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Mini-review

Origins of DNA methylation defects in Wilms tumors

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ABSTRACT

Wilms tumor is an embryonic renal cancer that typically presents in early childhood and accounts for 7% of all paediatric cancers. Different genetic alterations have been described in this malignancy, however, only a few of them are associated with a majority of Wilms tumors. Alterations in DNA methylation, in contrast, are frequent molecular defects observed in most cases of Wilms tumors. How these epimutations are established in this tumor is not yet completely clear. The recent identification of the molecular actors required for the epigenetic reprogramming during embryogenesis suggests novel possible mechanisms responsible for the DNA methylation defects in Wilms tumor. Here, we provide an overview of the DNA methylation alterations observed in this malignancy and discuss the distinct molecular mechanisms by which these epimutations can arise.

1. Introduction

Wilms tumor (also known as nephroblastoma) is the most frequent embryonic renal cancer, accounting for 7% of all childhood cancers. It generally affects about 1 in 10,000 children, with incidence differences observed for distinct ethnicities [1]. This disease typically occurs in early childhood, with a peak incidence between 3 and 4 years of age, and the majority of cases are sporadic, with only 1%–2% familial cases [2]. Different congenital disorders are associated with a high predisposition to Wilms tumor, including the WAGR syndrome (Wilms tumor, aniridia, genitourinary anomalies and intellectual disability), and the overgrowth disorders of Beckwith Wiedemann syndrome (BWS), Simpson–Golabi–Behmel syndrome (SGBS) and Perlman syndrome (PS), among others [3–5]. A diagnosis of Wilms tumor is generally performed under the 10 years of age for sporadic tumors, or earlier for syndromic and familial Wilms tumor. However, the diagnosis of this disease is extremely hard when only one cellular component is present in the tumor. For instance, Wilms tumors with more prevalence of blastemal cellular components are difficult to distinguish from other embryonic tumors, such as neuroblastoma and lymphoma [6].

According to protocols from the International Society of Paediatric Oncology (SIOP), Wilms tumors are usually treated by a combination of nephrectomy and chemotherapy, which usually results in a very good overall survival (up to 90%). However, a subgroup of children with Wilms tumor shows high-risk histology and relapsed tumors and do not respond to conventional treatments. Additionally, these therapies are extremely harsh and, given the young age of the patients, can have drastic consequences throughout the lifetime of the survivors [1,7]. Therefore, there is a dire need to identify new therapies for this disease.

Historically, the somatic genetic mutations reported for Wilms tumor were limited to aberrations of the genes *WT1*, *TP53*, *MYCN*, genes that regulate the canonical Wnt signaling pathway (*CTNNB1* and *WTX*), and microRNA processing genes (including *DROSHA*, *DICER1*, *DGCR8*, *XPO5* and *TARBP2*) [8–11]; however, recent comprehensive genomic analyses of large cohorts of patients with Wilms tumor have identified numerous different mutations that involve about forty genes [1,12]. A detailed and complete description of the genetic changes that can affect Wilms tumors is nicely reviewed by Treger and colleagues [1]. Interestingly, the majority of gene point mutations generally affect only a small subset of patients [1]. In contrast, DNA methylation alterations represent the most frequent molecular defects found so far in Wilms tumor [13–17].

DNA methylation at the CpG dinucleotide is the best-characterized epigenetic mark, and it is largely reprogrammed between generations in mammals [18]. Specifically, almost all methylation marks from the parents are erased in primordial germ cells and then established *de novo* during gametogenesis. After fertilization, a second wave of demethylation takes place in the pre-implantation stage, and re-methylation occurs during and after embryo implantation [19]. Strikingly, some DNA regions that are methylated in the female and male gametes are

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protected from this pre-implantation erasure of methylation. The protected regions include the imprinting control regions (ICRs) that display differential methylation marks on the maternal and paternal alleles and are responsible for the gamete-of-origin-dependent-expression of imprinted genes [20]. For most mammalian genes, both parental alleles are transcriptionally expressed (or repressed); for imprinted genes, in contrast, only one allele (either the maternal or the paternal) is ever expressed. Even though only few hundred imprinted genes have been described so far, it has been well demonstrated that this imprinting plays a key role in many biological processes, including development, growth and cell cycle [21].

During the last decade, we and others have identified key regulators required for DNA methylation reprogramming during mammalian embryogenesis [22–24]. This work has revealed novel possible mechanisms responsible for the methylation defects in Wilms tumor. In this review, we provide an overview of the DNA methylation alterations in this malignancy and discuss the distinct mechanisms by which the epimutations can arise.

2. DNA methylation defects at imprinted genomic loci

DNA methylation alterations in Wilms tumor mainly affect the 11p15.5 and 11p13 chromosomal regions.

