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# Localization and distribution of wolframin in human tissues

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## 1. ABSTRACT

Wolframin is a transmembrane glycoprotein of 890 aminoacids, encoded by *WFS1* gene. *WFS1* mutations are responsible for Wolfram syndrome, an autosomal recessive disorder. In the present paper, we first characterized the polyclonal wolframin antibody by dot blot. Secondly, we verified antibody specificity by western blotting using different human cell lines. Thirdly, we studied wolframin localization in human foetal (14-35 weeks) and adult tissues by immunohistochemistry. Wolframin expression was distributed in many organs, with different tissue and cell localization and expression levels. In foetal systems, wolframin expression was faint at 14-16 weeks and increased when development proceeded. In adult human tissues a variable positive staining was observed in both simple and stratified epithelia. A moderate wolframin expression was observed in liver and in the endocrine portion of the pancreas. In conclusion, our data suggest that this protein may have important roles in a number of different tissues, including many that are not known to be affected by *WFS1*-linked diseases. The immunopositivity in adult human tissues suggests that it may function maintaining physiological cellular homeostasis.

## 2. INTRODUCTION

Wolfram Syndrome (WS), also called with the acronym DIDMOAD (diabetes insipidus, diabetes mellitus, optic atrophy and deafness; 1-3) is a rare autosomal recessive disorder characterized by juvenile onset insulin deficient diabetes mellitus, neurodegeneration (1) and optic atrophy (1, 4). About 60% of the patients shows various degrees of hearing impairment by 20 years of age (5, 6), and frequently endocrinological, psychiatric, and urological symptoms (5, 7). The variety and multitude of symptoms are consistent with the disease representing a progressive neurodegenerative disorder affecting the central and peripheral nervous systems, such as ataxia, nystagmus, peripheral neuropathies and mental retardation (8, 9). WS patients usually die from central respiratory failure as a result of brainstem atrophy and/or from complication of urinary tract atony in their third or fourth decade (1, 10).

The *WSF1* gene, discovered in 1998 (2, 3), maps to chromosome 4p16.1 and contains eight exons spanning 33.4 kb of genomic DNA, of which exon 1 is noncoding (11). The product of the *WFS1* gene, wolframin, is an integral, endoglycosidase H-sensitive membrane

glycoprotein of 890 amino acids with a molecular weight of about 100 kd (9) and primarily localizes at the endoplasmic reticulum (ER) (12, 13). Secondary structure predictions identify three structural domains: a hydrophobic central domain comprising 9–10 membrane-spanning segments flanked by a hydrophilic domain at the N-terminus in the cytosol and a hydrophilic carboxy tail in the ER lumen (2, 3, 14, 15). Subsequent functional studies showed that wolframin is important in the regulation of intracellular $Ca^{2+}$ homeostasis (12, 16), contributes to cell cycle progression (17), and it is produced under conditions of troubled homeostasis by the activation of ER-stress/unfolded protein response (UPR) pathways (15, 17-22). Proteins with sequence similarity are now found in public databases of other organisms, but little is known about their functions, suggesting that wolframin belongs to a novel family of multispanning transmembrane proteins (14, 18).

Several studies have demonstrated that complete inactivation of the gene on both alleles is the cause of WS (2, 3, 23). Mutations studies in *WFS1* have identified a broad range of mutations, including nonsense, missense and splice variations (2, 3, 6, 24, 25) and it has been shown that these mutations are distributed over the entire coding region and usually result in loss of function of the encoded protein (7, 26). Heterozygous mutations in the *WFS1* gene have also been associated with autosomal dominant low frequency sensorineural hearing loss (AD-LFSNHL) (3, 27-29). The corresponding mutations are mainly missense mutations in exon 8, probably resulting in gain of function of the protein (30, 31). Furthermore, to further emphasize the pleiotropic effects of impaired wolframin function, mutations in *WFS1* have also been found in association with a dominant hearing loss and optic atrophy syndrome (5, 32), as well as in patients with increased predisposition to autoimmune diseases (33) and neuropsychiatric disorders (11).

Only through a deeper understanding of the normal biological functions of wolframin we can obtain important informations of its clinical implications in human disorders. However, functional studies of a protein like wolframin, which has such a heterogeneous array of related manifestations, are best directed if preceded by a careful characterization of protein expression patterns in developing and adult normal tissues. Therefore, to gain insight into the pathophysiology of wolframin-related diseases, in the present study, we have first characterized a polyclonal wolframin antibody in order to test its specificity. We have then defined the immunohistochemical localization and distribution of wolframin in normal foetal human tissues from the $14^{th}$ until the $35^{th}$ week of development and in normal adult human tissues.

## 3. MATERIALS AND METHODS

### 3.1. Generation of a polyclonal antibody against wolframin

The rabbit polyclonal immune serum against wolframin was produced by immunizing rabbits matching the wolframin protein sequence. One epitope (EQDKIEPPRAPRPQAD) corresponds to the N-terminal (amino acids 39-49) region of protein sequence. The second epitope (KVFQDSKAWENFRTLTD) recognizes the central region of the protein (amino acids 364- 381). The third epitope (QLSPARRHVKIEQDWR) corresponds to the C-terminus of wolframin (amino acids 852-868). These epitopes were used from a company (Invitrogen corporation, Carlsbad, CA, USA) to generate rabbits polyclonal antibodies following standard procedure used to generate antibody. In order to evaluate the title of immunoglobulin against the peptide used, the serum from the each group of rabbits immunized with the three epitopes, was tested with ELISA. The serum with the higher title, corresponding to the antibody directed against the N-terminal region of the protein, was used for the biochemical characterization (Dot Blot and Immunoblotting).

