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# *WT1* Alternative Splicing: Role of Its Isoforms in Neuroblastoma

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**Abstract** Wilms tumor 1 (*WT1*), a tumor suppressor gene, was originally identified in the homonymous renal neoplasm but is also involved in other cancers. Its function is still unclear, since it acts both as a pro- and an anti-oncogene. At least 14 WT1 transcriptional variants have been described; yet most investigations have focused on a small number of isoforms. We describe their structural features and review the evidence of their involvement in cancer with emphasis on neuroblastoma. In future, full characterization of all WT1 isoforms is expected to identify new molecular tumor markers and/or therapeutic targets.

**Keywords** WT1 · Alternative splicing · Isoforms · Neuroblastoma

## Introduction

Wilms tumor 1 gene (*WT1*) maps on the short arm of human chromosome 11 and has been one of the first tumor suppressor genes to be cloned (Call et al. 1990; Gessler et al. 1990; Hung et al. 2016; Goyal et al. 2016; Naitoh et al. 2016). It takes its name from Wilms tumor, a renal tumor that largely affects children, but is also involved in several other cancers.

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*WT1* plays a crucial role in fetal life, due to its involvement in the development of the organs of mesodermal origin (kidney, spleen, gonads, and cardiovascular system) and in the proliferation of some nervous system progenitor cells. It promotes kidney differentiation by blocking cell cycle progression, whereas in other tissues it sustains cell proliferation (Moore et al. 1998; Moore et al. 1999). In healthy adults it is found only in podocytes, highly specialized cells forming the visceral layer of the Bowman's capsule in the kidney (Hohenstein and Hastie 2006), whereas it is widely expressed in tissue from patients suffering from a variety of pathological conditions, including tumors.

Its function is the subject of extensive debate, since *WT1* acts as an oncogene in some neoplasms and as an oncosuppressor in others, including Wilms tumor. Interestingly in neuroblastoma, a neuroepithelial tumor of embryonic origin that also largely affects children, it seems to act in both ways (Sebire et al. 2005; Wang et al. 2011; Kletzel et al. 2015; Maugeri et al. 2016; Masserot et al. 2016). Neuroblastoma is characterized by a highly heterogeneous clinical behavior, as it can either progress to more aggressive cancer or regress spontaneously to less malignant ganglioneuroblastoma or ganglioneuroma. *WT1* is expressed in tumor tissue both in the nuclear and the cytoplasmic compartment (Niksic et al. 2004; Parenti et al. 2013; Magro et al. 2015) and its level of expression is related to tumor malignancy (Maugeri et al. 2016).

*WT1* encodes a protein having an N-terminal region rich in lysine and glutamine residues and a C-terminal region composed of four consecutive zinc finger motifs that are highly homologous to those of early growth response-1 (Egr-1) transcription factor (Rauscher et al. 1990). These domains have led to the classification of *WT1* as a transcription factor, where the N-terminal region interacts with transcriptional co-activators and the C-terminal tract binds to GC-rich DNA regions (Rauscher et al. 1990), WTE sites (Nakagama et al.

1995), or (TCC)n motifs (Wang et al. 1993). Through these regions, *WT1* regulates the transcription of target genes such as Bcl-2, Bcl-xL, Bfl-1, and c-myc, which are involved in cell growth or apoptosis (Mayo et al. 1999; Han et al. 2004; Simpson et al. 2006; Bansal et al. 2012).

Despite the existence of multiple WT1 isoforms—generated through alternative splicing—only a few have been investigated (Oji et al. 2002; Koesters et al. 2004; Luna et al. 2013). The cloned variants deposited in the various databases (NCBI, Ensembl, UniProt) are listed in Table 1. The published evidence has suggested to us that their expression profile may be associated to a specific cellular phenotype. Immunolocalization studies performed in tissue samples from cancer patients have found a variable staining pattern— cytoplasmic, nuclear, or both—depending on whether the antibody employed is directed against an epitope on the C or the N-terminus (Carpentieri et al. 2002; Nakatsuka et al. 2006; Schittenhelm et al. 2010; Bisceglia et al. 2011; Salvatorelli et al. 2011; Magro et al. 2014a, b, 2015; Salvatorelli et al. 2015).

