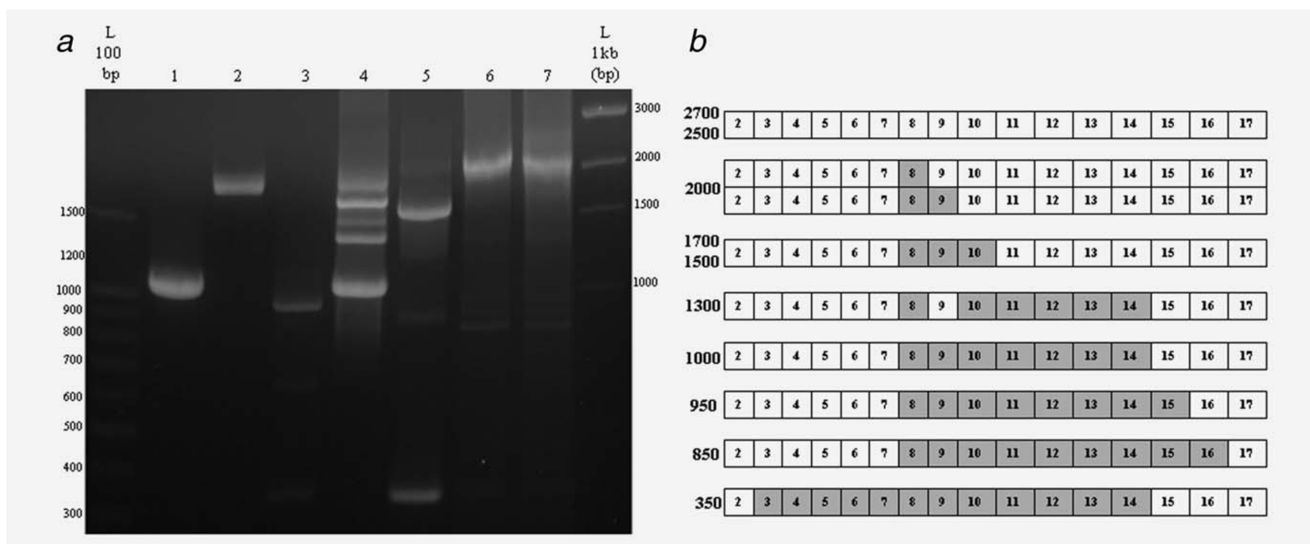


Figure 3. LongRange RT-PCR results with primer pair F2/R17. Panel (*a*) shows agarose gel 1.5% bands using PCR master mix with (Q) or without (N) Q solution obtained in 1 = breast cancer (BC) n°1 with N, 2 = BC n°1 with Q, 3 = BC n°2 with N, 4 = BC n°2 with Q, 5 = subcutaneous adipose tissue (AD) with N, 6 = thyroid tissue (TT) with N and 7 = TT with Q. Panel (*b*) shows a schematic representation of novel TPO mRNA isoforms found sequencing PCR products obtained with LongRange RT-PCR using primer pair F2/R17. Numbered squares from 2 to 17 represent TPO gene exons. White and grey squares represent, respectively, expressed and missed exons.

Figure 4. WB representative results; 40-min exposure time. Panel (*a*): 105–110 kDa band expression using ab76935 obtained in breast cancer tissue (1, 2), breast peritumoral tissue (3, 4), primary kidney cancer (5), pancreatic adenocarcinoma (6, 7), abdominal fat (10), subcutaneous fat (11), knee fat (12) and orbital fat (13); thyroid tissue (9, 15) is used as positive control and it is separated from other samples by empty spaces (8, 14). Panel (*b*): 105–110 kDa band expression using normal ab76935 obtained in breast peritumoral tissue (1, 2), hepatic methastasis of kidney cancer (3), breast cancer (4, 5, 6) and thyroid tissue (8), separated from other samples by an empty space (7). Panel (*c*): 105–110 kDa band expression using TPO absorbed ab76935 obtained in the same samples of panel B: the signal is strongly reduced or eliminated. Panel (*d*): complete WB results in thyroid tissue using normal ab76935; it shows the doublet at 105–110 kDa and three additional bands at 70, 60 and 30–35 kDa. Panel (*e*): complete WB results in the same thyroid tissue of panel D using TPO absorbed ab76935: the 105–110, 70 and 60 kDa bands are strongly reduced, whilst the 30–35 kDa band is not significantly modified. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