### 3.2. Dot blot

Dot blots were used as qualitative assay for antibody characterization. The peptides, complementary at the N- terminal, medium and C-terminal region of the wolframin protein sequence, were dissolved in the TBS buffer (150 mM NaCl, 20 mM Tris-HCl pH 8) and spotted directly on the nitrocellulose membrane. The membrane was incubated with 1:2000 wolframin rabbit polyclonal antibody for 30 min at room temperature. After washing with 0.1% Tween-20 TBS, the filter was incubated with 1:5000 peroxidase-conjugated anti-rabbit immunoglobulins for 30 min at room temperature. It was extensively washed and finally analyzed using the ECL system.

### 3.3. Human cell lines

All the human cell lines used to perform the western blot analysis have been purchased from ATCC (LGC Standards Middlesex 0LY UK): human stomach (AGS), human biphasic mesothelioma (MST0-211H), human melanoma (Colo-38), human liver (NeHepLxHT), human placenta (BeWo).

### 3.4. Protein extraction and western blotting analysis

The cells were lysed in lysis buffer [50mM Tris-HCl (pH7.4), 5mM EDTA, 250mM NaCl, 50mM NaF, 0.1% Triton X-100, 0.1mM Na3VO4, 1mM phenylmethylsulfonyl fluoride and 10 ug/ml leupeptin] for 30min in ice. Total extracts were cleared by centrifugation for 30 min at 4°C at 10000 rpm and assayed for the protein content by Bradford's method. Fifty µg of protein from each cell lysates were separated by a 8% SDS-PAGE and transferred to PVDF membranes and the filters were stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. Blots were blocked overnight with 5% non-fat dry milk, and then incubated with wolframin rabbit polyclonal antibody, in TBS (150 mM NaCl, 20 mM Tris-HCl pH 8) for 2 h at room temperature at a 1:500 dilution. After washing with 0.1% Tween-20 PBS, the filter was incubated with peroxidase-conjugated anti-rabbit immunoglobulins for 1 h at 22°C at a 1:5000 dilution, extensively washed, and analyzed using the ECL system (Amersham, Milan, Italy). Hsp70 (Heat Shock Proteins) antibody (Santa Cruz, CA) was used to estimate equal protein loading.

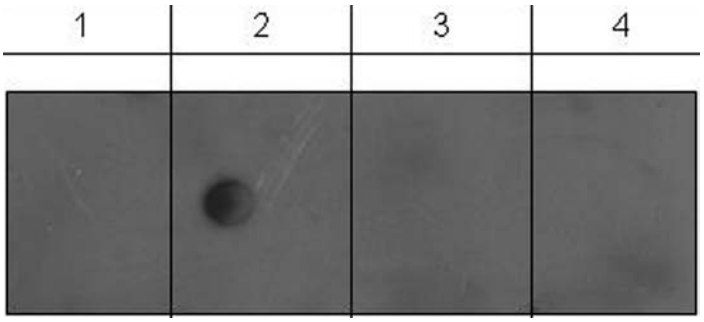


**Figure 1.** Dot Blot. The peptides, dissolved in TBS buffer, were spotted directly of the nitrocellulose membrane. The membrane has been incubated with anti wolframin antibody.1: TBS buffer, 2: peptide complementary at N-terminal region, 3: peptide complementary at Medium region, 4: peptide complementary at C-terminal region

### 3.5. Normal foetal and adult human tissues

Normal human foetal tissues from autopsy after spontaneous or therapeutic abortion and normal human tissues from autopsy were obtained from the Section of Anatomic Pathology of the Second University of Naples, Italy. Tissues were formalin-fixed and paraffin-embedded. Representative sections of each specimen were cut at a thickness of 3 μm and stained with hematoxylin-eosin and were examined by a pathologist to confirm the histological preservation of the microanatomic structure. For each tissue examined, at least three specimens from two different individuals were analyzed.

A panel of different human foetal tissues from the $14^{th}$ to the $35^{th}$ week of development was studied. Human tissue arrays (HA1, HB1, HC1 fetus, 14-35 weeks, AA9 adult tissues, Superbiochips, Korea) were also used. Protocols involving human tissues in this research were approved by our institutional human research studies committee.

### 3.6. Immunohistochemistry

Immunohistochemistry was carried out essentially as described previously (34, 35). Briefly, all sections were deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all the subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide and blocked with PBS-6% non-fat dry milk (Biorad) for 1 hr at room temperature. Slides then were incubated at 4°C overnight with an affinity-purified rabbit polyclonal immune serum raised against wolframin at a 1:100 dilution and then with diluted anti-rabbit biotinylated antibody (Vector Laboratories) for 1 hr. All the slides then were processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Novared (Vector Laboratories) was used as the final chromogen and hematoxylin was used as the nuclear counterstain. Negative controls for each tissue section were prepared by substituting the primary antiserum with the respective pre-immune serum. All samples were processed under the same conditions.

Observations were performed using a Zeiss Axioskop microscope; images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system and Adobe Photoshop. The expression level of wolframin-stained cells per field (250 X) at light microscopy was calculated and compared in different specimens by two separate observers (A.B. and F.B.) in a double blind fashion and described as: absent (-); very low (+/-); low (+); moderate (++); high (+++). Any disagreement was resolved by reevaluation of the sections and achievement of a consensus between the observers.

## 4. RESULTS

### 4.1. Antibody characterization

Antibody characterization was performed by the qualitative assay Dot Blot. Three peptides, complementary at the N- terminal, Medium and C-terminal region of the wolframin protein sequence, were spotted directly on the nitrocellulose membrane. The membrane was incubated with wolframin rabbit polyclonal antibody. This antibody specifically recognized the N-terminal region of wolframin sequence (Figure 1).

### 4.2. Western blot analysis

To further verify the specificity of wolframin antibody, we performed a western blot analysis using different cell lines. We observed that wolframin antibody recognized a protein of a molecular mass of ~100 kDa (Figure 2). Moreover, we observed modulation of wolframin expression among different cell lines. Particularly, we showed intense wolframin expression in a human liver cell line (NeHepLxHT), moderate wolframin expression in both human stomach (AGS) and human placenta (BeWo) cell lines. Very low expression was observed in a human melanoma cell line (Colo-38).

