A Congenital Diaphragmatic Hernia and 46,XX Disorder of Sex Development Caused by a *WT1* Pathogenic Variant

Daries M[1], Moniez S[2], Cartault A[3], Sartor A[4], Aubert-Mucca M[5], Mouttalib S[6], Mallet D[7] and Pienkowski C[8*]

[1]Centre de référence des Pathologies Gynécologiques Rares, Unité d'Endocrinologie Pédiatrique, Hôpital des Enfants, CHU Toulouse, 31059 Toulouse, France

[2]Centre de référence des Pathologies Gynécologiques Rares, Unité d'Endocrinologie Pédiatrique, Hôpital des Enfants, CHU Toulouse, 31059 Toulouse, France

[3]Centre de référence des Pathologies Gynécologiques Rares, Unité d'Endocrinologie Pédiatrique, Hôpital des Enfants, CHU Toulouse, 31059 Toulouse, France

[4]Centre de Diagnostic anténatal, Hôpital Paule de Viguier, CHU Toulouse, 31059 Toulouse, France

[5]Service de Génétique Médicale, Hôpital Purpan, CHU Toulouse, 31059 Toulouse, France

[6]Service de Chirurgie viscérale pédiatrique, Hôpital des Enfants CHU Toulouse, 31059 Toulouse, France

[7]Laboratoire de Biochimie et Biologie Moléculaire Grand Est, UM Pathologies Endocriniennes Rénales Musculaires et Mucoviscidose, Groupement Hospitalier Est, Hospices Civils de Lyon, Bron, France.

[8]Centre de référence des Pathologies Gynécologiques Rares, Unité d'Endocrinologie Pédiatrique, Hôpital des Enfants, CHU Toulouse, 31059 Toulouse, France

*Corresponding author:

Pienkowski Catherine,
Centre de référence des Pathologies
Gynécologiques Rares,
Unité d'Endocrinologie Pédiatrique, Hôpital
des Enfants , CHU Toulouse, 330 Avenue
de Grande Bretagne, 31300 Toulouse, France,
Tel : +33-5-34-55-85-56,
Fax : +33 5 34 55 85 58,
E-mail: pienkowski.c@chu-toulouse.fr

Abbreviations:

DSD: Disorder of Sex Development; CDH: Congenital Diaphragmatic Hernia; GCT: Germ Cell Tumour

1. Abstract

WT1 is an important gene in gonadal differentiation process, especially in the male differenciation. It is known in some syndromic and non-syndromic pathology. This gene is associated with under-virilization in 46,XY patient. Here we report a 46,XX case presenting with external genital virilization, diaphragmatic hernia and wilms tumor. A screening on a Next Generation Sequencing (NGS) panel of 11 genes involved in 46,XX disorder of sex development (DSD) revealed the heterozygous *de novo WT1* nonsense variant NM_024426.4 c.1468C>T p.(Gln490*). A gain-of-function effect seems to be predicted for his variant affecting the fourth zinc finger region of the protein, as previously described. This case report confirms the implication of *WT1* gene in 46, XX DSD and expands its phenotype with the association of diaphragmatic hernia.

2. Introduction

Most of 46,XX disorder of Sex development (DSD) are due to androgen excess, with congenital adrenal hyperplasia as the main cause. Less frequently, 46,XX DSD are associated with disorder of gonadal development such as ovotesticular DSD or a genetic involvement caused by the translocation of *SRY* or a *SOX9* du-

plication [1, 2]

Concerning other determining genes, *WT1*, gene [MIM*607102] (Wilm's tumor suppressor gene 1), located at chromosome band 11p13, is involved in various embryological process stages such as in early gonadal differentiation [3] , kidney formation [4] and diaphragm development formation [5]. Heterozygous *WT1* variants have either been reported in individuals with nonsyndromic Wilms tumor [MIM#194070] [4] or nephrotic syndrome type 4 [MIM#256370], and in association with syndromic forms in Frasier Syndrome [MIM#136680] or Denys-Drash Syndrome [MIM#194080]. Moreover, the contiguous gene deletion at 11p13 is well-known as WAGR Syndrome [MIM#194072], causing the association of Wilms tumor, aniridia, genitourinary anomalies and mental retardation syndrome (aniridia due to *PAX6*, other features probably due to *WT1*).