Furthermore, WT1 protein shows a discrete distribution in embryonic organs: exclusively in the nucleus of urogenital apparatus progenitor cells and only in the cytoplasm of cardiovascular and nervous system germ cells (Pritchard-Jones et al. 1990; Sharma et al. 1992; Armstrong et al. 1993; Mundlos et al. 1993; Ramani and Cowell 1996; Charles et al. 1997; Parenti et al. 2013, 2015; Salvatorelli et al. 2015). Based on these data, it may be hypothesized that *WT1* regulates gene transcription when it is expressed in the nucleus, whereas it participates in post-transcriptional events when it is expressed in the cytoplasm. Such compartmentalization could well reflect the different actions and functions of the diverse WT1 isoforms.

Here we describe the features of the *WT1* transcript variants that have already been cloned and review the literature regarding their involvement in tumors, especially neuroblastoma.

## Alternative Splicing and the *WT1* Gene Isoforms

*WT1*, a complex gene measuring approximately 50 kb in length, comprises 10 exons and 9 introns. Various mRNA transcripts are translated into different isoforms by alternative splicing (Haber et al. 1991; Tadokoro et al. 1992).

During precursor mRNA (pre-mRNA) splicing, a crucial stage of post-transcriptional regulation, introns are removed and exons joined to form mature mRNA, which is exported to the cytoplasm for translation into a protein (Crick 1979; D'Agata et al. 2000; Hastings and Krainer 2001; Jurica and Moore 2003; Hollander et al. 2016). Four highly conserved regions in the intronic sequences are involved in the splicing process: two splice sites (a donor and an acceptor) respectively at the 5′ (GU) and the 3′ (AG) end and a branching point in the intron followed by a polypyrimidine tract (Hertel and Graveley 2005). The spliceosome, a molecular complex consisting of small nuclear ribonucleoproteins (snRNP) and of non-snRNP factors, recognizes these sites and enables the synthesis of mature mRNA through a number of steps (Collins and Guthrie 2000; Das et al. 2000; Maroney et al. 2000; Hertel and Graveley 2005). Different transcript variants are produced by the exclusion of whole exons and/or introns during alternative splicing (D'Agata and Cavallaro 2004; Scuderi et al. 2014; La Cognata et al. 2014; D'Amico et al. 2015; Maugeri et al. 2015).

As regards *WT1*, an alternative splicing site is found on exon 5, which encodes 17 amino acids; another is located on the 3′ end of exon 9, which codes for the amino acids lysine, threonine, and serine; this tract is also referred to as KTS region (Haber et al. 1991). Notably, *WT1* pre-mRNA has three different alternative start codons including a CUG and an AUG, respectively, upstream and downstream of the regular AUG (Bruening and Pelletier 1996; Scharnhorst et al. 1999). This combination of alternative splicing sites and start codons results in generation of at least 12 transcript variants, although in theory up to 24 isoforms can be formed through subsequent RNA editing events. Moreover, additional mRNA variants (AWT1) are generated by an alternative promoter found on the first intron of *WT1*, which starts transcription from exon 1a (Dallosso et al. 2004). A further variant, Ex4a(+), containing the additional exon 4a downstream of exon 4, has recently been cloned (Schnerwitzki et al. 2014; Tatsumi et al. 2015).

Fourteen variants are found in public repositories (Ensembl, NCBI, UniProt) and have lately been given a new ID code (Maugeri et al. 2016). Their list, accession number, nucleotide and amino acid composition, isoelectric point, and molecular weight as well as the articles describing any variants are reported in Table 1.

To identify the structural differences among the transcripts, we aligned their nucleotide sequence variants using the CLC Sequence viewer 7 software. WT1.6, the most widely studied isoform, was arbitrarily designated as the canonical sequence.