**Table 1.** Wolframin expression in human fetal tissues

| Tissue | | | Degree of Expression | | |
|---|---|---|---|---|---|
| | | | 15th | 25th | 35th |
| **Skin** | | | | | |
| Hair follicles | | | ++ | +++ | +++ |
| Epidermis | basal layers | | + | ++ | ++ |
| | | mature layers | + | +++ | +++ |
| **Respiratory system** | | | | | |
| Bronchus | | epithelia | - | +/- | ++ |
| | | glands | - | +/- | ++ |
| Pneumocytes | | | - | +/- | ++ |
| **Gastrointestinal system** | | | | | |
| Tongues | | epithelia | +/- | ++ | +++ |
| | | muscles | +/- | ++ | +++ |
| Stomach | | epithelia | +/- | +/- | +/- |
| | | muscles | +/- | ++ | +++ |
| Small intestine | | epithelia | + | ++ | ++ |
| | | muscles | + | + | + |
| Colon | | | + | ++ | +++ |
| Liver | | hepathocytes | ++ | ++ | +++ |
| Pancreas | | esocrine | - | ++ | +++ |
| | | endocrine | - | + | +++ |
| **Urinary system** | | | | | |
| Kidney | | glomeruli | +/- | +/- | +/- |
| | | proximal tubules | +/- | +/- | ++/- |
| | | distal tubules | +/- | +/- | +/- |
| | | collecting ducts | +/- | +/- | +/- |
| **Endocrine system** | | | | | |
| Adrenal gland | | cortical | - | - | - |
| | | chromaffin | - | - | + |
| **Cardiovascular system** | | | | | |
| Myocardium | | | - | +/- | ++ |
| **Blood and lymphoid tissue** | | | | | |
| Spleen | | | + | + | ++ |
| Thymus lymphocytes | | | +/- | ++ | ++ |

- : absent, +/- : very low, + : low , ++ : moderate, +++: high

**4.3. Wolframin localization in foetal human tissues**

Wolframin showed variable tissue distribution and several levels of expression in the examined organs (Table 1). In the integumental system, wolframin had low immunoreactivity in the epithelial layers of the skin at the 18$^{th}$ week (fig. 3 a) and progressively increased to a high level, localizing mainly to the outer layers of epidermis from the week 24 (Figure 3 b). In addition, beginning from the 24$^{th}$ gestational week until to the 35$^{th}$ week, we observed moderate/high wolframin immunoreactivity in the hair follicles, in the adipose tissue, and in muscle fibers of the dermis (Figure 3 c).

In the muscular system, wolframin was expressed at very low level in the striated fibers at the 18th week (Figure 3 d). Its level of expression progressively increased to a moderate level at the 26th week (Figure 3 e), reaching a high expression level at the 35th week (Figure 3 f). In the respiratory system, lungs appeared completely negative at 14-16 weeks when it was characterized by a glandular phase (Figure 3 g). Beginning from the 28th week (Figure 3 h), the alveolar epithelium showed moderate wolframin immunoreactivity until the 31st-35th weeks (Figure 3 j). In the cardiovascular system, the cytoplasm of cardiomyocytes exhibited very low wolframin immunopositivity at the 21st week (Figure 3k); immunoreactivity increased to moderate levels of expression at the 26th week (Figure 3 l) until reached higher expression levels at the 33-35th gestational week (Figure 3 m).

In the digestive system, wolframin was expressed at very low levels in the epithelium and striated muscles of the tongue at 14-16 weeks (Figure 4 a). The immunoreactivity increased to a moderate level at the 24th week with a localization in epithelial cells of the tongue (Figure 4 b). Then wolframin expression markedly increased to high levels at the 33-35th weeks in the outer layer of the epithelium and in the cytoplasm of striated muscle fibers (Figure 4 c). In the stomach, very low wolframin immunopositivity was almost exclusively localized to the smooth muscles beginning from week 18 (Figure 4 d), and reached high levels from the 24th (Figure 4 e) to the 28th week (Figure 4 f). In the small intestine, low wolframin immunoreactivity was present in the smooth muscles at the 14th week (Figure 4 g), whereas moderate expression levels were seen in the epithelial lining from gestational week 24 (Figure 4 h) until the 35th week (Figure 4 j). In the colon, low wolframin immunoreactivity was observed at the 18th week in the epithelium, becoming moderate to high at the 33-35th week. In the liver, low/moderate wolframin expression was seen in the hepatocytes at the 18th gestational week (Figure 4 k). This immunoreactivity increased to a moderate level at the 21st week (Figure 4 l), until reached a high expression level at the 33-35th weeks (Figure 4 m).

No immunoreactivity was observed in the pancreas between the 14th and 18th week. Moderate wolframin

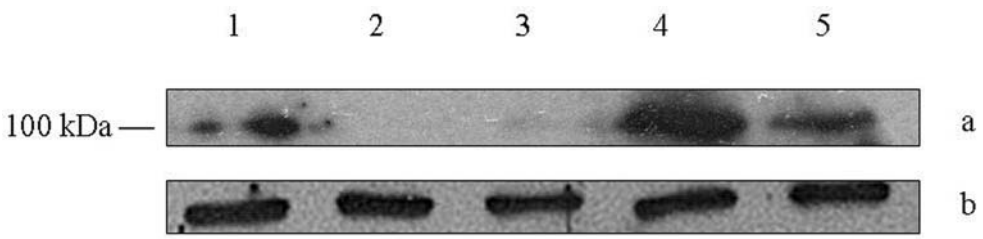


**Figure 2.** Western blot analysis of wolframin antibody. (a) pattern of expression of wolframin in different human cell lines; a representative panel of different human cell lines including: lane 1 – human stomach (AGS), lane 2 – human biphasic mesothelioma (MST0-211H), lane 3 – human melanoma (Colo-38), lane 4 – human liver (NeHepLxHT), lane 5 – human placenta (BeWo); is shown. (b) HSP70 was used for normalization of the protein amounts loaded.