2.1. The 11p15.5 chromosomal region

The 11p15.5 genomic region contains a cluster of imprinted genes functionally divided into two domains. Domain 1 includes two imprinted genes, *IGF2* and *H19*. *IGF2* is transcriptionally active on the paternal allele and encodes an embryonic growth factor, whereas *H19* is transcriptionally active on the maternal allele and encodes a non-coding RNA with a tumor suppressor function [25]. The normally imprinted expression of both genes is fine-tuned by the allele-specific methylation at a genomic region located between the two genes known as *H19/IGF2*:IG-DMR, also known and herein indicated as imprinting center 1 (IC1; Fig. 1A). Absence of DNA methylation at the maternal IC1 enables the insulator factor CTCF (CCCTC-binding factor) to bind, thereby creating a chromatin boundary that insulates *IGF2* from the enhancers located downstream of *H19*. In contrast, DNA methylation at paternal IC1 prevents CTCF binding, thereby allowing enhancer access and *IGF2* expression. During early development, paternal DNA methylation extends from the IC1 into the *H19* promoter, leading to repression of *H19* on the paternal allele [26] (Fig. 1A). Hence, maintenance of the differential methylation patterns at the IC1 normally assures that *IGF2* is only expressed from the paternal allele, and *H19* only from the maternal allele (Fig. 1A). In Wilms tumor, the gain of methylation (hypermethylation) of the maternal IC1 prevents CTCF from binding to it and from abolishing the enhancer-blocking activity of this genomic region. Consequently, *IGF2* becomes transcriptionally activate also on the maternal allele (Fig. 1B–D). In addition, hypermethylation of the maternal IC1 extends into the *H19* promoter, silencing *H19* expression. Thus, IC1 hypermethylation in Wilms tumor is associated with biallelic *IGF2* expression and *H19* silencing due to loss of imprinting (LOI) (Fig. 1B–D) [16,27]. Interestingly, the same epigenetic defect has been found in the premalignant precursors of Wilms tumor (termed nephrogenic rests) as well as in non-neoplastic tissue of patients affected by BWS with a high predisposition to Wilms tumor, demonstrating that LOI at the *IGF2* and *H19* genes is an early event in the malignant transformation [27,28]. Although IC1 hypermethylation in Wilms tumor has been observed in numerous studies, how this epimutation arises is not completely clear. In about half of Wilms tumors with LOI of the *IGF2/H19* genes, the active maternal 11p chromosomal region or the whole maternal chromosome 11 is lost from the tumor cells, and the inactive and methylated paternal 11p or the whole paternal chromosome 11 is duplicated (paternal uniparental disomy, pUPD; Fig. 1B). Thus, in these cases, the patients show a gain of IC1 methylation due to cytogenetic alterations [16,29,30]. In contrast, in less than 5% of Wilms tumors, the IC1 hypermethylation is associated with a microdeletion or microinsertion at the maternal IC1 allele (Fig. 1C) [16]. Binding of the transcription factors CTCF, SOX2 and OCT4 to the maternal IC1 is normally required to maintain its unmethylated profile [31,32]. It was therefore proposed that loss of SOX2/OCT4 binding sites in the deleted genomic region, or reduced CTCF binding affinity to the mutated IC1 genomic region, could be responsible for the epigenetic defect (Fig. 1C) [20]. Intriguingly, the pUPD of 11p15 chromosome region and the constitutional IC1 hypermethylation are found in a mosaic form in most BWS patients with Wilms tumor, suggesting that these (epi)mutations arise during embryonic development [27]. However, in some syndromic Wilms tumor, microdeletion of the IC1 is not associated with a gain of methylation of the same genomic region. For these cases, Prawitt and colleagues have demonstrated that at least one additional genetic lesion is required for the clinical phenotype to be manifested [33]. Why genetic defects at the IC1 are associated with methylation alteration in only some cases is still an open question. Moreover, it is also not clear why some of these cases are sporadic rather than syndromic tumors. According to the international consensus statement of BWS, patients affected by Beckwith–Wiedemann spectrum (BWSp) include those covering classical BWS phenotype without a molecular diagnosis and BWS-related phenotypes with a 11p15.5 molecular anomaly [34]. The patients affected by isolated Wilms tumor with a constitutional 11p15 defect may be considered as BWSp [16,34].

For another half of Wilms tumors with LOI of the *IGF2/H19* genes, hypermethylation at the IC1 occurs in the absence of *in cis* deletions/insertions and of point mutations (Fig. 1D) [27,35]. The origin of the DNA methylation defect in these patients is unknown. Given the strong association observed between loss of heterozygosity (LOH) at 16q and LOI at 11p15.5 in a series of Wilms tumor cases, it was initially proposed that the CTCF haploinsufficiency caused by 16q LOH could be responsible for the LOI of the 11p15.5 chromosomal region [36,37]. However, numerous studies have not observed the same genetic/epigenetic association [15,38,39]. Brown and colleagues, for example, show that the LOI of the *IGF2/H19* genes occurs before the 16q LOH in a series of Wilms tumor, suggesting that mechanisms other than the CTCF haploinsufficiency are responsible for the IC1 methylation defect [15]. Of note, exposing pregnant mice to the widely-used organophosphate pesticide chlorpyrifos-methyl leads to increased DNA methylation patterns of the *H19* locus in primordial germ cells and embryonic tissues, suggesting that environmental factors play a role in establishing this epimutation [40]. Indeed, Wilms tumor has been significantly associated with exposure to home pesticides during childhood [41,42]. Further experiments have shown that exposing mouse preimplantation embryos to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin increases DNA methylation at the IC1 [43]. Altogether, these data suggest that environmental factors (especially pesticides and contaminants) could contribute to the IC1 methylation defect in Wilms tumor (Fig. 1D).

Finally, it was recently proposed that the oxidized form of 5-methylcytosine (5-hydroxymethylcytosine, 5hmC), synthesized by the TET proteins on the mouse maternal IC1 allele, could protect this genomic region from *de novo* DNA methylation in the post-implantation embryo [44]. Indeed, Jaenisch and colleagues demonstrated that the progeny of Tet1-and Tet-2-deficient female mice show a global loss of 5hmC and more than 65% methylation at IC1, raising the possibility that the gain of maternal IC1 methylation in Wilms tumor can be the direct consequence of the loss of maternal 5-hmC levels (Fig. 1D) [44]. However, more studies are required to further investigate this mechanism.