To date, most studies have examined exclusively the *WT1* variants containing or lacking exon 5 and the KTS region, besides the canonical sequence (Loeb et al. 2001; Oji et al. 2002; Oji et al. 2003; Koesters et al. 2004; Oji et al. 2004a, b; Luna et al. 2013). Yet, transcripts differing in the initial part of the N-terminal domain and, in some cases, also in the C-terminal domain have also been cloned. These nucleotide regions found in the 14 splice variants are shown in Figs. 1, 2, 3, and 4.

The canonical sequence (WT1.6), containing both exon 5 and the KTS region, is the +/+ variant; WT1.7, which contains exon 5 but not the KTS region, is the +/− variant; WT1.8 contains the KTS region and lacks exon 5 and is the −/+ variant; and finally WT1.9, which lacks both regions, is the −/− transcript. WT1.1 and WT1.4 mRNAs are similar to WT1.6, except that they have a longer N-terminal domain,

**Table 1** *WT1* gene transcripts and isoforms

| ID code | NCBI ID mRNA protein | Ensembl ID | UniProt ID | Length (bp) | Length (aa) | Mw | pI | Reference |
|---|---|---|---|---|---|---|---|---|
| WT1.1 | AY245105.1 / | / | P19544-7 | 3026 | 522 | 56,88 | 9.10 | |
| WT1.2 | NM_024426 NP_077744 | ENST00000332351 | J3KNN9 | 3037 | 517 | 56,26 | 9.16 | Bruening et al. 1992; Jinno et al. 1994; Sharma et al. 1994; Bruening and Pelletier 1996; Mitsuya et al. 1997; Wagner et al. 2003; Maki et al. 2017; Ramanathan et al. 2017. |
| WT1.3 | NM_024424 NP_077742 | ENST00000448076 | H0Y7K5 | 3028 | 514 | 55,95 | 9.11 | Bruening et al. 1992; Jinno et al. 1994; Sharma et al. 1994; Bruening and Pelletier 1996; Mitsuya et al. 1997; Wagner et al. 2003; Maki et al. 2017; Ramanathan et al. 2017 |
| WT1.4 | AY245105.1 / | / | P19544-8 | 3026 | 505 | 55,21 | 9.11 | |
| WT1.5 | NM_000378 NP_000369 | ENST00000452863 | A0A0A0MT54 | 2977 | 497 | 54,27 | 9.12 | Bruening et al. 1992; Jinno et al. 1994; Sharma et al. 1994; Bruening and Pelletier 1996; Mitsuya et al. 1997; Wagner et al. 2003; Maki et al. 2017; Ramanathan et al. 2017 |
| WT1.6[a] | AY245105.1 AAO61088 | / | P19544-1 | 3026 | 449 | 49,19 | 9.23 | |
| WT1.7 | CH471064 EAW68220 | / | P19544-4 | 3014 | 446 | 48,87 | 9.18 | |
| WT1.8 | CH471064 EAW68221 | / | P19544-3 | 2972 | 432 | 47,51 | 9.24 | |
| WT1.9 | CH471064 EAW68226 | / | P19544-2 | 2963 | 429 | 47,19 | 9.19 | |
| WT1.10 | / | ENST00000379077 | H0Y3F0 | 3032 | 317 | 33,04 | 5.76 | |
| WT1.11 | NM_001198551 NP_001185480 | ENST00000379079 | P19544-6 | 2480 | 302 | 34,45 | 9.53 | Bruening et al. 1992; Jinno et al. 1994; Sharma et al. 1994; Bruening and Pelletier 1996; Mitsuya et al. 1997; Wagner et al. 2003; Maki et al. 2017; Ramanathan et al. 2017 |
| WT1.12 | NM_001198552 NP_001185481 | ENST00000530998 | P19544-9 | 2438 | 288 | 33,09 | 9.59 | Bruening et al. 1992; Jinno et al. 1994; Sharma et al. 1994; Bruening and Pelletier 1996; Mitsuya et al. 1997; Wagner et al. 2003; Maki et al. 2017; Ramanathan et al. 2017 |
| WT1.13 | / | ENST00000527882 | H0YED9 | 551 | 178 | 20,20 | 10.25 | |
| WT1.14 | AB971668.1 BAR92607 | / | / | 3183 | 249 | 25,97 | 5.61 | Tatsumi et al. 2015 |

[a] WT1.6 corresponds to the canonical sequence

because the start codon is found upstream of the one of the canonical isoform. WT1.2 diverges from WT1.1 by a few initial nucleotides; WT1.3 is a “+/−” transcript, and WT1.5 a −/− splice variant (Maugeri et al. 2016).

