

# Thyroid Transcription Factor-1 (TTF-1): protein expression is not exclusive to lung and thyroid tissue.

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## Abstract

Thyroid Transcription Factor-1 (TTF-1) is a member of the homeodomain transcription factor family, is a tissue specific transcription factor, and plays a role in regulating proteins expressed within the thyroid, lung and brain. These include thyroglobulin, thyroid peroxidase, Clara cell secretory protein and surfactant proteins. Human TTF-1 (38 kD) is a single polypeptide of 371 amino acids sharing 98 percent homology with the equivalent rat and mouse proteins. TTF-1 functions by binding to specific recognition sites in a manner that may be regulated by both the redox and phosphorylation status of the protein. In addition to its role as a tissue-specific transcriptional activator in adult organs, TTF-1 may also function in organogenesis. Gene targeting studies have shown TTF-1 to be essential for the proper development of the thyroid and lungs and abnormal expression may underline a number of congenital abnormalities.

Novocastra™ clone SPT24 was developed to a 123 amino acid sequence of the N-terminal region of the TTF-1 molecule. The antibody is effective in both manual and automated immunohistochemistry on formalin fixed, paraffin embedded tissue sections.

Novocastra clone SPT24 was evaluated on 428 normal and tumor tissues. Expression was noted particularly in the follicular epithelial cells of the thyroid, type II pneumocytes and Clara cells of the lung, thyroid and lung tumors.

Novocastra clone SPT24 also demonstrated TTF-1 expression in colon adenocarcinomas and thymomas. Whilst not widely documented, RT-PCR analysis confirmed that cases detected with the Novocastra clone SPT24 positively expressed fragments of TTF-1 RNA. Novocastra clone SPT24 has a genuinely strong affinity for TTF-1 protein, proven by its ability to identify genuine TTF-1 expression in previously unrecorded tumors. Other clones, such as clone 8G7G3/1, when used at dilutions of 1:50 or lower also demonstrated staining in the same areas of tumor that were positive with Novocastra clone SPT24.

The responsible validation of any antibody clone, including TTF-1, is vital in assessing its potential diagnostic use. Specifically, awareness of the full range of normal tissue and tumor expression expected for an antibody is pivotal to diagnostic application. Interpretation needs to take into account expression of a panel of appropriate immunohistochemical antibodies in addition to clinicopathological features and most importantly tumor morphology before reaching a diagnosis

## Introduction

Carcinomas arising from lung and thyroid show frequent TTF-1 expression.<sup>1</sup> As lung is one of the most common sites of metastasis, TTF-1 is considered as a reliable marker to distinguish between primary lung carcinoma and metastases within the lung, especially when dealing with an adenocarcinoma or a large-cell carcinoma.<sup>1</sup> It is also considered as a reliable marker in the differential diagnosis between pleural localization of a peripheral lung carcinoma and malignant mesothelioma.<sup>1</sup>

In normal tissue, TTF-1 is reported to be expressed in epithelial cells of thyroid and type II pneumocytes and Clara cells in lung.<sup>1</sup>

In cancerous tissue, TTF-1 has been detected in pulmonary adenocarcinoma<sup>2</sup>, large-cell carcinoma<sup>2</sup>, small cell carcinoma of lung<sup>3</sup>; medullary thyroid carcinoma<sup>4,5</sup> and hepatocellular carcinoma.<sup>6</sup>

## Aim

The introduction of sensitive antibodies and polymer detection systems has lowered the threshold of immunohistochemical detection of some proteins. This has resulted in the realization of more widespread expression patterns by a number of proteins. The aim of this characterization was to review the expression of TTF-1 protein using Novocastra clone SPT24 and a sensitive polymer detection system in a broad range of tissues. RT-PCR was also used to identify the presence of TTF-1 mRNA transcripts in order to provide supporting evidence for protein expression in tissues in which TTF-1 has not been previously described

## Materials and Methods

### Analysis of TTF-1 RNA Transcripts

#### RNA extraction

RNA was extracted from 2 x 20 µm thick FFPE tissue sections using the PureLink FFPE total RNA isolation kit (Invitrogen, California) according to the manufacturer's protocol. This procedure included a xylene-free protocol for deparaffinization followed by proteinase K digestion at 60 °C for up to 3 hours. The tissue lysate was further processed by selectively binding the RNA to a silica-based membrane spin cartridge, followed by several washing steps and an elution step in 75 µl RNase free water.