The expression of imprinted *IGF2* is further controlled by two differentially methylated genomic regions located at its promoter (*IGF2*:alt-TSS-DMR) or within the exon 9 of the gene (*IGF2*:Ex9-DMR). In the kidney, these regions are normally methylated on the paternal chromosome, from which *IGF2* is expressed (Fig. 1A) [45,46]. We demonstrated that the gain of methylation on the maternally inherited IC1

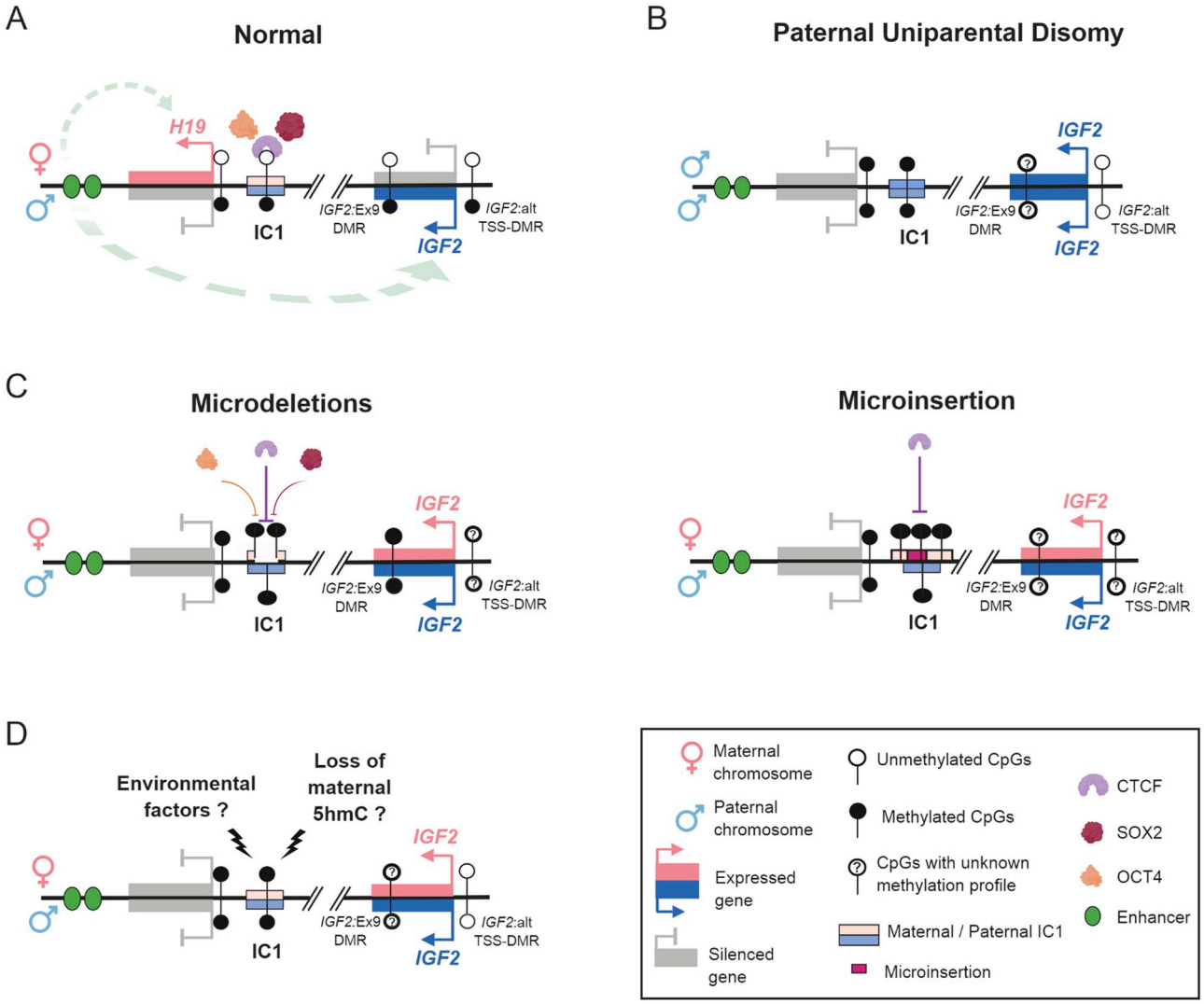

Fig. 1. Gain of DNA methylation at IC1 in Wilms tumor. (A) Schematic representation of the normal methylation profile at IC1, *IGF2*:Ex9-DMR, *IGF2*:alt-TSS-DMR and the parent-of-origin-specific allelic expression of the *IGF2* and *H19* genes. (B–D) Possible mechanisms responsible for the epimutation at IC1 include paternal uniparental disomy (B), genetic alterations (C) and environmental factors or loss of maternal 5-hydroxymethylcytosine (5hmC) (D). In all cases, the gain of methylation at the maternal IC1 is associated with biallelic expression of *IGF2* and silencing of *H19*.

can be accompanied by the same epigenetic defect at *IGF2*:Ex9-DMR or loss of methylation at the paternal *IGF2*:alt-TSS-DMR in syndromic Wilms tumors, indicating that the epimutation can occur on both parental alleles rather than *in cis* (Fig. 1B–D) [45,46]. On the other hand, BWS patients without Wilms tumor show a gain of methylation on the maternal chromosome at both IC1 and *IGF2*:alt-TSS-DMR, demonstrating that in non-neoplastic cells the methylation defect occurs only on one parental allele. Altogether these data indicate that sporadic epimutations can occur via distinct mechanisms in neoplastic and non-neoplastic cells [45].

