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No Oncogenic Role for WT1 in Peripheral Nerve Sheath Tumors

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ABSTRACT

This study aimed to investigate the protein and messenger RNA expression of Wilms' tumor gene 1 (WT1) in normal nerve root tissue and peripheral nerve tumors. The cauda equina nerve roots from 8 autopsy cases and tumor specimens consisting of 19 neurofibromas, 10 schwannomas, and 11 malignant peripheral nerve sheath tumors (MPNSTs) were studied with immunohistochemical staining, Western blotting, and real-time reverse transcription-polymerase chain reaction (RT-PCR). Immunohistochemical studies including double-immunofluorescence demonstrated the expression of WT1 protein in the cytoplasm of S-100-immunoreactive cells in all specimens examined. In Western blotting analysis, antibodies to WT1 protein yielded a 52-kDa band in all specimens examined. Scoring of WT1 protein immunostaining and semiquantitative analysis of WT1 messenger RNA with real-time RT-PCR revealed no significant differences between cauda equina, neurofibroma, Schwannoma, and MPNST. This study confirmed the cytoplasmic expression of WT1 protein in S-100-immunopositive Schwann cells of peripheral nerves, neurofibromas, and schwannomas as well as Schwann-like cells of MPNSTs. However, we did not find expression of WT1 to be significantly higher in peripheral nerve tumors than in normal peripheral nerves. These findings suggest that WT1 is independent of tumorigenesis and malignant transformation in neurofibroma, schwannoma, and MPNSTs. (Jikeikai Med J 2011 ; 58 : 95-102)

Key words : Wilms' tumor gene 1, S-100, Schwann cells, malignant peripheral nerve sheath tumor

INTRODUCTION

Wilms' tumor gene 1 (WT1) was originally identified as a tumor suppressor gene based on its mutational inactivation in many malignancies, including Wilms' tumor[1-5]. However, overexpression of the wild-type WT1 gene and protein in a variety of neoplasms, including leukemia[6], ovarian cancer[7], breast cancer[8], melanoma[9], mesothelioma[10,11] and desmoplastic round-cell tumor[12], together with the tumor-inhibiting effects of WT1-antisense oligomers suggest that WT1 plays an oncogenic role in tumor formation[13,14]. In normal tissues, WT1 plays important roles in cellular development, survival, and proliferation[15,16] WT1 is expressed in the mesothelium, glomerular podocytes, and mesangial cells of the kidney ; CD34-positive hematopoietic stem cells ; Sertoli cells of the testis ; stromal cells, surface epithelium, and granulosa cells of the ovary ; myometrium and endometrial stromal cells of the uterus [17,18] ; and peripheral nerves[19].

Neurofibroma is a well-demarcated intraneural or dif-

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fusely infiltrative extraneural tumor consisting of a mixture of 3 cell types : Schwann cells, perineurial-like cells, and fibroblasts. Schwannoma is a typically encapsulated benign tumor of peripheral nerves that is derived from and composed of mature Schwann cells[20]. In peripheral nerves and in these tumors, S-100 protein is expressed by Schwann cells and is a specific marker of nerve sheath differentiation[21]. Expression of S-100 protein in malignant peripheral nerve sheath tumor (MPNSTs) correlates with Schwann cell-like differentiation of tumor cells[22]. The definition of MPNST (the presence of S-100-immunopositive Schwann-like cells) is based on a malignant tumor arising from a peripheral nerve or in extraneural soft tissue if it shows nerve sheath differentiation[20]. Immunohistochemical and molecular studies have demonstrated the expression of WT1 protein and messenger (m) RNA in these tumors[19,23-25].

In the present study, we examined the cytoplasmic expression of WT1 protein in S-100-immunopositive cells of peripheral nerves, neurofibromas, schwannomas, and MPNSTs. We also investigated the oncogenic role of WT1 in peripheral nerve sheath tumors.

Materials and Methods

Specimens

Peripheral nerve specimens comprised the cauda equina nerve roots of 8 autopsy cases (postmortem interval : range, 2-17 hours ; mean±SEM, 9.30±2.07 hours) without neurological disease or neuropathologic abnormalities. Peripheral nerve tumor specimens comprised 10 schwannomas, 19 neurofibromas, and 11 MPNSTs obtained at surgical resection. The clinical data are summarized in Table 1. This study was approved by the ethics committee of The Jikei University School of Medicine (Permission No. 22-076-6253).