7/8 BC, 4/8 PT, 2/4 AD, 1/1 PKC, 1/1 MKC and 1/3 PC (Fig. 4*a*). To test the specificity, we absorbed the antibody using recombinant fragments of TPO spanning the region of the epitope recognized by the antibody. As shown in Figures 4*b* and 4*c*, the 105–110 kDa signal in TT was greatly reduced and that in the other tissues completely eliminated by the treatment. In contrast, absorption with LPO did not reduce the signal (data not shown).

Figures 4*d* and 4*e* illustrate an entire WB of TT probed with the TPO monoclonal antibody ab76935 and demonstrates proteins of apparent MW 70, 60 and 30–35 kDa, in addition to the doublet at 105–110 kDa. Absorption with

recombinant TPO strongly reduced not only the 105–110 band corresponding to complete TPO protein (TPO 1) but also the signals at 70 and 60 kDa, indicating that they could be smaller isoforms of TPO or degraded fragments of TPO 1; the 30–35 kDa band was not affected by absorption. Absorption with LPO did not reduce the signal (data not shown). The 70 and 60 kDa bands were also present in breast tissues BC/PT and PKC in addition to proteins of 50 and 150 kDa, all of which were reduced by absorption of TPO monoclonal antibody ab76935 with recombinant TPO (Supporting Information Fig. S2).

The WB results indicate that breast tissues contain low levels of TPO protein, including TPO 1, but in view of the various PCR data which demonstrate that AD also expresses TPO transcripts, we sought to identify the cellular location of the TPO protein using indirect IHC. Figure 5 demonstrates weak TPO immunoreactivity in a small proportion of breast epithelium, obtained in 2/2 BC tissues analyzed.

Discussion

Our experiments demonstrate that TPO mRNA and protein are not confined to thyroid cells but are also present in other tissues, albeit it at low levels. Our results confirm those of Lai *et al.*[30] who demonstrated TPO expression in orbital tissue.

TPO expression in BC tissue could explain both the known association between BC and ATD and the protective role of serum TPOAb in patients with aggressive BC. We hypothesize that TPO could be a common antigen between thyroid and BC tissues; as even trace amounts could suffice to trigger B lymphocyte activity although further studies of T lymphocyte immunoreactivity in both thyroid and BC tissues are necessary to validate this hypothesis.

Further investigation is also needed to determine whether TPO expression is confined to BC, especially as we found TPO in PT tissue, although neoplastic features may have been missed. TPO is expressed in several fat depots, and as fat is abundant in the breast this could explain PT TPO expression. A recent review has also reported that adipocytes within mammary gland carcinomas are dynamic cells that may contribute to human BC progression.[31] However, adipose tissue does not account for all the TPO we detected in breast as IHC indicates TPO protein in neoplastic breast epithelium.

An increased prevalence of serum TPOAb and other thyroid autoantibodies has been found in several other cancers including kidney,[32] pancreas,[33] lung[34] and gastric[35] and are associated with better (kidney) and worse (pancreas) prognosis, suggesting a role for these antibodies in tumor prognosis.

Our work revealed a new variant, lacking exons 14 and 16 (15 encodes the membrane spanning region) which is most abundant in breast tissue. LongRange PCR revealed numerous other isoforms, in several tissues tested, in which exons 2–7 were conserved, but 8–16 (encodes enzyme activity) were spliced out. Surprisingly we did not detect previously described isoforms such as TPO 2, TPO 3, and so forth. There are two possible explanations: the first and simplest being primer selection and the second the existence of additional START codons in some TPO isoforms. Future experiments will investigate whether mRNA transcripts exist which are the equivalent of the various lower MW bands we found using WB Exons from 5 to 12 encode the MPO-like sequence which contains important catalytic residues; probably TPO isoforms that lack exons in this region are not functional, as already demonstrated for TPO 2 that lacks exon 10[21] and TPO 5 that lacks exon 8.[22] The absence of functional activity