The second imprinted domain of the 11p15.5 chromosomal region contains at least six imprinted genes (five of which display maternal-specific expression) and a long non-coding RNA gene that is only expressed on the paternal chromosome (*KCNQ1OT1*) [47]. The allele-specific expression of all genes in this domain is regulated by an imprinting control region known as *KCNQ1OT1*:TSS-DMR, also known and herein indicated as imprinting center 2 (IC2), which corresponds to the promoter of *KCNQ1OT1* located within the intron 10 of *KCNQ1* (Fig. 2A). IC2 is differentially methylated between the two parental chromosomes. The absence of DNA methylation on the paternal allele allows the *KCNQ1OT1* transcript to be expressed, which in turn represses *in cis* the expression of the flanking imprinted genes [47]. In contrast, methylation at the maternal IC2 represses *KCNQ1OT1*, with the consequent expression of the flanking imprinted genes on the maternal chromosome, including the growth-inhibitor gene *CDKN1C* (Fig. 2A). Most Wilms tumors lose the heterozygosity of this imprinted domain (Fig. 2B); in contrast, a small subset of sporadic Wilms tumors loses DNA methylation at the IC2 (Fig. 2C) [48]. Additionally, a recent study described two BWS patients with Wilms tumor or nephrogenic rests who displayed a loss of methylation at the IC2, further supporting a role for the imprinted genes located at this domain in Wilms tumor development [13]. How the DNA methylation defects at IC2 arises in Wilms tumor is still unknown. A lack of transcription across this genomic region results in failure to establish methylation at the same region in the mouse female germline [49]. We recently demonstrated that genetic mutations in a subset of BWS patients reduce *KCNQ1* transcription across IC2, with consequent loss of methylation at this genomic region [50]. Although a direct role of *KCNQ1* transcription in the regulation of imprinting methylation maintenance at IC2 has not been demonstrated, the low transcriptional levels of *KCNQ1* in Wilms tumor with hypomethylation at the IC2 could suggest that a similar mechanism can be responsible for this methylation defect in non-syndromic Wilms tumor.

Environmental stimuli can also affect the methylation profile of IC2.

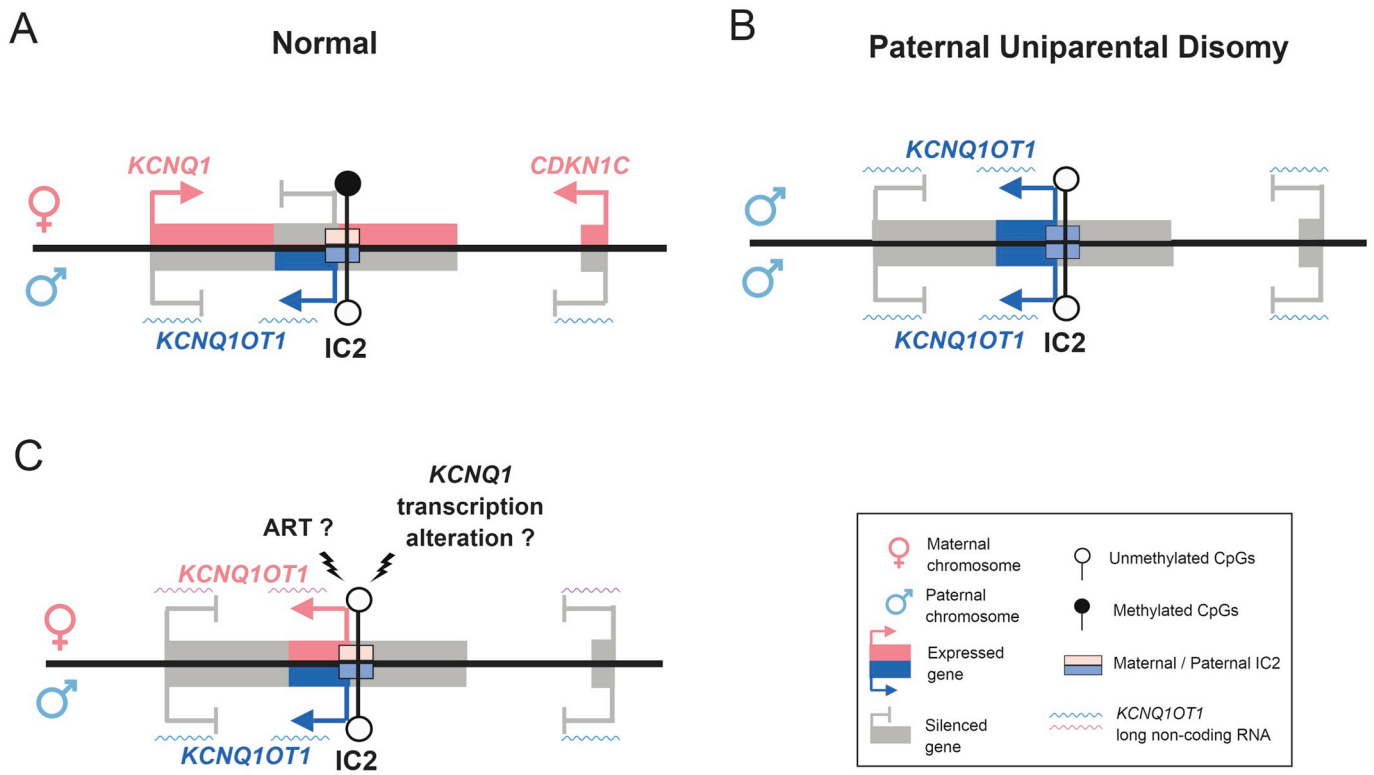

Fig. 2. Loss of methylation at IC2 in Wilms tumor. (A) Schematic representation of the normal methylation at IC2, and parent-of-origin-specific allelic expression of *KCNQ1OT1*, *KCNQ1* and *CDKN1C* genes. (B, C) The possible mechanisms responsible for epimutations at IC2 include paternal uniparental disomy (B) and environmental insults (such as hormonal stimulation, embryo manipulation and/or cryopreservation during ART) or *KCNQ1* transcription alteration (C). Loss of methylation at IC2 results in biallelic expression of *KCNQ1OT1* and silencing of *KCNQ1* and *CDKN1C* in Wilms tumor.