## PCR Amplification

Before conducting RT-PCR the total RNA extracted from each FFPE tissue sample was quantified by means of Qubit assay using the Quant-iT RNA assay kit (Invitrogen). The quality of the RNA extracted from each FFPE specimen was assessed by conducting a control RT-PCR test on the RNA extracted using primers specific for a 69 bp (base pair) fragment of the house keeping gene GAPD (forward: 5'-CTCTCTGCTCCTCCTGTTTCGAC3'; reverse: 5'TGAGCGATGTGGCTCGGCT-3'). TTF-1 RNA was reverse transcribed into cDNA using a specific reverse primer R5 (5'-GCTCGCCGGGCCATGAAGC-3') and the Reverse Transcription System Kit (Promega, USA) in accordance with the manufacturer's protocol. Amplification of a 93 bp fragment encoding for a region bridging the exon boundary between exon 1 and 2 of TTF-1 (Genbank accession No NM\_001079668.1) was carried out by PCR using a second TTF-1 specific primer (5'-ACCATGAGGAACAGCGCCTCTG-3') and half of the reverse transcription mix as a template in a 50 µl PCR reaction mix (2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.2 µM primer, 2.5 U Taq-DNA polymerase and 1x RT transcription buffer reaction mix (Promega, USA)). A total of 30 cycles of PCR (94°C/30 seconds, 55°C/30 seconds and 72°C/45 seconds) was performed.

## Cloning and Sequencing

The PCR products were verified by agarose gel electrophoresis, gel purified and then cloned into the pGEM-Teasy vector (Promega, USA). Clones were identified by digestion with the restriction enzyme EcoR1 and their identity confirmed by DNA sequencing (Beckman Coulter Genomics, Takeley, Essex, UK).