Immunohistochemical studies

After fixation with 10% neutral phosphate-buffered formalin, paraffin-embedded tissue sections 3 μm thick were cut and stained with hematoxylin and eosin. For immunohistochemical studies, a monoclonal antibody against the N-terminal of WT1 (6F-H2 [Dako, Carpinteria, CA, USA]), a rabbit polyclonal antibody against C-terminal of WT1 (C-19 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]), and 2 antibodies against S-100 (monoclonal antibody S-100 217 [Abnova Corporation, Taipei, Taiwan]), and a rabbit polyclonal antibody (Dako) were used. After deparaffinization, immunohistochemical staining was performed with an automated stainer (BenchMark XT, Ventana Medical Systems, Inc., Tucson, AZ, USA). Epitope retrieval was performed with a cell conditioning solution (CC1 ; Ventana Medical Systems, Inc.), and immunostaining was performed with a diaminobenzidine detection system (i-VIEW, Ventana Medical Systems, Inc.) according to the manufacturer's instructions. The sections were counterstained with hematoxylin. Negative controls omitting the primary antibody and a positive control using Wilms' tumor tissue sections were included in each run. For the WT1-immunostained sections, WT1 protein immunostaining was scored as 0 (negative : no staining), 1 (very weak : small spots), 2 (weak : small spots and faint strings), 3 (moderate : string-like emanating from the nuclear circumference), 4 (strong : diffuse staining), or 5 (very strong : concentrated dense bands).

Co-localization of WT1 and S-100 in nerve roots, neurofibromas, schwannomas, and MPNSTs was investigated with double-immunofluorescence staining with anti-WT1 and anti-S-100 antibodies. Sections were incubated in a solution of 0.01% Tween 20 (Sigma-Aldrich, St Louis, MO, USA), 5% bovine serum albumin, 10% normal goat serum, and 0.01 M phosphate-buffered saline (PBS) for 30 minutes at room temperature and were then incubated with primary antibodies diluted with blocking solution overnight at 4°C. Alexa488- and Alexa555-conjugated anti-immunoglobulin antibodies (Invitrogen, Carlsbad, CA, USA) were used, and fluorescence labeling was captured with an LSM 510 confocal microscope (Zeiss, Jena, Germany).

Western blotting

To identify WT1 protein with Western blotting, extracts of nerve roots and tumor tissues were prepared by adding lysis buffer [0.05 M Tris, pH 7.4, 120 mmol/L NaCl, 0.5% Triton, and EDTA-free protease inhibitor cocktail (Roche, Denver, CO, USA)]. One volume of 2X sample buffer (Sigma-Aldrich) was added to the extract supernatant (protein content, 2 mg/mL), and the sample was heated at 100°C for 5 minutes. The protein samples were resolved with 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose transfer membranes (Whatman Schleicher & Schuell, Dassel,

Table 1. Clinical data of the cases examined.

Case	age	gender	diagnosis	ICH (score)	Western blotting	real timePCR
1	37	female	MPNST	done (1)		
2	56	male	MPNST	done (2)	done	done
3	17	male	MPNST	done (2)		
4	51	female	MPNST	done (2)		
5	34	male	MPNST	done (3)		
6	66	male	MPNST	done (4)	done	done
7	47	male	MPNST	done (4)	done	done
8	15	male	MPNST	done (4)		
9	66	male	MPNST	done (5)	done	done
10	68	female	MPNST	done (5)	done	done
11	24	female	MPNST	done (5)		
12	27	male	Neurofibroma	done (1)	done	done
13	61	male	Neurofibroma	done (1)		
14	10	male	Neurofibroma	done (1)		
15	61	female	Neurofibroma	done (1)		
16	68	female	Neurofibroma	done (1)		
17	29	male	Neurofibroma	done (1)		
18	61	female	Neurofibroma	done (1)		
19	16	male	Neurofibroma	done (2)	done	done
20	32	male	Neurofibroma	done (2)	done	done
21	35	female	Neurofibroma	done (2)	done	done
22	18	female	Neurofibroma	done (2)	done	done
23	5	female	Neurofibroma	done (2)	done	done
24	66	male	Neurofibroma	done (2)		
25	33	female	Neurofibroma	done (2)		
26	15	male	Neurofibroma	done (3)		
27	22	female	Neurofibroma	done (3)		
28	24	male	Neurofibroma	done (4)		
29	62	male	Neurofibroma	done (4)		
30	56	female	Neurofibroma	done (5)		
31	46	female	Schwannoma	done (3)	done	done
32	46	male	Schwannoma	done (3)		
33	20	male	Schwannoma	done (3)		
34	60	female	Schwannoma	done (3)		
35	44	female	Schwannoma	done (4)	done	done
36	61	female	Schwannoma	done (4)		
37	32	male	Schwannoma	done (5)	done	done
38	56	male	Schwannoma	done (5)	done	done
39	29	female	Schwannoma	done (5)		
40	47	male	Schwannoma	done (5)		
41	74	male	Cauda Equina	done (2)	done	done
42	62	male	Cauda Equina	done (2)	done	done
43	89	male	Cauda Equina	done (2)	done	done
44	59	female	Cauda Equina	done (2)	done	done
45	98	male	Cauda Equina	done (2)		
46	31	male	Cauda Equina	done (2)		
47	75	male	Cauda Equina	done (2)		
48	62	female	Cauda Equina	done (2)		