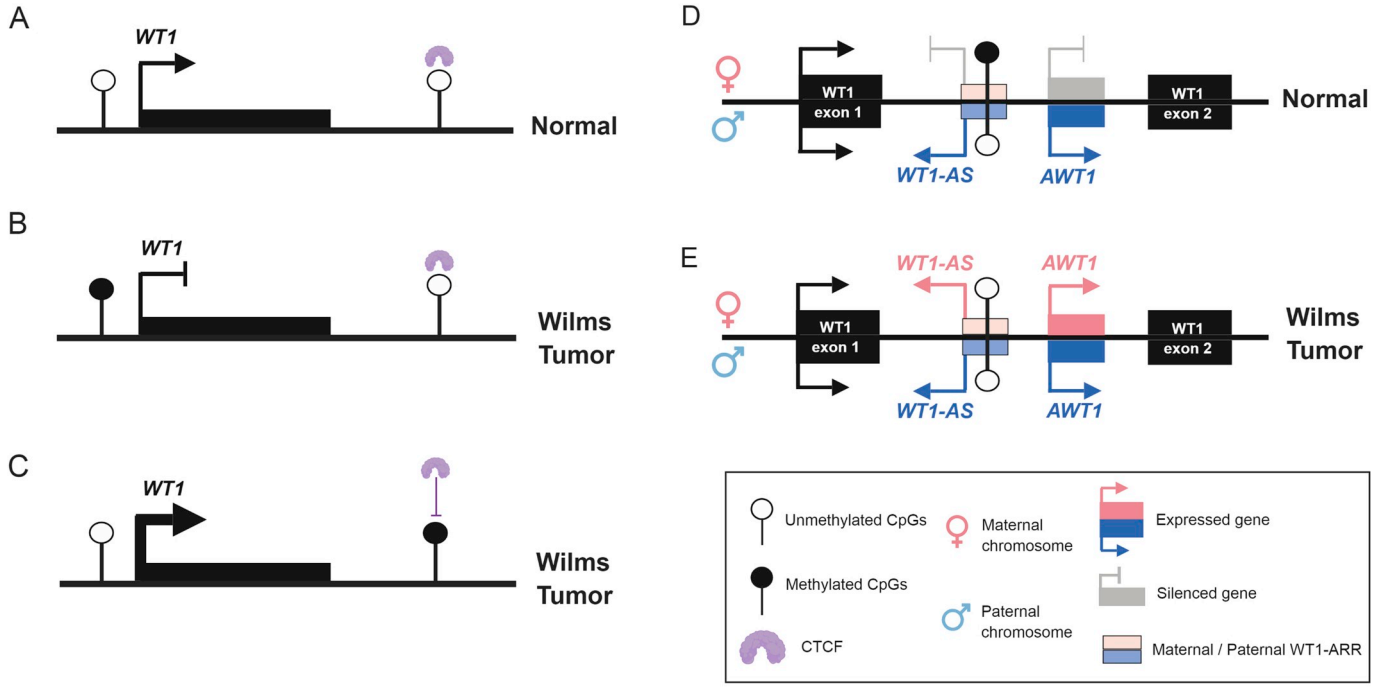

Fig. 3. DNA methylation defects at the 11p13 chromosomal region in Wilms tumor. (A) Schematic representation of the methylation profile at *WT1* locus associated with its expression status. (B) Gain of methylation at the *WT1* promoter reduces *WT1* expression in a subset of patients with WT. (C) Gain of methylation at CTCF binding sites downstream of the *WT1* gene is associated with *WT1* overexpression. (D) DNA methylation profile at the WT1-ARR and allele–specific expression of *AWT1* and *WT1-AS* in normal kidney. The WT1-ARR is ~50% methylated in normal kidney suggesting that it is methylated only on one parental allele, however, the maternal allele–specific methylation profile has not yet been reported (E) Loss of methylation at WT1-ARR results in biallelic expression of *AWT1* and *WT1-AS*.

In this context, different studies have reported a strong association between assisted reproduction technology (ART) and loss of methylation at IC2 in different imprinting disorders, suggesting that the hormonal stimulation, embryo manipulation and/or cryopreservation during ART can contribute to the IC2 methylation defects [51]. However, epidemiological studies that verify an association between ART and Wilms tumor are still lacking.