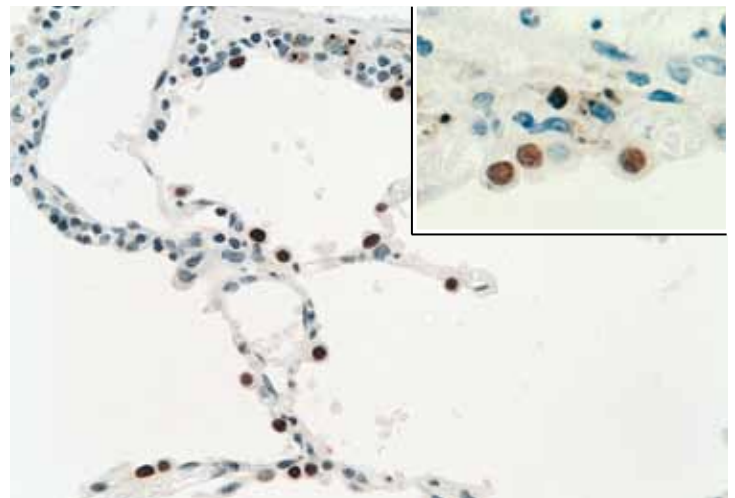
## Manual Immunohistochemistry

Manual immunohistochemical validation was performed on a range of normal and tumor tissues, in the form of whole tissue sections and tissue micro-arrays (TMA), using the mouse monoclonal TTF-1 antibody, Novocastra clone SPT24, in conjunction with the Novolink™ Polymer Detection System RE7140-K (250 tests). Briefly, whole tissue paraffin sections, 4 µm thick, were cut onto Leica Microsystems charged coated slides (S21.2113.A). To facilitate adhesion, these slides were then dried overnight at 37°C and finally baked for 1 hour at 56°C. TMA's were prepared as directed by the individual suppliers. Sections were deparaffinized in xylene and rehydrated through graded alcohols. Heat induced epitope retrieval was performed using Epitope Retrieval Solution pH6.0 (RE7113) in a Prestige stainless steel pressure cooker for 8 minutes at full pressure. Endogenous peroxidase activity was blocked by incubation for 5 minutes in Peroxide Block solution. Slides were washed in 50mM Tris buffered saline (TBS, pH7.6) for 5 minutes, incubated with Protein Block for 5 minutes and washed in TBS for a further 5 minutes. Sections were then incubated for 30 minutes at 25°C with TTF-1 primary antibodies, Novocastra clone SPT24 and Dako clone 8G7G3/1 both diluted 1:50 in Antibody Diluent (RE7133). Following two sequential 5 minute wash steps in TBS, sections were incubated in Post Primary for 30 minutes at 25°C. Two sequential 5 minute wash steps in TBS were again performed and sections incubated for 30 minutes at 25°C with Novolink Polymer. Two further sequential 5 minute wash steps in TBS were performed and bound peroxidase visualized using DAB chromogen. The DAB working solution was prepared by adding 50 µL of DAB chromogen per mL of DAB Substrate Buffer.

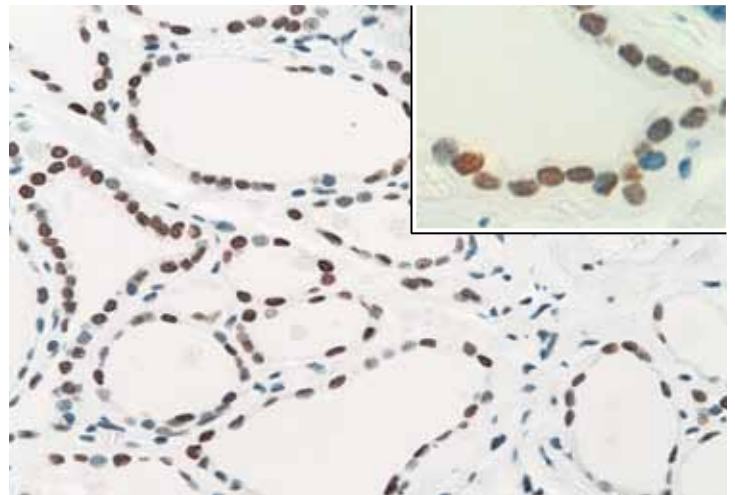
Slides were washed in water and then sections were counterstained with Hematoxylin. Finally sections were dehydrated, cleared and mounted in DPX.

## Automated Immunohistochemistry

Automated immunohistochemical validation was performed on a range of normal and tumor tissues, in the form of whole tissue sections and tissue micro-arrays (TMA), using the automated Leica BOND™ system, Leica BOND Dewax Solution (AR9222), Leica BOND Epitope Retrieval Solution 1 (AR9961) and Leica BOND Polymer Refine Detection (DS9800). Epitope retrieval was performed for 20 minutes followed by \*IHC Protocol F using TTF-1 primary antibody, Novocastra clone SPT24 (Leica BOND Ready-to-Use PA0364).



**Figure 1.** Immunohistochemical staining for TTF-1 using Leica Novocastra clone SPT24 on normal lung (x400 original magnification). Leica BOND automated system staining of a paraffin section. Insert shows nuclear staining localization in type II pneumocytes.



**Figure 2.** Immunohistochemical staining for TTF-1 using Leica Novocastra clone SPT24 on normal thyroid (x400 original magnification). Leica BOND automated system staining of a paraffin section. Insert shows nuclear staining localization in thyroid follicular epithelium.