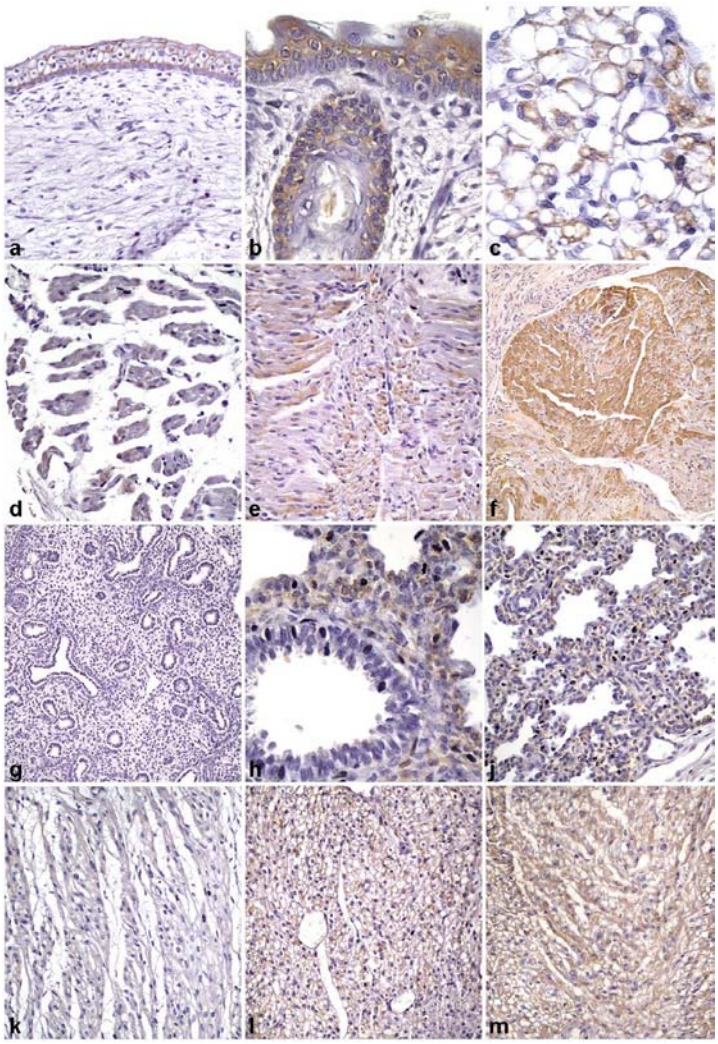


**Figure 3.** (a) wolframin low expression in the epithelial layers of the skin at the 18th week, 150 X; (b) high wolframin expression in the outer layers of epidermis and in the hair follicles at the 24th week, 640 X; (c) moderate wolframin expression in the adipose tissue of the skin at the 33rd week, 640 X; (d) wolframin localization at very low level in the striated fibres at the 18th week, 300 X; (e) moderate wolframin expression in the muscle at the 26th week, 300 X; (f) high wolframin level in the muscle at the 35th week, 150 X; (g) negative wolframin expression in the lung at the 14th week, 150X; (h) moderate wolframin expression in the alveolar epithelium of the lung at the 28th week, 640 X; (j) moderate expression for wolframin in the lung at the 31st week, 300 X; (k) very low wolframin expression in the heart at the 21st week, 300 X; (l) moderate expression for wolframin in the heart at the 26th week, 300 X; (m) high wolframin expression in the heart at the 33rd week, 300 X.