2.2. The 11p13 chromosomal region

The human chromosomal region 11p13 contains a cluster of genes involved in the mammalian development of several organs of the urogenital system [52]. In 1990, Rose *et al.* demonstrated that genetic deletion of this genomic region is associated with the WAGR syndrome [53]. This study described the first genetic locus associated with Wilms tumor, called Wilms tumor 1 (*WT1*) [53]. In humans, this gene is biallelically expressed during kidney development and its imprinting status in other tissues is still controversial; while it was initially reported that *WT1* is mainly expressed on the maternal allele in fetal brain and uterus [54], it was recently showed that *WT1* is generally low expressed on both alleles in all analyzed tissues, including brain and uterus [21]. *WT1* encodes a zinc-finger transcription factor involved in the regulation of different growth-inducing genes, including *IGF2*, early growth response (*EGR1*) and platelet-derived growth factor A (*PDGFA*) [55–57]. Some WT1 isoforms may have a role in RNA processing rather than in transcription regulation [58]. *WT1* was originally described as a tumor suppressor gene involved in Wilms tumorigenesis; however, different studies have demonstrated a possible oncogenic role for *WT1* in this malignancy [59,60]. Indeed, cases of Wilms tumor have been reported as having loss-of-function mutations of *WT1* or hyperexpression of *WT1* [48,61]. In both cases, the genetic alterations and/or DNA methylation defects at the *WT1* promoter only affect a small percentage of children with Wilms tumor (Fig. 3B) [48, 60-62]. In contrast, hypermethylation at CTCF binding sites localized downstream of the *WT1* gene is more frequently associated with the overexpression of *WT1* in a larger subset of Wilms tumor patients (Fig. 3C) [61,63]. Given that one role of CTCF is to protect different genomic regions against DNA methylation [64,65], Zitzmann and colleagues suggested that a gain of DNA methylation at these genomic sites in Wilms tumor could simply be the consequence of a lower CTCF binding affinity to DNA [63].

A truncated variant of WT1 (AWT1) and a non-coding WT1 antisense RNA (*WT1-AS*) are transcribed from two different promoters located in intron 1 of the *WT1* gene [66,67] (Fig. 3D). The *AWT1* gene has a different first exon but the same exons 2–10 as *WT1*; thus, the AWT1 protein lacks the transcriptional repression domain present at the N-terminal of WT1, and generally acts as a potent trans-activator of WT1-targeted gene promoters [66]. Intriguingly, *WT1-AS* overlaps with the 5′-end of the WT1 sense transcript and has a possible regulative role in *WT1* gene expression [68,69]. Dallosso and colleagues suggested that both *AWT1* and *WT1-AS* could be affected by imprinting regulation in the adult kidney, with expression restricted to the paternally inherited allele [66]. The same authors proposed that a *cis*-acting genomic element located within the *WT1* antisense regulatory region (WT1-ARR) might regulate the monoallelic expression of both genes via DNA methylation [66,67] (Fig. 3D). The WT1-ARR element, indeed, is ~50% methylated in normal kidney, as observed for the imprinted DMRs where this epigenetic mark is associated with only one parental allele [67]; and a large subgroup of Wilms tumors patients show loss of methylation at this genomic region associated with the biallelic expression of *WT1-AS* and *AWT1* (Fig. 3E). The methylation defect does not affect the *WT1-AS* or *AWT1* promoters, nor other CpG islands located at the 5′-end of the *WT1* gene, suggesting a key role of WT1-ARR in the monoallelic expression of *WT1-AS* and *AWT1* [66]. Interestingly, the WT1-ARR is already slightly hypomethylated in nephrogenic rests as compared to the normal kidney; however, in this case the epigenetic defect is not associated with the biallelic expression of *WT1-AS* and *AWT1* [70], suggesting that loss of methylation at WT1-ARR is an early event in Wilms tumorigenesis, but that a second “hit” is required to complete the erasure of methylation for the biallelic expression of *WT1-AS* and *AWT1* as well as the neoplastic progression.

During cellular differentiation, the establishment of DNA methylation at a large group of intragenic CpG islands depends on the transcriptional activity running across them [71], raising the possibility that the *WT1* downregulation found in a subset of Wilms tumor could be responsible for the loss of methylation at WT1-ARR.

2.3. LOI at multiple genetic loci?

Whether LOI in Wilms tumor specifically affects the imprinted genes located on chromosome 11or multiple genetic loci is still being debated. In 2007, Bjornsson and colleagues analyzed the methylation and expression status of 38 imprinted genes in a series of Wilms tumors with LOI at *IGF2* and found that the methylation defects did not affect genomic regions besides the 11p15.5 chromosomal region [72]. Similarly, in a different series of Wilms tumors, other studies excluded the involvement of methylation defects at the imprinted gene cluster localized at 14q32 chromosomal region [73,74]. In contrast, other studies have shown the involvement of different imprinted loci in some Wilms tumors. For instance, Dekel and colleagues showed that multiple paternally-expressed imprinted genes (e.g., *MEST, PEG3, NNAT, PEG10, DLK1, MEIS1* and *MEIS2),* located in genomic regions other than 11p15.5, were up-regulated in a subset of Wilms tumors compared to normal tissue. The deregulated expression of these genes contributes to the Wilms tumor phenotype by inhibiting cell differentiation and maintaining cell proliferation [75]. A further study described a series of Wilms tumors with hypermethylation at the *IGF2/H19* locus and overexpression of five imprinted genes *(MEST, NNAT, DLK1, RTL1* and *MEG3)* located in multiple genomic regions [76]. A second group of Wilms tumors, additionally, showed hypermethylation at the 11p15.5 locus and loss of methylation at two potential regulatory sites located at *NNAT/BLCAP* imprinted locus [77]. Loss of methylation was highest for three putative CTCF binding sites located upstream the *NNAT* gene and was associated with upregulation of both *NNAT* and an alternative transcript of the *BLCAP* imprinted gene [77]. Altogether, these data indicate that Wilms tumors can show a widespread failure of imprinting, raising the possibility that this imprinting error could be caused by the deregulation of a common superordinate regulatory mechanism of imprinted genes. Interestingly, different reports recently described familial Wilms tumors with a loss-of-function mutation in the *KAP1* gene [78–80]. Given the key role that the KAP1 protein plays in the maintenance of DNA methylation at imprinted loci [23,24], we speculate that *KAP1* mutations may be responsible for the generalized imprinting defects.