Tissue	IHC Staining Results
Adrenal	Negative
Bone Marrow	Negative
Breast	Negative
Bronchus	Negative
Cecum	Negative
Cerebellum	Negative
Cerebrum	Weak nuclear staining in glial cells
Cervix	Negative
Colon	Negative
Endometrium	Negative
Esophagus	Negative
Eye	Negative
Fallopian Tube	Negative
Heart	Negative
Ileum	Negative
Kidney	Negative
Liver	Negative
Lung	Strong nuclear staining in type II pneumocytes and Clara cells
Lymph Node	Negative
Mesothelium	Negative
Muscle, Skeletal	Negative
Ovary	Negative
Parathyroid	Negative
Pancreas	Negative
Peripheral Nerve	Negative
Pituitary	Negative
Placenta	Negative
Prostate	Negative
Rectum	Negative
Salivary Gland	Negative
Spleen	Negative
Skin	Negative
Stomach	Negative
Spinal Cord	Negative
Testis	Negative
Thymus	Negative
Thyroid	Strong nuclear staining in follicular epithelial cells
Tongue	Negative
Tonsil	Negative
Umbilical Cord	Negative
Ureter	Negative
Uterus (myometrium)	Negative

**Table 1.** In positive tissues, only the tissue elements stated were positive with Leica Novocastra clone SPT24, all other tissue elements were negative. (Results from TMA tissue).

Abnormal tissue	IHC positivity
Lung - adenocarcinoma	26/32
Lung - small cell carcinoma	4/7
Lung - bronchioalveolar carcinoma	2/3
Thyroid - papillary carcinoma	6/6
Thyroid - follicular carcinoma	3/3
Thyroid - medullary carcinoma	4/4
Thyroid - follicular adenoma	1/1
Thyroid - Hashimoto's thyroiditis	1/1
Thymus - thymoma	19/59
Thymus - atypical carcinoid	1/5
Colon - adenocarcinoma	4/49
Desmoplastic small round cell tumor	1/1

**Table 2.** Immunostaining for TTF-1 in abnormal tissues (whole sections and TMA's)

The tissue involved in this study consisted of 31 whole tissue cases and 7 TMA's comprising 392 cases in the form of 1mm and 1.5mm cores. All scoring was carried out by senior scientist(s) at Leica Biosystems Newcastle Ltd experienced in the assessment of immunohistochemical staining and tissue histology. In normal tissues, nuclear staining of tissue elements was noted and the staining intensity recorded as negative, weak, moderate or strong. In tumor tissues, any nuclear staining of tumor elements was classified as "positive" regardless of the staining intensity.

## Results

### Immunohistochemistry

#### Normal Tissues

Table 1 shows the staining for TTF-1 in normal tissues (TMA). Novocastra clone SPT24 detected the TTF-1 protein in the nucleus of type II pneumocytes and Clara cells of the lung (Figure 1), in the nucleus of follicular epithelial cells of the thyroid (Figure 2) and in glial cells of cerebrum. (Total cases = 140).

#### Abnormal Tissues

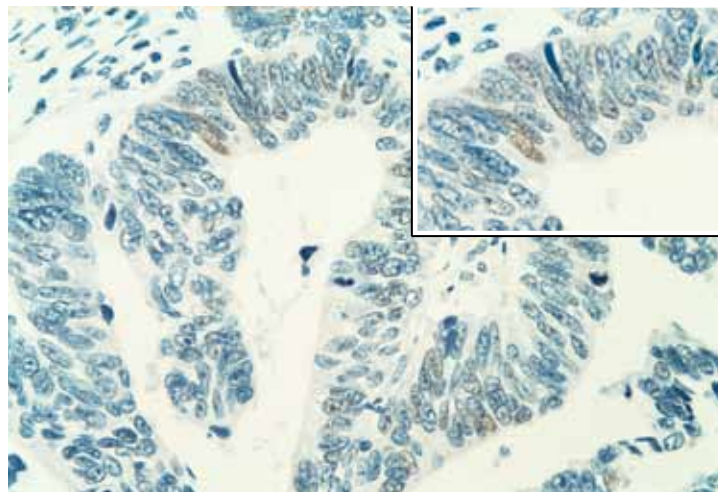
Novocastra clone SPT24 detected the TTF-1 protein in 72/171 abnormal tissues (whole sections and TMA's). Staining was particularly noted in the nucleus of tumors of the lung, thyroid, thymus and colon (Table 2). No staining was observed in an additional 112 abnormal tissues (Table 3).