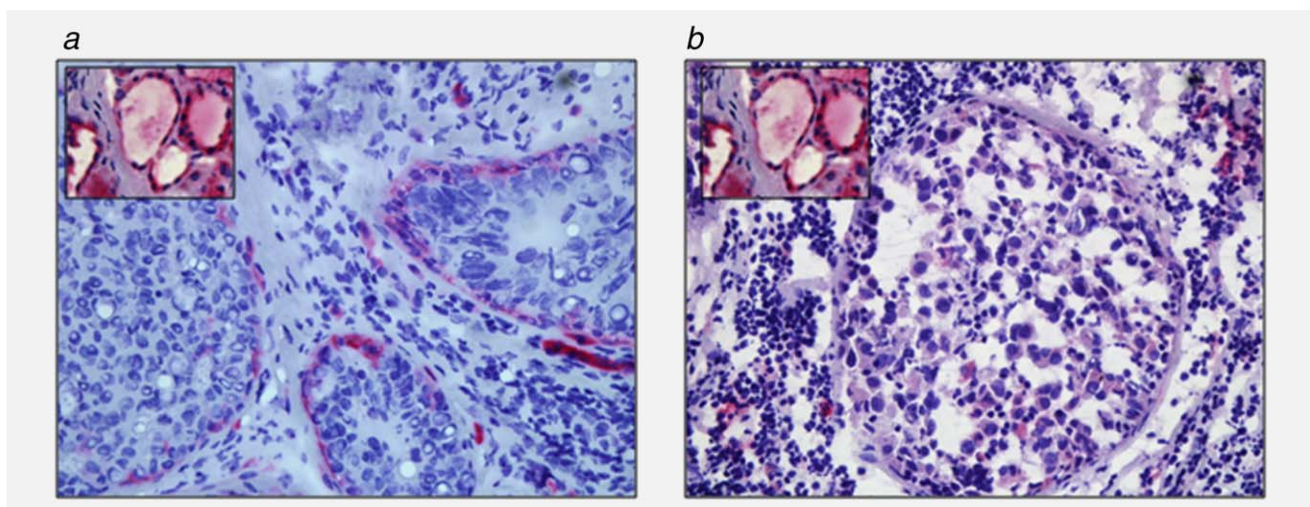


Figure 5. Indirect immunohistochemistry results using TPO mouse monoclonal ab12500 as primary antibody. Panels (*a*) and (*b*) show two different breast cancer tissues (BC); in the small square is represented thyroid tissue (TT) as positive control. In TT, the TPO signal (red) is strongly positive and located into thyreocytes that express TPO, disposed around the colloid follicles; in BC the TPO signal (red) is weaker but present and located in breast epithelium cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

does not influence the antigenic role: to this purpose TPO protein sequence must just contain those short linear peptides processed and presented to T-lymphocytes by antigen presenting cells *via* major histocompatibility complex (MHC) molecules. Many authors identified TPO epitopes recognized by T lymphocytes in the MPO-like sequence.[36–40] Furthermore, two T-lymphocytes epitopes have been found also in C-terminal part of TPO protein, in particular in the transmembrane region[38,39] and in the intracellular region.[38]

B-lymphocytes epitopes are different, as a few are linear (C21, C2)[41,42] but the majority are conformational,[36,43–46] therefore strongly related to tertiary and quaternary protein structures. TPO isoforms that lack some exons probably fold into a different conformation when compared with the full-length TPO1 and hence may lose their antigenic property for B cells and autoantibodies.

In conclusion, TPO no longer seems to be thyroid specific: mRNAs and proteins for known TPO isoforms are weakly but clearly expressed in BC and other tissues. In particular TPO expression in BC could explain at least in part the high frequency and protective role of serum TPOAb in BC patients, hypothesizing an enhancement of specific T and B lymphocytes immunoreactivity, with potential implications in translational medicine. Moreover, we have found many novel TPO mRNA isoforms lacking several exons both in thyroid and other tissues examined, tumoral or not. Some of these TPO mRNA isoforms could be translated into the correspondent lower MW proteins: further studies are needed to investigate their function and immunogenicity and if they can be used as tissue specific markers.

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