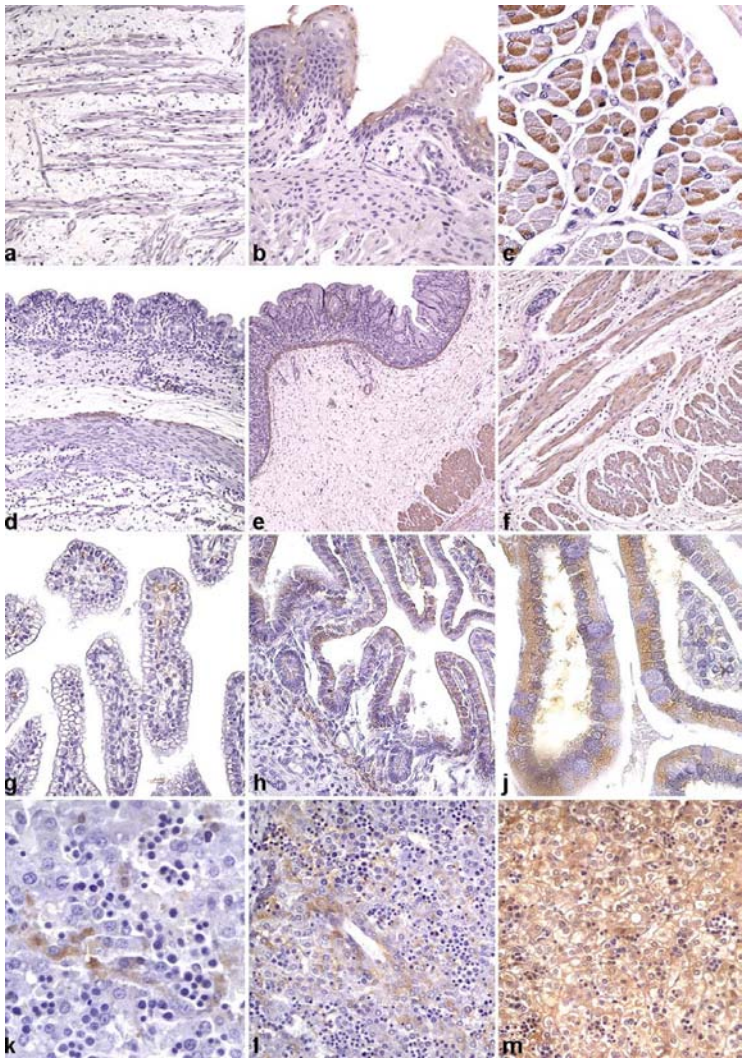


**Figure 4.** (a) wolframin very low expression in the epithelium and muscles of the tongue at the 14th week, 150 X; (b) moderate wolframin expression in the outer layers of tongue epithelium at the 24th week, 300 X; (c) high wolframin expression in the muscles of the tongue at the 33rd week, 640 X; (d) wolframin very low expression in the smooth muscles of the stomach at the 22nd week, 150 X; (e) moderate wolframin expression in the smooth muscles of the stomach at the 24th week, 300 X; (f) high wolframin level in the smooth muscles of the stomach wall at the 28th week, 150 X; (g) low wolframin expression in the stroma of small intestine villi at the 14th week, 300X; (h) moderate wolframin expression in the epithelium of the small intestine at the 24th week, 300 X; (j) moderate expression for wolframin in the epithelium of the small intestine at the 35th week, 640 X; (k) low/moderate wolframin expression in the liver at the 18th week, 640 X; (l) moderate expression for wolframin in the hepatocytes at the 21st week, 300 X; (m) high wolframin expression in the liver at the 35th week, 300 X.

expression started only at the 19-20th weeks with localization at the ductal and peri-insular level (Figure 5 a). Expression decreased at these loci at the 28th week (Figure 5 b) until the 33rd-35th week, when moderate wolframin immunopositivity became detectable in the exocrine portion of the pancreas (Figure 5 c). In the remainder of the endocrine system, other than high wolframin immunoreactivity localized in the pancreatic islets at the 35th week (Figure 5 d), we observed no wolframin immunopositivity in the adrenal gland at the 14th week (Figure 5 e). Wolframin was low expressed only in the chromaffin cells of adrenal gland medulla beginning from the 28th gestational week (Figs. 5 f).

In the urinary system, kidneys appeared almost completely negative for wolframin expression between the 14th and the 24th week of gestation (Figure 5 g, h), whereas low wolframin immunoreactivity was observed in the basal part of the proximal tubules from the 31st to the 35th week (Figure 5 j).

Finally, in the lymphoid system, the spleen showed a low wolframin immunopositivity localized in rare intravascular elements at the 14-16th weeks and in the smooth muscles at the 19th week (Figure 5 k). Wolframin immunoreactivity became moderate in the smooth muscles of the spleen beginning from the 31-32nd weeks (Figure 5

**Table 2.** Wolframin protein expression in normal adult human tissues

| Tissue | | | Degree of Expression |
|---|---|---|---|
| **Skin** | | | |
| Hair follicles | | | - |
| Sweat glands | | | - |
| Sebaceous glands | | | - |
| Epidermis | basal layers | | + |
| | | mature layers | - |
| **Respiratory system** | | | |
| Bronchus | | epithelia | +/- |
| | | glands | - |
| Pneumocytes | | | - |
| Mesothelium | | | - |
| **Gastrointestinal system** | | | |
| Salivary glands | | | - |
| Esophagus | epithelia | | ++ |
| | | muscles | + |
| Stomach | | epithelia | - |
| | | muscles | + |
| Small intestine | | | + |
| Large intestine | | | + |
| Gall bladder | | | - |
| Liver | | hepathocytes | ++ |
| Pancreas | | esocrine | - |
| | | endocrine | + |
| **Endocrine system** | | | |
| Thyroid | | | ++ |
| Adrenal gland | | cortical | ++/- |
| | | chromaffin | - |
| **Urinary system** | | | |
| Kidney | | glomeruli | + |
| | | proximal tubules | + |
| | | distal tubules | ++ |
| | | collecting ducts | ++ |
| Bladder | | | +/- |
| Prostate | | | +/- |
| **Reproductive system** | | | |
| Breast | | epithelia | - |
| Uterus | | proliferative endometrium | - |
| | secretive endometrium | | + |
| Salpinx | | | - |
| Vagina | | epithelia | +++/- |
| Testis | | Leydig cells | ++/- |
| | | germ cells | - |
| Ovary | | granulosa | - |
| | | germ cells | - |
| **Cardiovascular system** | | | |
| Myocardium | | | - |
| **Blood and lymphoid tissue** | | | |
| B lymphocytes | | | - |
| T lymphocytes | | | - |
| Spleen | | | - |
| Thymus lymphocytes | | | - |
| **Nervous system** | | | |
| Astrocytes | | | - |
| Oligodendroglia | | | - |
| Microglia | | | - |
| Purkinje cells | | | ++/- |
| Neurons | | | ++/- |

- : absent, +/- : very low, + : low , ++ : moderate, +++: high

l). In the thymus, moderate wolframin immunoreactivity was observed in the Hassel corpuscles beginning from gestational week 19-20th (Figure 5 m).

### 4.4. Wolframin localization in adult human tissues

Wolframin showed variable tissue distribution and several levels of expression in the examined human adult tissues (Table 2). In the integumental system, we observed that epithelial cells, either from simple or stratified epithelia, showed variable positive staining for wolframin depending on examined organs. In the skin, we observed low wolframin immunoreactivity confined almost exclusively to the basal layer of epidermis, compared to undetectable levels in the outer layers (Figure 6 a). Vice versa, in the other stratified epithelia, such as the esophagus and the cervix (see below), we observed moderate levels of immunoreactivity for wolframin in the mature and outer layers.