3. DNA methylation defects at non-imprinted genomic regions

Other genomic regions besides imprinted loci can present methylation defects in Wilms tumors. These neoplasms generally show a global loss of methylation as compared to both normal kidney and nephrogenic rests [81], which may play a key role in malignant transformation. Studies of tumorigenesis in rodents fed methyl-deficient diets demonstrate a causative role of loss of DNA methylation in cancer progression [82]. Interestingly, global DNA hypomethylation in Wilms tumor is not associated with significant changes in DNA methyltransferases levels, suggesting that the epimutation is not due to aberrant *de novo* DNA methylation [83]. In accordance, TET proteins mediate loss of methylation in different kind of cancers, with an active demethylation process during carcinogenesis [84–86]. Recent studies have shown that the WT1 protein recruits the TET2 demethylases to DNA, promoting DNA demethylation in brain tumors and leukemia [87,88]. These data raise the possibility that the *WT1* loss of mutations found in a subset of Wilms tumors can contribute to the DNA

hypomethylation during tumorigenesis.

Satellite DNA (Sat 2 and Sat α) and the long interspersed element-1 (LINE-1) are the genomic regions mainly affected by loss of methylation in Wilms tumor. These regions are normally methylated DNA repetitive sequences that ensure genome stability. Their methylation profile is totally lost in Wilms tumor, which increases genome instability in the cancer cells [89,90]. Interestingly, more severe LINE1 hypomethylation is associated with anaplastic Wilms tumors and higher tumors relapse, suggesting that LINE1 methylation levels could be used as an independent prognostic marker for this malignancy [91]. The cause of the methylation defect at these genomic elements is still unknown. The role of KAP1 protein in the regulation of methylation levels at repetitive elements now raises the question whether the loss-of-function mutations of *KAP1* in Wilms tumors contribute to this epimutation [78–80,92].

In addition to imprinted genes and DNA repetitive sequences, multiple differentially methylated regions (DMRs) have been identified in Wilms tumor [93–97]. Loss of methylation at growth-induced genes is associated with their overexpression, as demonstrated for the hypomethylation at the 5′-ends of the *GLIPR1/RTVP-1*, *SIX2* and *MYCN* genes [98–100]. On the other hand, hypermethylation at tumor suppressor genes promoters (e.g., the protocadherin cluster, *RASSF1A*, *HACE1*, *P16*, *WWOX* and *RECK*) is generally associated with gene silencing [101–105] (Table 1). Intriguingly, it was recently demonstrated that hypermethylation at the *RECK* tumor suppressor gene promoter is closely associated with metastasis of Wilms tumor, and treatment with curcumin (a phenolic molecule produced by some plants) strongly reduces *RECK* methylation and abolishes the progression and metastasis of Wilms tumor cells [105]. The potential for this kind of treatment should be further studied for Wilms tumor.

Even though alterations of methylation (and thus expression) of many of different genomic regions have been shown to play key roles during carcinogenesis of different cancer types [106–109], whether the epigenetic defect is the cause or consequence of the altered transcription is still not clear [71,110]. Recent evidence underscored the causal effect of the WT1 protein in Wilms tumorigenesis [111]. WT1 directly regulates *DNMT3A* gene expression, and its depletion from Wilms tumor cells decreases DNMT3A binding at the protocadherin genomic cluster as well as at *RASSF1A* tumor-suppressor gene promoters, thereby reducing the methylation profile at these genomic sites [111]. Therefore, the *WT1* overexpression found in a subset of Wilms tumor patients may cause *de novo* methylation at tumor suppressor gene promoters by increasing levels of DNMT3A.

In addition to the DNA methylation defects, different genomic regions can also be affected by copy-number alterations (CNAs) in Wilms tumor [112]. Different studies reported recurrent somatic copy-number gain or loss of distinct chromosomal regions, including the LOH of 1p, 11q, 16q, and 22q, deletions at 12q, 17p and 18q, as well as, gain of 1q and 2p24 [112–114]. Interestingly, the combined LOH of 1p and 16q is generally associated with poor prognosis and higher tumor relapse [113]. Similarly, the gain of 1q genomic region correlates with death in Kenyan patients with Wilms tumor, and the gain of 17p13 region (where the *TP53* gene is located) is associated with anaplastic tumor [115,116]. The CNAs can affect the expression of oncogenes and/or tumor suppressor genes by altering their dosage or by breaking genomic regions that regulate their transcription [115]. However, if the DNA methylation is regulated by *cis*-acting mechanisms, the CNAs should not affect the genome methylation. Intriguingly, Sun and colleagues recently observed that the DNA methylation is redistributed along the genome upon CNAs in several types of cancer, suggesting that *trans*-acting mechanisms activated by CNAs can regulate DNA methylation [117]. Nonetheless, if this mechanism can occur also in Wilms tumors is still an open question.

4. Molecular differences between unilateral and bilateral Wilms tumor

Wilms tumor is generally present in only one kidney of an organism (unilateral), however, it can affect both kidneys (bilateral) in about 5–7% of cases [118]. In contrast to the unilateral tumor, the bilateral disease is frequently due to early disruption of renal development caused by germline genetic or epigenetic alteration [119]. A higher frequency of bilateral Wilms tumors, indeed, has been reported in children with WAGR and BWS syndrome, which shown constitutional (epi)mutations [120,121]. These (epi)mutations frequently alter the normal mesenchymal to epithelial transition that occurs during renal development, enabling the presence of retained embryonic tissue in the normal kidney (nephrogenic rests). Therefore, almost all children with bilateral diseases show larger nephrogenic rests compared to unilateral tumors [122].