ICH, immunohistochemistry ; PCR, polymerase chain reaction.

Germany). Membranes were blocked and incubated overnight at 4°C with the anti-WT1 antibodies (C-19, 1 : 1,000 ; 6F-H2, 1 : 30,000). The bound antibodies were visualized by incubation with a horseradish peroxidase-conjugated secondary antibody (1 : 10,000 ; Millipore, Bedford, MA) followed by enhanced chemiluminescence (ECL Plus system ; GE Healthcare, Buckinghamshire, UK) and by exposure to Hyperfilm-ECL (GE Healthcare).

Sequence analysis and reverse transcription polymerase chain reaction

For analysis of the expression of WT1 mRNA in the tissues, RNA was isolated from nerve roots and tumor tissues with an isolation reagent (Isogen, Nippon Gene, Tokyo, Japan) and was converted into random-primed complementary (c) DNA with reverse transcriptase (RT ; Superscript II, Invitrogen, San Diego, CA, USA). By means of primers (WT1F, CCACAGCACAGGGTACGA GAGCGATAACCA, and WT1R, TGGCCACCGACAGCT GAAGGGCTTTTCAC), all isoforms of WT1 cDNA were amplified, and cycle sequencing was performed directly on the purified polymerase chain reaction (PCR) products with BigDye terminator chemistry (Model 3100 genetic analyzer, BigDye Terminator v3.1 Cycle Sequencing Kit ; Applied Biosystems LLC, Foster City, CA, USA) according to the manufacturer's recommendations.

Real-time RT-PCR was performed using the Thermal Cycler Dice Real Time System TP800 (TaKaRa Bio Inc., Shiga, Japan). In each reaction, 100 ng of total RNA was amplified using the One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa Bio) and primers (AGCACAGGGTAC GAGAGCGATAAC and TATTGCAGCCTGGGTAAGCACA for WT1, GGATAGCCTCAGCGTCAACGA and CCG AGTCCTGATTCACATCCAA for S-100) according to the manufacturer's recommended protocol. The RT-PCR conditions were 42°C for 5 minutes and 95°C for 10 seconds, followed by 55 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Subsequently, a melting curve program was applied with continuous fluorescence measurement. Beta-actin and YWHAZ (human housekeeping gene primer set, TaKaRa Bio) were used as internal standards. Semiquantitative analyses of WT1 mRNA expression relative to internal controls and to S-100 were performed according to the standard curve method using the crossing point of the amplification plots (Thermal Cycler Dice Real Time System Multiplate RQ TP860 software version 3.00A, TaKaRa Bio).

Statistical analysis

Data are expressed as means±SEM. Scores of WT1-immunostained sections were analyzed using the Kruskal-Wallis one-way analysis of variance by ranks. The semiquantitative data of real-time PCR were analyzed with one-way analysis of variance followed by a post hoc Tukey-Kramer test. All statistical analyses were performed with JMP software (version 8.0.2 ; SAS Institute Inc, Cary, NC, USA). A p value less than 0.05 was considered to indicate significance.