WT1.10 and WT1.14 differ at the 5′ end (Fig. 4). As shown in Fig. 3, they share a 153 nucleotide insertion downstream of exon 4, corresponding to exon 4a. The latter exon bears a stop codon whose presence could expose such variants to nonsense-mediated mRNA decay, a process where mRNA degradation is triggered by nonsense mutations to prevent abnormal or truncated protein formation. However, the degradation has been excluded in these transcripts by demonstration

**Fig. 1** Exon 5 alignment of *WT1* transcript variants. WT1.5, WT1.1, WT1.9 and WT1.12 variants lack exon 5

that WT1.14 is translated into a truncated protein lacking four zinc finger domains (Tatsumi et al. 2015).

Variants WT1.11 and WT1.12, the former lacking the KTS region and the latter lacking exon 5, can be considered as AWT1 variants. Their transcription starts from exon 1a, following the splice out of exon 1. These transcripts encode a ~33-kDa molecular weight protein having an N-terminal domain that is shorter than that of the other isoforms (Dallosso et al. 2004; Royer-Pokora 2013). Finally, WT1.13 is the smallest cloned variant. With regard to the translated protein, the Ensembl database reports the annotation: "5′ truncation in transcript evidence prevents annotation of the start of the CDS." This suggests that further investigation is required to confirm its expression.

To summarize, the WT1.1, WT1.2, WT1.4, WT1.6, WT1.10, WT1.13, and WT1.14 transcripts contain exon 5 as well as the KTS region, both of which are absent in WT1.5 and WT1.9; variants WT1.3, WT1.7, and WT1.11 contain exon 5 but not the KTS region, and WT1.8 and WT1.12 contain the KTS tract but not exon 5.

## Involvement of the WT1 Isoforms in Tumors, with Emphasis on Neuroblastoma

*WT1* plays a role in a wide range of conditions, where it seems to exert conflicting effects. It is rarely detected in healthy tissue, whereas it is commonly expressed in several cancers including leukemia (Luna et al. 2013) and lung (Oji et al. 2002), colon (Koesters et al. 2004), pancreatic (Oji et al. 2004a), breast (Loeb et al. 2001; Oji et al. 2004b), and thyroid (Oji et al. 2003) tumors. Since its discovery, *WT1* has been

**Fig. 2** Exon 9 alignment of *WT1*transcript variants. WT1.3, WT1.5, WT1.7, WT1.9 and WT1.11 variants lack 9 nucleotides on exon 9 encoding KTS tract

**Fig. 3** Exon 4a alignment of *WT1* transcript variants. Only WT1.10 and WT1.14 variants possess exon 4a

considered as a tumor suppressor gene with a mutation in Wilms tumor, WAGR (Wilms tumor, aniridia, genitourinary malformations, mental retardation) (Gessler et al. 1990), and Danys-Drash (Pelletier et al. 1991; Patek et al. 1999) and Frasier syndrome (Barbaux et al. 1997; Klamt et al. 1998). However, an oncogenic role has also been hypothesized in some solid tumors and leukemia (Oji et al. 2002; Bansal et al. 2012; Luna et al. 2013), leading it to be considered both as an oncosuppressor and an oncogene (Yang et al. 2007). Even though such conflicting actions may depend on changes in promoter methylation (Loeb et al. 2001; Brocato and Costa 2013; Guillaumet-Adkins et al. 2014; Zitzmann et al. 2014; Abete et al. 2015; Sahnane et al. 2015; Mžik et al. 2016), a correlation with the tissue- and cell-specific expression of the