Tumor tissue	No of cases evaluated
Lung – squamous cell carcinoma	18
Lung – large cell carcinoma	5
Malignant Mesothelioma	5
Breast – carcinoma	6
Ovary – thecoma	1
Ovary – juvenile granulosa cell tumor	2
Ovary – serous carcinoma	3
Ovary – mucinous carcinoma	2
Ovary – germ cell tumor	1
Ovary – clear cell carcinoma	1
Endometrium – adenocarcinoma	1
Endometrium – clear cell carcinoma	1
Endometrium – stromal sarcoma	1
Cervix – adenocarcinoma	2
Testis – seminoma	3
Testis – mixed germ cell tumor	1
Testis – embryonal carcinoma	1
Prostate – adenocarcinoma	1
Prostate – benign prostatic hyperplasia	1
Bladder – transitional cell carcinoma	2
Bladder – small cell carcinoma	1
Kidney – renal cell carcinoma	5
Kidney – transitional cell carcinoma	1
Adrenal – adenoma	1
Adrenal – pheochromocytoma	1
Liver – hepatocellular carcinoma	3
Liver – cholangiocarcinoma	2
Liver – adenoma	1
Pancreas – adenocarcinoma	3
Pancreas – endocrine tumor	2
Stomach – adenocarcinoma	4
Small bowel – adenocarcinoma	1
Small bowel - carcinoid	4
Brain – astrocytoma	1
Brain – choroid plexus papilloma	1
Squamous cell carcinoma - skin	2
Squamous cell carcinoma - penis	2

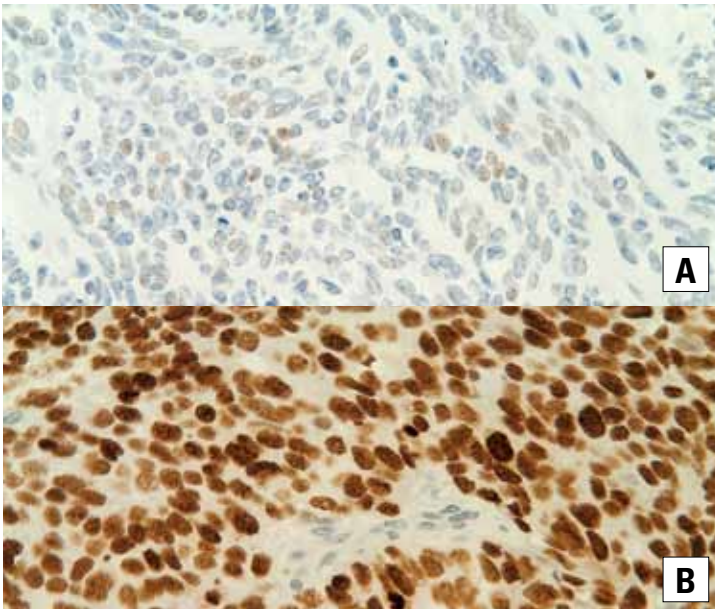
**Table 3. Continues**

Tumor tissue	No of cases evaluated
Squamous cell carcinoma - esophagus	2
Squamous cell carcinoma - larynx	1
Squamous cell carcinoma - tongue	2
Squamous cell carcinoma - cervix	1
Skin – basal cell carcinoma	1
Malignant Melanoma	3
Large B-cell lymphoma	1
Thymic tumor – metastatic	1
GIST	1
Synovial sarcoma	1
Leiomyosarcoma	1
Ewing's sarcoma	1
Spindle cell rhabdomyosarcoma	1
Omental fibrous tumor	1
Ganglioneuroma	1
Paraganglioma	1

**Table 3.** Abnormal tissues showing negative immunostaining for TTF-1 (whole sections and TMA's)



**Figure 3.** Immunohistochemical staining for TTF-1 using clone SPT24 on colonic adenocarcinoma (x400 original magnification). Leica BOND automated system staining of a paraffin section. Insert shows weak nuclear staining localization in tumor cells (TMA).