RESULTS

Immunohistochemistry

Immunohistochemical staining showed that WT1 protein was expressed in the cytoplasm of all cauda equina nerve roots, neurofibromas, schwannomas, and MPNSTs. Equivalent results were obtained with both antibodies. No obvious nuclear staining was detected. In the cauda equina nerve roots of all autopsy cases, WT1 showed a weak staining pattern (score 2, faint, Fig. 1c) in the myelin sheath and cytoplasm of Schwann cells. In the neurofibromas, the immunostaining patterns for WT1 were as follows : very weak in 7 cases (score 1, small spots, Fig. 1a), weak in 7 cases (Fig. 1b), moderate in 2 cases (score 3, string-like), strong in 2 cases (score 4, diffuse), and very strong in 1 case (score 5, concentrated). In the schwannoma speicmens, the WT1 staining patterns were moderate in 4 cases, strong in 2 cases (Fig. 1e), and very strong in 4 cases. In the MPNST specimens, WT1 expression was focally accentuated but was not associated with tumor cell density, mitotic activity, or perinecrotic areas. Immunostaining was very weak in 1 case, weak in 3 cases, moderate in 1 case (Fig. 1d), strong in 3 cases, and very strong in 3 cases (Fig. 1f). Table 2 summarizes the WT1 protein expression scores for each histological subtype. The score data in each group were not normally or logistically distributed, except for those of MPNSTs. No significant difference was detected among the groups.

On confocal microscopic analysis of 1-μm-thick optical sections stained for double immunofluorescence, WT1 was expressed only in S-100-immunopositive cells in all cauda

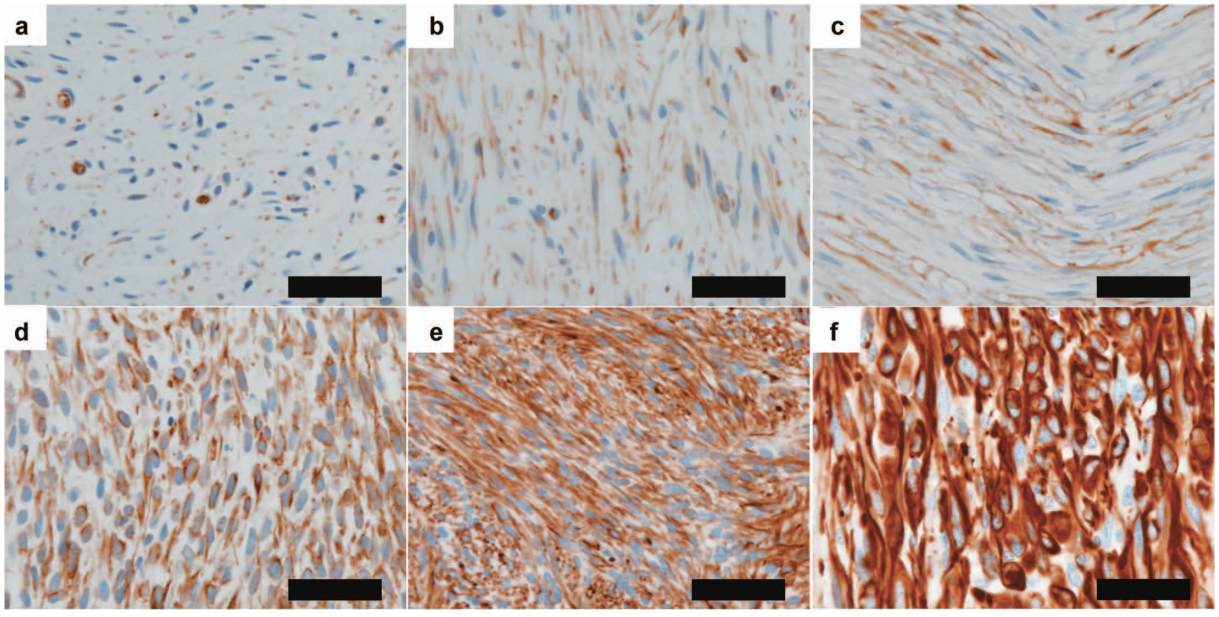

Fig. 1. Immunohistochemical staining for WT1.
a : very weak staining ; small spots in neurofibroma (case 19, score 1). b : weak staining ; small spots and faint strings in neurofibroma (case 26, score 2). c : weak staining ; small spots and faint strings in cauda equina nerve roots (case 42, score 2). d : moderate staining ; string-like in MPNST (case 6, score 3). e : strong staining ; diffuse in schwannoma (case 37, score 4). f : very strong staining ; concentrated dense band in MPNST (case 10, score 5). Scale bars = 50 μm (×400)