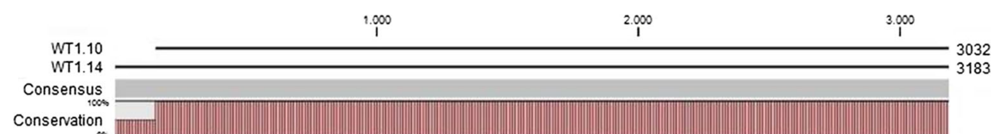


**Fig. 4** WT1.10 and WT1.14 transcript variant alignment. The sequence of these variants differs at the 5′ end

*WT1* isoform cannot be ruled out. Indeed, the different roles played by different variants suggest a definite cellular phenotype.

Since most studies of WT1 isoforms have been confined to establishing the presence/absence of specific splicing regions, they have been unable to discriminate among the different variants. For instance, Oji et al. (2002) and Luna et al. (2013) assessed them respectively in lung cancer and acute myeloid leukemia (AML). Using probes for exon 5 and the KTS region, they identified the +/+, −/+, +/−, and −/− variants, which included several transcripts. All variants were found to be overexpressed in both tumors except −/− transcripts, which were underexpressed. These findings suggest that the scanty expression of the −/− isoforms (WT1.5 and WT1.9) might depend more on the small number of variants (only 2) than on downregulation. Furthermore, Luna et al. (2013) found that blood progenitor cells in AML patients also expressed WT1.11 and WT1.12 (AWT1 isoforms).

The Ex4a(+) variant, cloned by Tatsumi et al. (2015), is found in non-cancerous kidney cells and is downregulated in some tumors. By inhibiting the transcriptional activation of the anti-apoptotic gene Bcl-xL, it could regulate the oncogenic action of other WT1 isoforms which conversely induce this gene (Tatsumi et al. 2015).

Some studies have suggested that in neuroblastoma*WT1* acts as an oncosuppressor as well as an oncogene (Haber et al. 1993; McMaster et al. 1995; Menke et al. 1997; Smith et al. 2000; Fraizer et al. 2004; Sebire et al. 2005; Wang et al. 2011; Kletzel et al. 2015; Masserot et al. 2016; Maugeri et al. 2016). Neuroblastoma is a childhood tumor occurring more often in boys (Spix et al. 2006; Gatta et al. 2012). It is a rare solid tumor characterized by a highly variable clinical behavior that may either regress spontaneously or progress to an aggressive form resistant to chemotherapy as well as radiation therapy. Outcome is related to conversion to a benign form, including ganglioneuroblastoma and ganglioneuroma, or to progression to a highly malignant form with metastases and a poor prognosis. The degree of malignancy is reflected in discrete histological features, undifferentiated cells with poor stroma being characteristic of aggressive cancer and differentiated cells with abundant stroma being found in benign forms (Maris et al. 2007; Salvatorelli et al. 2015).

Although the etiopathogenic mechanism of neuroblastoma development is unknown, epidemiological studies have identified some risk factors, which include maternal exposure to pesticides, volatile hydrocarbons, tobacco, and codeine-containing drugs during pregnancy or lactation (De Roos et al. 2001; Schüz et al. 2001; Cook et al. 2004; Heck et al. 2009).

The tumor originates from the cells of the neural crest, which are arranged as two cords on the sides of the neural tube. During development, these cells migrate to various sites in the thoracic and abdominal regions, differentiating into tissue-specific cells; this explains why the tumor rarely forms in the brain. The most common sites of origin are the adrenal glands and the paravertebral ganglia and involve formation of masses that induce abdominalcompression. Tumor development in the posterior mediastinal ganglia gives rise to respiratory symptoms. Occasionally, neuroblastoma forms in the neck, giving rise to Bernard-Horner syndrome, or in the pelvis, affecting the urinary and/or anorectal sphincter (Caron and Pearson 2005; Brodeur and Maris 2006; De Bernardi et al. 2008).

The prognosis is related to patient age and tumor stage: it is favorable in newborns and usually adverse in adolescents (Gatta et al. 2014). Surgical resection of localized tumor is associated to 85% survival, whereas metastasis and amplification of the MYCN gene, a biomarker of neuroblastoma, predicts a poor prognosis (Luksch et al. 2016).