The molecular alterations mainly associated with bilateral rather than unilateral Wilms tumors are germline mutations at *WT1* locus, the constitutional LOI at 11p15.5, duplication of the 2p24.3, *de novo* translocation t(5; 6) (q21; q21) and mosaic variegated aneuploidy [123–125]. Despite germline mutations have been reported in several other genes (*DICER1*, *DROSHA*, *DGCR8*, *XPO5*; *DIS3L2*, *SIX1/2*, *KAP1)*, it is still unknown whether these mutations are linked with bilateral disease [79, 126-128]. Interestingly, while the gain of methylation at IC1 can be associated with either unilateral or bilateral Wilms tumors, the LOH of 11p, in contrast, is less frequent in bilateral than unilateral tumors, suggesting that this cytogenetic defect is a later event in Wilms tumorigenesis [119,129]. In agreement with this hypothesis, different studies have previously reported children with bilateral Wilms tumors with LOH of 11p in only one tumor [129,130]. Whether the LOH of other chromosomal regions rather than 11p is differently linked with unilateral or bilateral Wilms tumors is still unknown.

5. DNA methylation as blood-based marker of Wilms tumor

Genetic and epigenetic analyses of circulating cell-free DNA (cfDNA) isolated from Wilms tumor patients' blood can be used to predict tumor prognosis and monitor tumor response to chemotherapy,

Table 1
Non-imprinted genomic regions affected by methylation defects in Wilms tumor.

Methylation Defect	Genomic Location of the DMR[a]	Associated Genes	References
Gain of methylation	Promoter	*CASP8, MGMT, NORE1A*, INK4a/ARF, *DAPK, CALCA, SLIT2, RASSF1A, WWOX, HIN-1, AURKC, RECK*	[83,93–96,101,104,105]
	Gene body	*PRRT1, MYO7A, TNFRSF12, RPS6KA4/MIR1237*	[83,96,133]
	Proximal CpG island	*ZNF311, HACE1, PCDHA@, PCDHB@, PCDHG@*	[97,102,103,133]
	5′-UTR or 3′-UTR	*MCJ, TP73*	[83,96]
Loss of methylation	Promoter	*MYCN, SIX2*	[99,100]
	Gene body	*MTHFR, GLIPR1/RTVP1, MYCN*	[83,98,100]
	Repetitive elements: LINE-1, Satellite 2, Satellite α	Not applicable	[89,90]

[a] DMR, differentially methylated region.

and can help to differentially diagnosis distinct cancers [131,132]. Pritchard-Jones and colleagues analyzed the methylome of cfDNA extracted from the blood of children with or without Wilms tumor and found a genomic region (DMR-2) close to the *PRRT1* gene that has relatively higher methylation levels in children with Wilms tumor prior to treatment. Additionally, the levels of the epimutation at this genomic region were shown to significantly increase after the pre-operative chemotherapy phase and to persist into the immediate postoperative period [133]. On the other hand, Song *et al.* showed that methylation at the *SIX2* gene promoter is significantly reduced in preoperative venous blood of patients with Wilms tumor compared to those without, and it is generally associated with low survival rates of children with Wilms tumor [99]. Together, these data suggest that methylation defects of DMR-2 and *SIX2* could be used as blood-based biomarkers of Wilms tumor, and that analyses of the methylation profiles in blood from patients with Wilms tumor can be a non-invasive tool to help diagnose this malignancy.

6. Concluding remarks and future perspectives

Different molecular mechanisms, including stochastic error, environmental stimuli, and genetic and cytogenetic aberrations contribute to altering the methylation profile of Wilms tumor cells. Even though (epi)mutations can affect both imprinted genes and non-imprinting genomic regions, it is most frequently associated with the imprinted 11p15.5 and 11p13 regions.

The conventional epigenetic drugs used to modify the DNA methylation profile (e.g., azacitidine, decitabine) affect the methylation of the whole genome, thereby ruling out their usefulness for a more specific clinical management of diseases characterized by targeted DNA methylation alterations [134]. The recent development of the CRISPR/dCas9 system now enables the epigenome of any organism to be manipulated in a locus-specific manner. For this, a catalytically inactive Cas9 (dCas9) is engineered to bring the enzymatic domain of DNA methyltransferases (DNMT3a) or demethylases (TET1) to a target genomic region recognized by the guide RNA via standard Watson-Crick base-pairing [135]. Editing of the DNA methylation defects associated with growth-related genes (such as *IGF2/H19*) could revert their expression to normal levels, which would thereby reduce the proliferation/size of Wilms tumors. Therefore, the CRISPR/dCas9 fused with Tet1 or DNMT3a catalytic domains represents a promising technology for personalized medicine of Wilms tumor in the future.

Abbreviations

ART, assisted reproductive technology; BWS, Beckwith–Wiedemann syndrome; cfDNA, cell-free DNA; CNAs, copy number alterations; CRISPR, clustered regularly interspaced short palindromic repeats; DMRs, differentially methylated regions; ICRs, imprinting control regions; LOI, loss of imprinting; LOH, loss of heterozygosity; PS, Perlman syndrome; SGBS, Simpson–Golabi–Behmel syndrome; SIOP, International Society of Paediatric Oncology; WAGR,Wilms tumor, aniridia, genitourinary anomalies and intellectual disability syndrome; WT1-ARR, WT1 antisense regulatory region; 5hmC, 5-hydroxymethylcytosine.

Author contributions

G.V. conceived the idea for the review, searched the literature and wrote the manuscript. Z.A. helped in the literature search, wrote the reference section and generated the table. B.A. generated the figures. J.R. and F.C. edited the final version of the manuscript.

Declarations of interest

None.

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