Running title

WT1 in neurons

Wilms' Tumor Gene 1 (WT1) overexpression in neurons in deep endometriosis: a pilot study

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Capsule

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Abstract

Innervation of deep endometriosis has recently been linked to its severe pain symptoms. We could demonstrate for the first time that WT1 is overexpressed in part of these nerves.

Key words: Wilms' tumor gene 1 (WT1), endometriosis, nerve growth factor (NGF)

Wilms' tumor gene 1 (WT1) is located on chromosome 11p13. It has many molecular functions (1), which are partially explained by different splicing of WT1 RNA, resulting in 36 protein isoforms. WT1 is thought to have a role in the regulation of transcription, RNA metabolism (possibly splicing) and in translation. It has a central role in embryonic development, for example in developing kidneys (2) while its overexpression in several malignancies suggests a role in tumorigenesis (3).

Experimental evidence to support a role for WT1 in neuron development is provided by Wagner *et al*, showing deficient formation of retinal ganglia and of the olfactory epithelia in WT1-deficient mice (4-5). Also, WT1 is suggested to play a role in neuronal pathology. Since degenerating brain neurons of Alzheimer patients overexpress WT1, a role in apoptosis is suggested (6).

Deep endometriosis is a major cause of severe pelvic pain, dysmenorrhoea, deep dyspareunia, and dyschesia. Large lesions present as adenomyotic nodules with sparse but active endometrial stroma and glands in hyalinous material with smooth muscles proliferation. Deep endometriosis is clinically and morphologically different from peritoneal lesions and from cystic ovarian endometriosis (7). Based on nerve growth factor (NGF) expression, Anaf *et al* (8) concluded that deep endometriosis is attracted towards richly innervated anatomical sites. The infiltration of endometriosis into the nerves and its expression of NGF were put forward as an explanation of the severe pain symptoms.

Since WT1 is associated with neuronal pathology, a pilot study was performed to evaluate WT1 expression in deep endometriosis.

Paraffin embedded tissue samples from 10 patients with deep endometriosis were collected from our central tissue bank after approval of the local ethical committee. To investigate the specificity of WT1 staining, we also stained 7 malignant tumoral tissue samples and 5 peripheral nerves, isolated from the axilla during breast surgery (intercostobrachial nerve) or during para-aortic lymph node resection (sympathetic part of autonomic plexus). Non pathological brain tissue (n=5) was collected as well, to serve as a negative control (6).

All patient samples were stained for WT1 and S100 in serial slices. The latter is a calcium binding protein of 20-30kD. All slices were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by H_2O_2 in methanol. We then used a heat-induced epitope retrieval system. We manually incubated in water with citrate at PH 6 (90-98°C). The slides were cooled and in case of S100 buffered with PBZ (phosphate buffer) + Tween, twice. Monoclonal mouse anti-human Wilms' Tumor 1 clone 6F-H2 or polyclonal rabbit anti-human S100 (DAKO Belgium NV, Heverlee, Belgium) was used as primary antibody (dilution respectively 1:400 and

1:3000). Slides were incubated with the antibody at room temperature. Slides were then washed with buffer and incubated with respectively Envion+System-HRP Labelled Polymer anti-mouse and Envision detection system peroxidase/DAB, Rabbit/Mouse (DAKO Belgium NV, Heverlee, Belgium). Finally, slides were stained with a brown reaction product (DAB) to identify positive staining. Immunohistochemical staining was interpreted semi quantitatively. Intensity of the staining ranged from negative (0), weak (1), moderate (2) to strong (3). Of each patient sample, one tissue slide was evaluated. Examination of immunohistochemical staining was performed independently by 2 investigators (A C en Ph M).

Using S100 as the golden standard for nerve identification, 70% of deep endometriotic nodule samples showed WT1 staining in 5-70 % of the nerves. In 20% of samples (2/10) 100% of nerves were WT1 positive (Fig 1 a-d). In total, 90% of the deep endometriosis samples contain nerves that express WT1 (1 out of 10 samples had to be omitted because it did not contain nerves (negative S100 staining)). In all tumoral tissues samples, 65% of all nerves stained WT1 positive (Fig 1 e-f). Nerves in normal brain tissue were negative. The isolated peripheral nerves were negative or very weakly WT1 positive.

Comparing the endometriosis patients clinically, women with only part of the nerves being positive had a spontaneous menstrual cycle, whereas women with 100% WT1 nerve positivity used a GnRH antagonist or a progesterone in continuous setting, prior to surgery.