The expression pattern of the WT1 isoforms in neuroblastoma has not been fully characterized. In a recent study, our group has identified 13 WT1 isoforms, although the analysis was limited to in vitro models mimicking a benign and a highly aggressive form (Maugeri et al. 2016). Although it is impossible to determine the expression profile of each isoform, upregulation was detected in less malignant cells, suggesting a tumor suppressor role. Variants WT1.1–9, whose molecular weight ranges from ~56 to ~47 kDa, were the most highly expressed. The study did not include the WT1.14 isoform, because it was the last to be cloned (Tatsumi et al. 2015). In line with our findings, Wang et al. (2011) have reported WT1 overexpression in ganglioneuroblastoma and suggested that it may act as a pro-differentiation and anti-proliferation factor involved in benign progression.

Other studies have hypothesized an oncogenic role for *WT1* in neuroblastoma. In 2005, Sebire et al. demonstrated its cytoplasmic expression in some Bcl-2-positive tumors and suggested that *WT1* may activate this endogenous anti-apoptotic gene through a transcriptional mechanism, thus providing resistance to chemotherapy (Mayo et al. 1999). In another study (Kletzel et al. 2015), investigation of *WT1*expression in the bone marrow, peripheral blood, or peripheral blood stem cells from young neuroblastoma patients sampled at the time of diagnosis, during treatment, and during clinical remission/relapse suggested a correlation with the level of tyrosine hydroxylase, a molecular marker of neuroblastoma (Naito et al. 1991; Parareda et al. 2005; Ootsuka et al. 2008; Avigad et al. 2009; Lee et al. 2010). During remission, WT1 levels were significantly reduced, albeit no data were provided regarding the isoform(s) involved. Amplification and PCR analysis of a 440 nucleotide tract corresponding to the 1350–1789 region of the canonical sequence excluded variant WT1.13. Then, amplification using the inner primers gave a 302 nucleotide product corresponding to the 1418–1719 region of WT1.6, which did not allow discriminating among the variants. However, the authors did not clarify

**Table 2** Expression and functionof WT1 isoforms in neuroblastoma

| Expression | Function | Isoforms | Reference |
|---|---|---|---|
| Neuroblastoma[a] | Oncogene | Undetermined[d] | Sebire et al. Appl Immunohistochem Mol Morphol 2005 |
| Neuroblastoma[a], ganglioneuroma, and neuroblastoma cell lines (NB1, NB19[b], NB69[b], and SK-N-SH[c]) | Pro-differentiation and anti-proliferative factor | WT1.1–WT1.14[d] | Wang et al. J Pediatr Surg 2011 |
| Bone marrow, peripheral blood, or peripheral blood stem cells of neuroblastoma patients | Oncogene | WT1.1–WT1.12 and WT1.14[e] | Kletzel et al. Transl Pediatr 2015 |
| Neuroblastoma cell line (SH-SY5Y[c]) | Tumor suppressor | WT1.1–WT1.14[d] | Maugeri et al. Genes Cancer 2016 |
| Neuroblastoma[a] and neuroblastoma cell lines (SJNB-1[b], SK-N-FI[b], SK-N-SH[c], GI-ME-N[c], SK-N-AS[c], SH-EP[c], SH-SY5Y[c], LA-N-1[c], IGR-N-91[b], IMR-32[b], IGR-N-835[b], NBLW-N[b], NBLW-S[b], SK-N-BE 2[c]) | Oncogene | WT1.1–WT1.14[e] | Masserot et al. Mol Oncol 2016 |

[a] Neuroblastoma aggressive form

[b] Non metastatic neuroblastoma cell lines

[c] Metastatic neuroblastoma cell lines

[d] Isoforms were established by aligning the recognized antibody to each WT1 sequence. It is indicated as "undetermined" when the epitope is unknown

[e] WT1 isoforms were detected by mRNA analysis

whether the patients in clinical remission included both those who had been cured and those with benign tumor.