**Figure 4.** Immunohistochemical staining for TTF-1 using Leica Novocastra clone SPT24 on thymoma (x400 original magnification). Leica BOND automated system staining of a paraffin section. (a) shows a case of thymoma with weak positive staining in a proportion of the tumor nuclei (b) shows a case of thymoma with moderate to strong positive staining in majority of the tumor nuclei (whole sections and TMA).

Abnormal tissue	IHC positivity
Thymus - thymoma Type A	1/5
Thymus - thymoma Type AB	1/4
Thymus - thymoma Type B1	6/22
Thymus - thymoma Type B2	4/10
Thymus - thymoma Type B3 (atypical thymoma)	1/4
Thymus - thymoma Type C (thymic carcinoma)	3/11
Thymus - thymoma (unclassified)	3/3

**Table 4.** Immunostaining for TTF-1 in thymoma (TMA)

## Discussion

TTF-1 Novocastra clone SPT24, was shown to be effective at a dilution of 1:50 using Heat Induced Epitope Retrieval solution pH6.0 (RE7113, Leica Microsystems) and the Novolink Polymer Detection System (RE7140-K, Leica Microsystems). Staining was unaffected by the position of the peroxide block step in the protocol or the use of TBS or PBS-based diluents and wash buffers. TTF-1, Novocastra clone SPT24 (Leica BOND Ready-to-Use PA0364) was also effective on the automated Leica BOND system using Leica BOND Epitope Retrieval Solution 1 and Leica BOND Polymer Refine Detection.

Novocastra clone SPT24 demonstrated TTF-1 protein expression in the nuclei of tumors in lung, thyroid, thymus and colon (whole sections and TMA's). Expression of TTF-1 in medullary, papillary and follicular carcinomas of thyroid<sup>5</sup>, and in non squamous cell carcinomas of lung<sup>2,3</sup> was expected and has been well documented.

The demonstration of TTF-1 protein expression in the nuclei of tumors in colon and thymus with Novocastra clone SPT24 is not widely documented.

Novocastra clone SPT24 demonstrated TTF-1 protein in the nuclei of 3/49 colon adenocarcinomas (TMA) (6%) (Figure 3). TTF-1 staining was weakly positive and present in only a proportion of the tumor nuclei. TTF-1 positive staining in primary colon adenocarcinomas (5%) has recently been identified and published.<sup>1</sup> Genuine expression of TTF-1 using Novocastra clone SPT24 has been cited in a small proportion (10%) of colonic adenocarcinoma metastases found in lung. The identification of this expression has been attributed to the sensitivity of Novocastra clone SPT24.<sup>1</sup>

Novocastra clone SPT24 also demonstrated TTF-1 protein in the nucleus of 19/59 thymomas, in whole sections and a TMA (Table 4). In 24% of thymomas (14/59), TTF-1 staining was weakly positive in a proportion of tumor nuclei (Figure 4a). In 8% of thymomas (5/59), moderate to strong staining was noted in the majority of tumor nuclei (Figure 4b). Reported expression of TTF-1 in thymoma and thymic carcinoma has previously been documented as being negative.<sup>7,8,9</sup>

The TTF-1 expression in cases of thymoma which have previously been reported as being TTF-1 negative is possibly a reflection of increased sensitivity in IHC detection.