Table 2. WT1 protein immunostaining score for peripheral nerve (cauda equina) and each histological subtype of peripheral nerve tumor

	Score-1	Score-2	Score-3	Score-4	Score-5
Cauda Equina	0	8	0	0	0
Neurofibroma	7	7	2	2	1
Schwannoma	0	0	4	2	4
MPNST	1	3	1	3	3

MPNST, malignant peripheral nerve sheath tumor.
See Figure 1 for details of the scoring

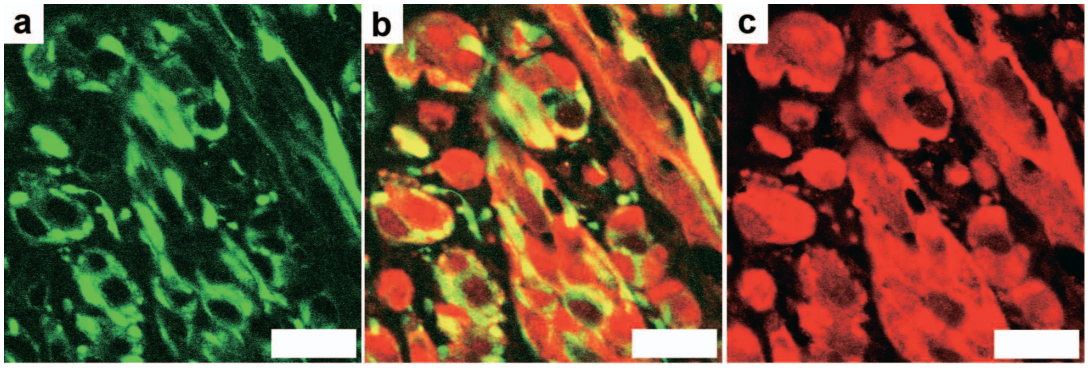

Fig. 2. Double immunofluorescence staining for WT1 and S-100 proteins.
a : WT1 immunostaining ; b : WT1 and S-100 double immunostaining ; c : S-100 immunostaining. WT1 protein is expressed only in S-100 protein–immunopositive cells in a case of MPNST (case 10). Scale bars = 20 μm (×1,000)

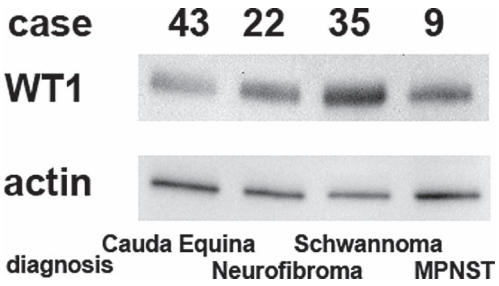

Fig. 3. Western blotting for WT1.
A 52-kDa band is shown for the protein extracts from cauda equina nerve roots, neurofibromas, Schwannomas, and MPNSTs.

equina nerve roots, neurofibromas, schwannomas, and MPNSTs (Fig. 2).

Western blotting

In Western blotting analysis, anti-WT1 antibodies recognized a 52-kDa band in the protein extracts from cauda equina nerve roots, neurofibromas, schwannomas, and MPNSTs (Fig. 3).

RT-PCR

Expression of WT1 mRNA was confirmed with sequence analysis of the PCR products (using WT1F and WT1R primers) amplified from the cDNA of cauda equina nerve roots, neurofibromas, schwannomas, and MPNSTs (data not shown). In addition, real-time RT-PCR revealed the expression profiles for WT1. The relative expression levels of WT1 normalized to the internal standards (YWHAZ and beta-actin) and to S-100 were not significantly different between cauda equina nerve roots, neurofibromas, schwannomas, and MPNSTs (Fig. 4).