In this study, we demonstrated that all innervated deep endometriotic nodules contain WT1 positive nerves. In 7/10 samples, WT1 is not expressed in all nerves, stained by S100, a marker for glia cells, neurons, Schwann cells, melanocytes, Langerhans cells and reticulum cells of lymphoid tissue (DAKO guidelines). In 2/10 deep endometriotic samples on the contrary all nerves expressed WT1. This expression is not specific for deep endometriosis, since in tumoral tissue, nerves are also WT1 positive. The difference between these benign and malignant tumor samples is the percentage of positive nerves. In deep endometriotic nodules the percentage ranges from 5 to 70 % depending on the sample, whereas in tumoral tissue all samples have 65% positivity. Surprisingly, WT1 expression is almost absent in isolated peripheral nerves. Negative WT1 staining in non pathological brain tissue confirms previous results (6).

The inhomogeneous WT1 expression in endometriotic nodules can be explained by the underlying molecular mechanism in neuron development and differentiation.

The activity of nerve growth factor (NGF), a chemotaxin, is critically dependent on the expression of its two receptors, trkA and p75, of which trkA is the high-affinity tyrosine kinase receptor. It is known that trkA is widely expressed in developing and adult central and peripheral nervous system (9). In addition, Anaf *et al* (8) confirmed this theory in deep endometriosis: trkA was highly overexpressed in all nerves that were invaded and/or surrounded by endometriosis or that were near endometriotic lesions, whereas its expression was absent in the endometriotic tissue itself. NGF was on the other hand mainly expressed in the endometriosis tissue and weakly in the nerves. However, the presence of the chemotaxin NGF in the neighbourhood of its receptor causes binding, providing an explanation for the pain symptoms in these patients (nociception). This pathway was further investigated by Liu *et al* (10). They were able to demonstrate a link between NGF and WT1. If PC12 cells were treated with NGF, which initiates differentiation of these cells into neuronal cells, WT1 was downregulated, only if the pathway downstream from the receptor was intact (trkA, Src, Ras). This molecular finding may explain why we observed no (or very weak) WT1 expression in neurons of non-pathological brain tissue and isolated normal peripheral. Since trkA is so widely expressed in neuronal tissue, WT1 expression is suppressed. Consequently, we can assume that if the NGF-trkA-Ras/Src pathway fails, WT1 will rise. In brain tissue of Alzheimer patients, Lovell *et al* (6) also observed neuronal WT1 positivity, which led to their conclusion that these neurons underwent a WT1-mediated apoptosis.

For the mechanism that can interrupt this cascade and result in WT1 positive nerves in deep endometriosis we only can speculate. Oestrogens might contribute to this. Singer *et al* (11) demonstrated that estrogens positively regulate Src and MAPK (mitogen-activated protein kinase, activated when NGF binds trkA). Since GnRH (Gonadotrophin Releasing Hormone) agonists and antagonists are known to suppress oestrogen production. They have become a standard treatment of deep endometriosis associated pain. Although today the relationship between WT1 expression and pain in deep endometriosis can only be speculative, it is attractive to mention the downregulation of the NGF-trkA-Ras/Src pathway by decreasing estrogen concentrations (Singer et al (11)). Consequently, NGF can not exercise its nociceptive function. This may explain why in two patients the number of WT1 positive nerves was equal to the number of S100 nerves. As mentioned before, one of them was treated with a GnRH-antagonist for 5 months, the other one with lynestrenol during 12 months, both prior to surgery (and thus tissue sampling). The long lasting oestrogen depletion may have interrupted the cascade, causing a rise in WT1.

Our impression that WT1 overexpression is more uniform and pronounced in tumoral tissue might be due to the fact that organ innervation is changed during cancerous transformation. Neuronal changes in the Auerbach plexus of colorectal cancer have indeed been proven by Sobaniec-Lotowska *et al* in 2004 (12).

To conclude, our findings demonstrate that WT1 is selectively expressed in neurons of deep endometriosis which is a unique finding for a benign disease. It is suggested that this results from a defect in the NGF-trkA-Src/Ras pathway, possibly influenced by oestrogens. Anaf et al (8) found that NGF can be linked to pain in endometriosis. By our findings, we hope to reveal more of this pathway, based on Liu's findings that link NGF and WT1 (10). We are aware of the small sample size of this study and larger studies are needed to further explore a true clinical correlation. It would also be interesting to set up molecular studies to better clarify the relationship of WT1 and the pathophysiology of deep endometriosis.

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Legend to figure

Figure 1. Cytoplasmic immunohistochemical staining of S100 and WT1. (A) S100 staining
of a neuron in a deep endometriotic nodule. (B) Identical WT1 staining (same nerve as in
(A)). (C) S100 staining of a series of nerves in an endometriotic nodule. (D) Absence of
WT1 positivity in the same nerves as in (C). (E) S100 staining of the Auerbach plexus in
colorectal cancer. (F) Weaker, but present WT1 staining in the same nerves as in (E).