From the literature published since 2002 it becomes apparent that expression of TTF-1 has now been identified as extending beyond the expected lung and thyroid tumors. This could possibly be due to a combination of the increased use of TTF-1 in a wider range of tumors and an increase in the sensitivity of IHC polymer detection systems. Expression of TTF-1 is now being identified in tissues and tumors previously thought to be negative. Our experience with Novocastra clone SPT24 in thymoma and colon adenocarcinomas being a case in point.

Recently Novocastra clone SPT24 has been identified in primary colon adenocarcinoma and colon adenocarcinoma metastases in lung<sup>1</sup>, uterine tumors<sup>10</sup> – especially in malignant mixed Müllerian tumor<sup>10</sup> (82%), ovarian tumors<sup>10</sup> and in hepatocellular carcinoma.<sup>6</sup> Novocastra clone SPT24 was identified as being the most sensitive TTF-1 antibody in combination with Leica BOND Polymer Refine Detection (DS9800) and the automated Leica BOND system.<sup>10</sup> Novocastra clone SPT24 was also identified as possessing consistent nuclear positivity and an absence of erratic cytoplasmic staining in comparison with other clones.<sup>11</sup>

Other clones, such as clone 8G7G3/1 and clone BGX-397A (available from a variety of suppliers), were found to be slightly less sensitive in the same studies.<sup>1,10,11</sup> However clone 8G7G3/1 when used at a dilution of 1:50 or lower demonstrated weak staining in the same areas of tumor that were positive with clone SPT24.<sup>1</sup>

The obvious question arising from this current Novocastra clone SPT24 characterization work and the recently published new TTF-1 expression data is whether the TTF-1-positivity in primary colon cancers and thymomas was specific and a result of true aberrant expression of TTF-1. To provide supporting evidence to answer this question, analysis of the mRNA present in TTF-1 positive cases of primary colonic cancer and thymoma was performed to identify the presence of TTF-1 RNA transcripts.

Six cases of primary colon adenocarcinoma (whole sections) which were positive with Novocastra clone SPT24 (two cases of which were also weakly positive with clone 8G7G3/1) were analyzed for the presence of TTF-1 RNA transcripts.<sup>11</sup> RT-PCR analysis showed that all six Novocastra clone SPT24 positive cases expressed fragments of TTF-1 RNA transcripts indicative of TTF-1 gene expression. Moreover, three smaller RNA fragments when sequenced provided evidence of the existence of a novel splice variant previously not described in the literature.<sup>11</sup>

A case of thymoma (whole section), which was positive with Novocastra clone SPT24 and also weakly positive with clone 8G7G3/1, was analyzed for presence of TTF-1 RNA transcripts. RT-PCR analysis showed that this Novocastra clone SPT24 positive case expressed fragments of TTF-1 RNA transcripts indicative of TTF-1 gene expression.

Overall, the SPT24 clone appears to have a genuinely strong affinity for TTF-1 protein, proven by its ability to identify genuine TTF-1 expression in tumors previously considered TTF-1 negative. Tumors now reported as TTF-1 positive, other than lung and thyroid tumors, include sub-sets of primary colorectal adenocarcinomas, thymomas, uterine tumors, ovarian tumors and hepatocellular carcinoma as well as its established expression in medullary, papillary and follicular carcinomas of thyroid, and in non squamous cell carcinomas of lung.

The responsible validation of any antibody clone, including TTF1, is vital in assessing its potential diagnostic use. Specifically, awareness of the full range of normal tissue and tumor expression expected for an antibody is pivotal to diagnostic application. Interpretation needs to take into account expression of a panel of appropriate immunohistochemical antibodies in addition to clinicopathological features and most importantly tumor morphology before reaching a diagnosis.

## Disclaimer

Unless specifically stated all references quoted refer to data on TTF-1 derived from established scientific publications involving various antibodies and techniques and **do not** refer to IHC staining expression directly attributable to Novocastra NCL-L-TTF-1 or Leica BOND Ready-to-Use PA0364.

## Acknowledgement

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