In the muscular system, we observed moderate wolframin immunopositivity localized to the cytoplasm of striated muscle fibers (Figure 6 b). Unlike striated muscles, in the cardiovascular system, no detectable wolframin immunoreactivity was observed in the heart. In the respiratory system, we observed very low wolframin immunoreactivity only in the outer layers of the nasal mucosa, whereas wolframin immunoreactivity in the bronchi and lungs was virtually undetectable (Figure 6 c). In the gastrointestinal system, no wolframin immunoreactivity was observed in the salivary glands and gallbladder. In contrast, moderate wolframin

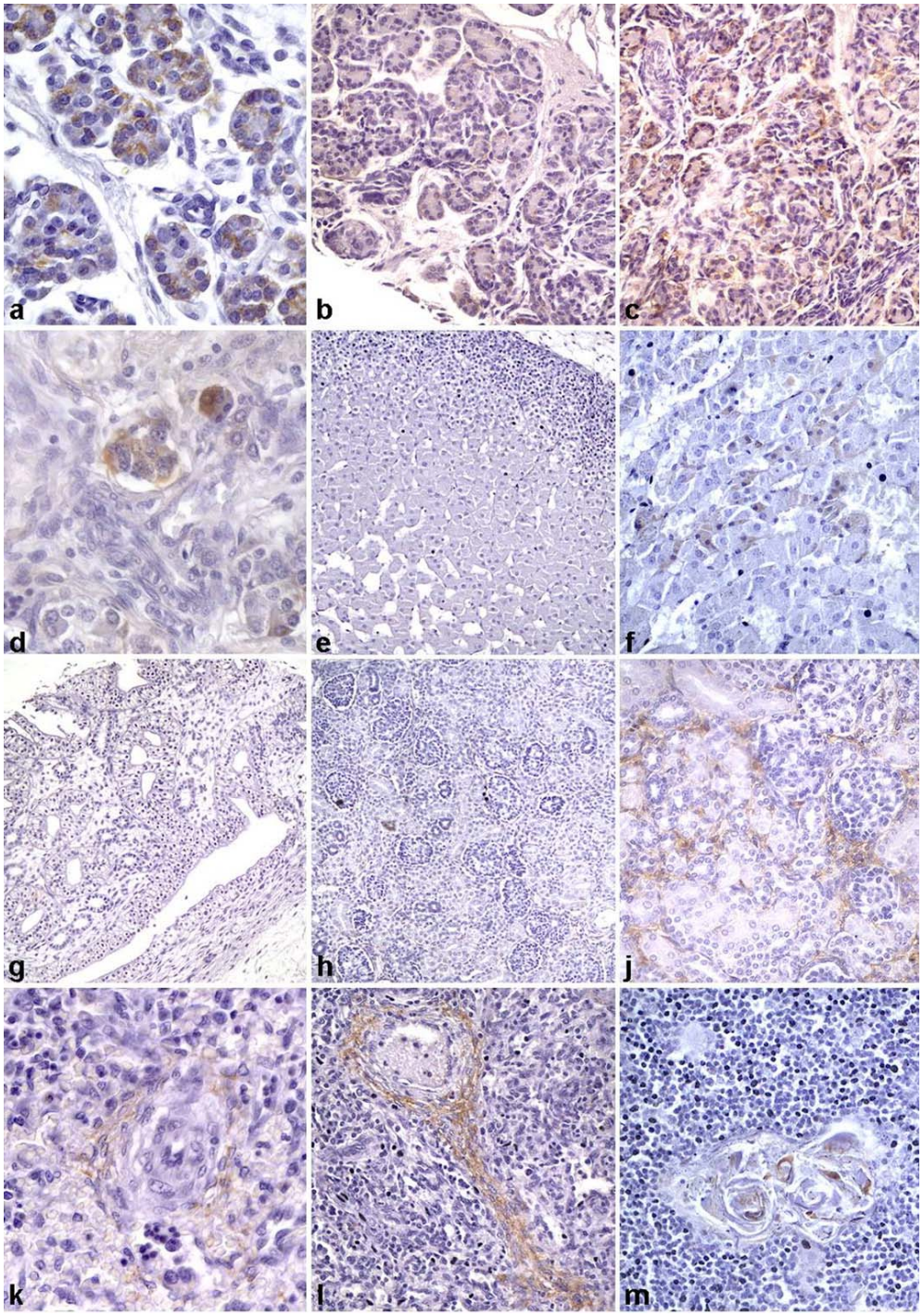


**Figure 5.** (a) moderate wolframin expression in the pancreatic acina at the 19$^{th}$ week, 640 X; (b) low wolframin expression in the exocrine pancreas at the 28$^{th}$ week, 300 X; (c) moderate wolframin expression in the pancreas at the 35$^{th}$ week, 300 X; (d) wolframin localization at a high expression level in the pancreatic islets at the 35$^{th}$ week, 640 X; (e) absent wolframin expression in the adrenal gland at the 14$^{th}$ week, 150 X; (f) faint wolframin level in the chromaffin cells of the adrenal gland medulla at the 28$^{th}$ week, 640 X; (g) absent wolframin expression in kidney at the 19$^{th}$ week, 150X; (h) negative expression for wolframin in the kidney at the 24$^{th}$ week, 150 X; (j) low expression for wolframin in the kidney tubules at the 35$^{th}$ week, 300 X; (k) low wolframin expression in the smooth muscles of the spleen at the 19$^{th}$ week, 640 X; (l) moderate expression for wolframin in the smooth muscles of the spleen at the 33$^{rd}$ week, 300 X; (m) moderate wolframin expression in the Hassle corpuscles of the thymus at the 24$^{th}$ week, 640 X.