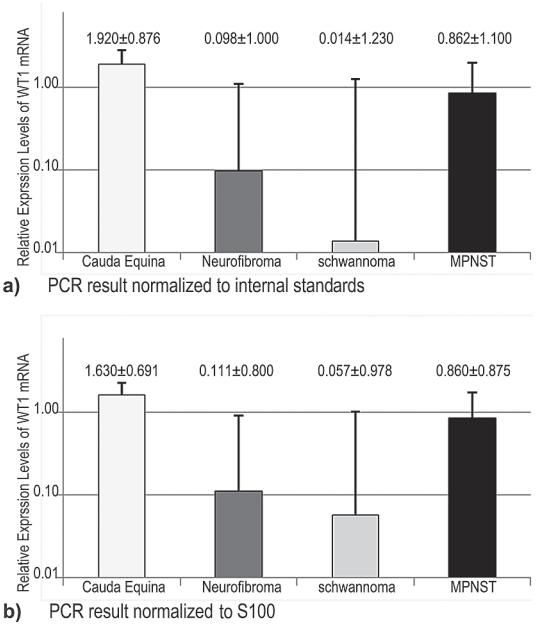

Fig. 4. Real-time RT-PCR.
Relative expression levels of WT1 mRNA, normalized to the internal standards (YWHAZ and beta-actin) (a) or normalized to S-100 (b) are not significantly different between cauda equina nerve roots, neurofibromas, Schwannomas, and MPNSTs.

DISCUSSION

Our Western blotting and RT-PCR data show that WT1 protein and mRNA are expressed in the tissues of peripheral nerves, neurofibromas, schwannomas, and MPNSTs. Immunohistochemical findings also confirmed that WT1 protein is expressed in the cytoplasm of S-100-immunopositive Schwann cells of peripheral nerves, neurofibromas, and schwannomas as well as in Schwann-like cells of MPNSTs. However, the scoring of WT1 protein immunostaining and semiquantitative analysis of WT1 mRNA with real-time RT-PCR showed that expression of WT1 is not significantly greater in peripheral nerve tumors than in normal peripheral nerves. These findings suggest that WT1 is independent of tumorigenesis and malignant transformation in peripheral nerve sheath tumors.

Nuclear expression appears to correlate with melanocytic atypia and melanoma[26]. Furthermore, a variety of neoplasms have been associated with high oncogenic expression levels of cellular WT1[25,27-30], which inhibits apoptosis[31-33]. WT1 has 4 major isoforms that arise from 2 alternative splice sites[34]. One form has 3 amino acids (KTS) inserted between zinc fingers 3 and 4, which enhances the affinity for RNA, and is likely to play a posttranscriptional role necessary for the development of the genitourinary tract[35], spleen[36], diaphragm[37], and cardiovascular progenitor cells[38] as well as mesothelial structures in the fetus[39]. The second alternative splice inserts 17 amino acids (exon 5) within the transactivation domain. WT1 targets genes encoding growth factors, differentiation markers, cell-cycle regulators, and apoptosis regulators[33]. WT1 has been suggested to govern cell differentiation and suppress cell proliferation in neuroblastoma[40]. The transcriptional regulatory properties of WT1 are complex and include both activator and repressor functions. Several binding part-

ners of WT1 have been proposed to regulate its function. Although it is unclear how WT1 manifests these distinct activities in Schwann or Schwann-like cells in peripheral nerve tissue, neurofibromas, schwannomas, and MPNSTs, the expression of WT1 protein and mRNA in these normal and tumor tissues may indicate a crucial role for WT1 in the survival and proliferation of these cells.

Subsequent to a report of WT1 peptide-based immunotherapy for leukemia[41], WT1 immunotherapy has been used for lung cancer[42], chronic myelomonocytic leukemia[43], renal cell carcinoma[44], and glioblastoma[45] with clinical benefits and no severe adverse effects, except for local injection site erythema[46]. The National Cancer Institute pilot project has recently ranked WT1 as the first antigenic target with high potential for immunotherapy[47]. A WT1 peptide vaccine consisting of a WT1 peptide-HLA complex has been reported to promote the induction of WT1-specific cytotoxic T-cell activation and helper T-cell responses[48,49]. The development of WT1 immunotherapy for MPNST is expected in the near future. However, future studies should evaluate the possible effects of WT1 immunotherapy on Schwann cells expressing WT1 protein, such as in demyelinating diseases of the peripheral nervous system.

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