Masserot et al. (2016) have recently found a correlation between the levels of WT1 and MYCN, a member of myc family transcription factors, in tumor tissue and neoplastic cell lines. The MYCN gene is considered as a marker of malignant neuroblastoma progression, because its overexpression is often related to a fatal outcome, yet 70–80% of MYCN-negative tumors have a poor prognosis (Hiyama et al. 1991; Schwab et al. 2003). Masserot et al. (2016) found that WT1 overexpression was associated to a poor prognosis in samples lacking MYCN amplification. However, the amplification

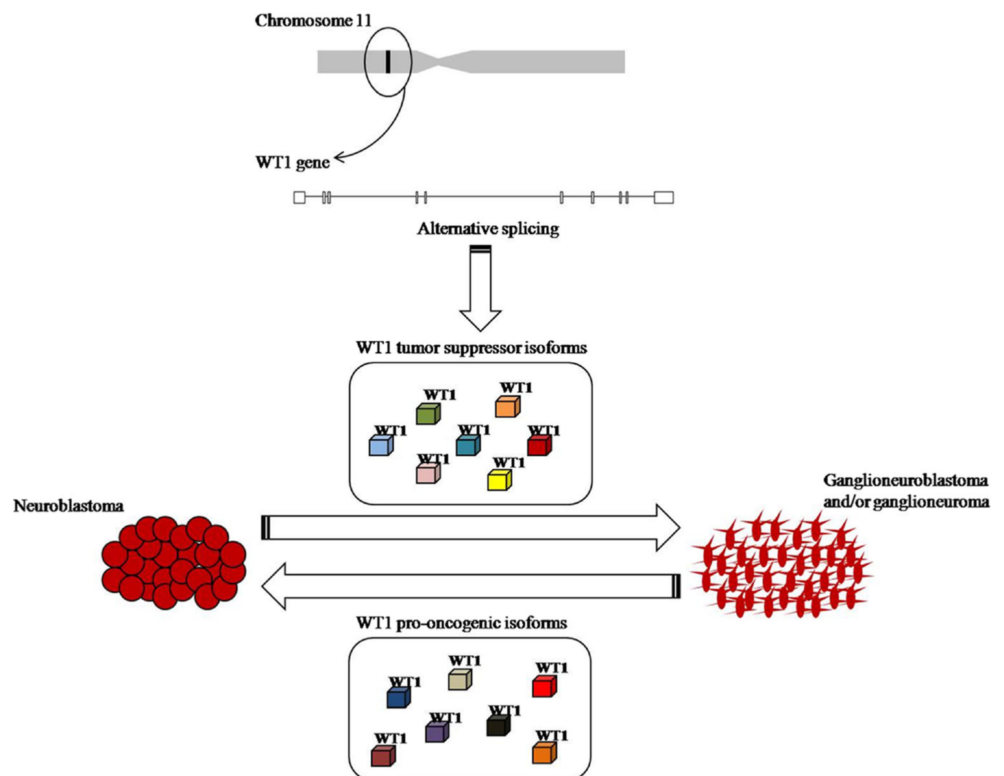


**Fig. 5** Expression profile of *WT1* gene during tumor malignancy progression

product, including the canonical sequence from 1218 to 1313, did not allow to discriminate among variants, since it is found in all isoforms.

As summarized in Table 2, the available data do not allow the establishment of the role played by each isoform or it cannot be ruled out that the conflicting results reported in different studies depend on differences in pathogenicity or analytical methods. The hypothesis may be advanced that the oncogenic and oncosuppressor action of the WT1 isoforms may correlate with tumor malignancy (Fig. 5).

## Conclusions

Generation of splice variants with a tissue- and cell-specific expression profile enhances the functional diversity of the gene. Although the over- or underexpression of some isoforms could play a key role in tumor phenotype, their high homology currently hampers discrimination. Most studies have confined the analysis to overall transcript expression. In future, full characterization of all WT1 isoforms is expected to identify new molecular tumor markers and/or therapeutic targets.

**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of interests.

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