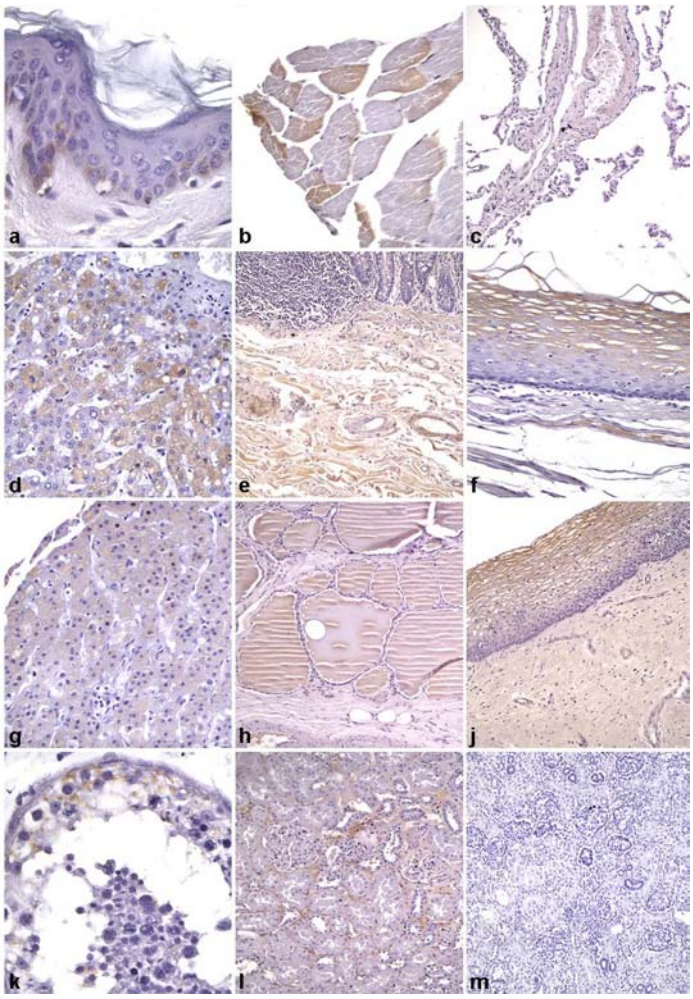


**Figure 6.** (a) low wolframin expression in the cytoplasm of cells lining the basal layer of the skin, 640 X; (b) moderate expression of wolframin in the striated muscle fibers, 300 X; (c) very low wolframin expression localized in the smooth muscles of lung, 150 X; (d) moderate immunopositivity for wolframin localized in the cytoplasm of hepatocytes, 300 X; (e) moderate wolframin immunostaining in the smooth muscles of colon, 150 X; (f) moderate wolframin expression observed in the outer layers of esophagus, 300 X; (g) low immunopositivity for wolframin localized in steroidogenic cells of fascicolata region of adrenal gland, 300 X; (h) moderate wolframin expression in thyroid colloid, 150 X; (j) moderate/high wolframin immunostaining in the outer layers of cervix epithelium, 150 X; (k) low wolframin immunopositivity localized in the Sertoli cells of testis, 640 X; (l) moderate wolframin expression level observed in the basal portion of kidney tubules together to a low expression for wolframin localized in the remaining part of kidney, 150 X; (m) representative negative control of kidney showed no immunostain, 150 X.

immunopositivity was found in hepatocytes (Figure 6 d). In the gastrointestinal tract, low wolframin immunoreactivity was localized almost exclusively in the smooth muscles of esophagus, stomach, duodenum, small bowel, and rectum, whereas wolframin was observed at moderate level of expression in the smooth muscles only in the colon (Figure 6 e). Moderate expression levels were observed in the mature and outer layers of the esophageal epithelium (Figure 6 f), whereas no detectable wolframin expression was found in the epithelium of the stomach, gallbladder and colon. The exocrine portion of the pancreas showed no detectable levels of wolframin expression, whereas the endocrine portion of the gland showed a low wolframin expression.

In the endocrine system, we observed a low level of wolframin expression in the cytoplasm of steroidogenic cells of the fascicolata region of the adrenal gland (Figure 6 g). Moreover, very low expression levels were observed in the glomerulosa and reticularis regions. On the contrary, the chromaffin portion of the gland appeared to show no immunoreactivity for wolframin. In the thyroid gland, a moderate level of wolframin expression was observed in the colloid in contrast with a very low expression level found in the follicular cells (Figure 6 h).

In the female reproductive system, low wolframin expression level was found in the glandular

epithelium of the secretive endometrium, in contrast to an undetectable expression level found in proliferative endometrium. A moderate/high wolframin level was observed in the outer epithelial layers of vaginal cervix (Figure 6 j). On the contrary, no detectable wolframin expression was found in the breast epithelium, salpinx, ovary, and myometrium. In the male reproductive system, a low wolframin expression level was found in the Sertoli cells (Figure 6 k) of the testis and in the epithelium of seminal vescicle, whereas an undetectable expression level was observed in the male germ cells.

In the urinary system, wolframin was expressed at a moderate level in the basal portion of both distal and collecting tubules of kidney cortex (Figure 6 l), while a low expression of the protein was shown in the proximal tubules and in the glomeruli. In the prostate and bladder, wolframin was expressed at a very low level, almost exclusively in the smooth muscles, whereas epithelia do not express the protein.

In the lymphoid system, no wolframin immunoreactivity was observed in several tissues, such as lymph nodes, spleen, tonsils, and thymus.

Figure 6 m shows a representative negative control of human kidney obtained by substituting the primary antiserum with the respective pre-immune serum.

## 5. DISCUSSION

Wolfram syndrome (WS) is a rare neurodegenerative disorder involving the central nervous system, peripheral nerves, neural sensory organs, and neuroendocrine tissue (8, 36, 37).

WSF1 protein, also called wolframin, is a hydrophobic protein consisting of 890 amino acids with a molecular mass of ~ 100 kDa. This protein is a type II membrane protein with nine putative transmembrane segments (14) and large hydrophilic regions at both termini. Wolframin localizes primarily at the endoplasmic reticulum (ER) in a $N_{cyt}/C_{lum}$ membrane topology (3, 13, 16, 21). Localization of wolframin in the ER allowed researchers to suggest that wolframin might play a role in membrane trafficking, protein processing, or regulation of ER calcium homeostasis (13, 14).

Although several studies have demonstrated the association of *WFS1* mutations with various phenotypes, including WS, the physiological functions of wolframin are still incompletely understood.

It has also been demonstrated that disruption of the *WFS1* gene in mice causes progressive beta-cell loss within the pancreas and impaired stimulus-secretion coupling in insulin secretion (18, 38). Recent studies in mice have demonstrated that the loss of wolframin expression leads to increased ER stress and induces cell death, especially in pancreatic beta cells (15, 17, 20, 21). This suggests that wolframin has a role in the ER stress response of normal cells. Based on this observation, a possible role of wolframin in protein biosynthesis, modification/folding, trafficking and/or regulation of $Ca^{2+}$ homeostasis has been suggested (12, 14).

Lastly, it has been suggested that accumulation of functional wolframin might also be critical for its function in several cells (14). Recent studies demonstrated that *WFS1* expression can be increased in certain tissues like the pancreas by increasing ER stress, but not in other tissues such as heart and skeletal muscle (39). Thus, it is possible that other tissues increase production of different proteins to combat ER stress, while the tissues affected by WS normally respond by increasing *WFS1* gene or protein expression levels (7).

Previous observations based on mRNA levels have demonstrated that *WFS1* is differently expressed in various tissues (2, 3). Studies of wolframin immunohistochemical expression in non-human pancreatic (9), cochlear (36), brain (9, 13) and ocular tissues (38) have been performed, documenting localization in cell populations and structures consistent with the classical WS phenotypic manifestations. However, little information is available about the localization of wolframin in other tissues, and little to no information is available for human tissues. In order to gain additional insight into the pathophysiology of *WFS1*-linked disorders, we undertook a study to characterize the expression patterns of wolframin in foetal and adult human tissues.

In the present study, we observed that wolframin was ubiquitously expressed in many human foetal organs and tissues. Wolframin expression was very low at the 14-16$^{th}$ week of gestation in most organs and tissues, and increased progressively during subsequent developmental stages. Intense wolframin immunoreactivity was observed in both striated and smooth muscle fibers. In addition, moderate expression levels were observed in both simple and stratified epithelia in all the organs examined in our study. These data suggest that wolframin is probably involved in the maintenance of a differentiated cell state in some tissues such as muscles and epithelia.

Our data confirm only in part the wolframin expression profiles observed in mouse tissues using antibodies raised against the hydrophilic N- and C-termini of the protein (14), unveiling previously unappreciated inter-species differences between human and mouse tissues. Specifically, we confirmed high levels of expression in muscles and the low immunoreactivity levels found in kidney and spleen seen in mouse (14). Protein levels found in the respective tissues generally correlate well with the transcript levels detected by northern blot analyses (2, 3, 14). Unlike this, we observed an intense wolframin expression in the human foetal colon and liver beginning from the 33$^{rd}$ week of gestation, which is in contrast to smaller amounts in liver found in mouse (14).

Moreover, we showed a unique pattern of expression in pancreas since the wolframin expression was faint at the 19-20$^{th}$ week and localized in the ductal and

peri-insular level. This immunoreactivity disappeared until the 35th week of gestation, when we observed intense wolframin immunoreactivity localized in endocrine islets of pancreas. These data confirm the northern blot analysis of pancreas showing that the WFS1 expression is restricted to pancreatic islet cells and is absent in the exocrine pancreas (2, 14), and are consistent with the diabetogenic effect of *WFS1* mutations. Recently, Xu *et al.* (40), using a gene array, have demonstrated that *WSF1* was highly expressed in pancreatic islet cells at E15.5 and E18.5 of mouse development and during the later mouse embryonic development when the pancreatic cells exhibit dramatic growth, differentiation and proliferation (40).

Therefore, taken together, our immunohistochemical expression data from human foetal pancreas and liver suggest that wolframin plays an important functional role both in pancreatic β-cells and hepatocytes, and support the contention that loss of wolframin function contributes to ER stress, which in turn may result in metabolic disorders such as the pancreatic β-cell damage that is characteristic of Wolfram syndrome (14).

We documented that wolframin is expressed in many adult human tissues and organs, although with different tissue distribution and expression levels. In particular, moderate wolframin expression levels were found in liver, adrenal glands, and kidney, which are organs that are not known to be affected either in WS or other *WFS1*-linked conditions. Our results in adult human tissues are in agreement with results obtained on human foetal tissues in which we observed a moderate/high wolframin expression in colon and liver beginning from the 33rd week of gestation. We observed that, as in foetal tissues, in the adult human pancreas wolframin expression exhibited moderate levels of expression in the endocrine portion of this organ. These results confirm the high wolframin level found in the postneonatal period in β-cells (40). These results are consistent with the role of *WFS1* mutations in diabetes mellitus, alone and within WS, and are in agreement with previous studies that demonstrated that *WFS1*-deficient pancreatic islets exhibited impaired insulin secretion in response to glucose, which was restored by re-expression of wolframin (9, 18, 19). In addition, wolframin overexpression in wild-type pancreatic islets resulted in an increase in glucose-induced insulin secretion (18). Therefore, it is possible that wolframin may be involved directly in the regulation of insulin secretion, conceivably by increasing apoptotic rate in β-cells. Moreover, since wolframin attenuates ER stress, maintains cell cycle progression and represses apoptotic pathway, specifically in pancreatic β-cells, it is possible to hypothesize its role in the maintenance of β-cells mass by balancing β-cells growth (differentiation and proliferation) and β-cells death (apoptosis) during pancreatic remodeling (40).

Lastly, recent studies have also shown that loss of wolframin function results in male sterility in wfs1 knock-out mice (41). This finding correlated with loss of Sertoli's cells and reduced spermatogonia. Our finding of physiologic expression of wolframin in Sertoli's cells in adult human tissues is in line with the findings by Noormets *et al.* (41), provides further explanation for the reported cases of hypogonadism in WS, and points to defective Sertoli's cell function as the main site for reduced fertility in human male carriers of *WFS1* mutations.

In conclusion, the presence of wolframin in most human normal foetal and adult tissues suggests that this protein may have a broad and complex range of functions in addition to regulation of glucose homeostasis in the endocrine pancreas and ion homeostasis hearing in the cochlea. Additional studies, including detailed investigations of ocular tissues, will be necessary to fully characterize the spectrum of functions subserved by wolframin and better comprehend the protean clinical manifestations associated with *WFS1* mutations.

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