Interestingly, two descriptions of 46, XX DSD associated with *WT1* variants were recently reported. The first case identified a missense variant by whole exome sequencing in a 46, XX case presenting with a syndromic form with male external genitalia, dysgenic testis, microcephaly and small uterus [6]. Secondly, Gomes *et al* identified a frameshift variant by target massively parallel sequencing in a 46, XX girl with atypical genitalia charactarized by clitoromegaly, single perineal opening, short blind-ending vagina, and bilateral testes with seminiferous tubules [7]. In both cases, *WT1* variants affected the fourth zinc-finger DNA-binding domain of the WT1 protein.

Here, we present an original case of a 46, XX DSD borned with Congenital diaphragmatic hernia (CDH) with genital virilization in which we identified a novel heterozygous *WT1* variant.

3. Materials & Methods

This study was designed in compliance with the tenets of Helsinki declaration and informed consent was obtained for all individuals included.

Molecular analysis consisted on a Massive Parallel Sequencing (MPS) panel of 11 genes involved in 46,XX DSD. Pathogenic and probably pathogenic variants were confirmed by Sanger analysis.

3.1. MPS Methods:

Custom capture probes were designed with SeqCap EZ Choice and NimbleDesign software (Roche, USA) targeting 11 genes involved in 46,XX DSD (more informations on demand). A library of all coding regions +/- 50 bp was prepared using the Kapa Nimblegen (Roche, USA) following the manufacturer's instructions. Paired-end 2X150-bp sequencing was performed on a NextSeq 500 (Illumina, San Diego, CA, USA). Sequence alignment to the human reference genome (hg19) and variants call and annotation were performed using an in-house bioinformatic pipeline Variant

classification followed ACMG recommandations [8] .

3.2. Sanger Methods:

A PCR amplification was performed, then PCR fragments were bidirectionally sequenced by capillary electrophoresis (3730xl sequencer, SeqScape software, Life Technologies). Sequence variations were numbered with the Adenine of the ATG initiation codon considered as the first nucleotide (NM_024426.4).

4. Results

The case is a one-year-old girl, the second child of unrelated parents. During pregnancy, ultrasound sonography revealed a left diaphragmatic hernia at 22 weeks of amenorrhea, then associated with a clitoridomegaly and an uterus (Figure 1). An amniocentesis was performed and showed antenatal karyotype was 46, XX. She was born after at 39 weeks of gestation with a weight of 3020 gr. Rapid surgical treatment of her hernia was done on day two, with succinate hydrocortisone in order to avoid a potential adrenal insufficiency. Post-operative recovery was uncomplicated. On clinical examination, she presented with a clitoromegaly Prader 2 characterized by a 2.5 cm erectile genital bud, a misplacement of the urinary meatus too low implanted and a single short urogenital opening (Figure 2), with a 5 mm common channel. Ultrasonography and MRI revealed a right hemi-uterus (16 x 7,5mm) without any ovarian structure but composed with an endometrial line, and a renal asymmetry (44 mm for the right one vs 30 mm for the left one). Biological testing at day 1 showed elevated testosterone (105 ng/dL) and normal adrenal hormones without adrenal insufficiency. Mini-puberty occurred between the first and the fifth months of life. At one month of age, elevated testosterone (140 ng/dL) was detected, with elevated inhibin B (109 pg/mL) levels, low estradiol (11 pg/mL) and low anti-mullerian hormone (11 ng/mL). Testosterone level varied from 159 ng/dL at three months to 63 ng/dL at five months. A male hormonal profile was confirmed by testosterone and inhibin levels showing persistent Leydig cells and Sertoli cells secretion (Table 1). Renal function was unremarkable without proteinuria. Control postnatal karyotype was 46,XX, with no evidence of *SRY* translocation and no chromosomal imbalance detected on the array-CGH. A screening on a Next Generation Sequencing (NGS) panel of 11 genes involved in 46,XX DSD revealed the heterozygous *de novo WT1* nonsense variant c.1468C>T p.(Gln490*). A right-kidney nephroblastoma was discovered at the age of three months, and treated by chemotherapy and nephrectomy. After an expert national meeting consensus, two decisions were made: first to keep the female gender assignment accepted by the family, and secondly not to perform surgery on the gonads for the moment. A right streak gonads with fallopian tube and uterine hypoplasia was detected during the nephrectomy laparoscopy.
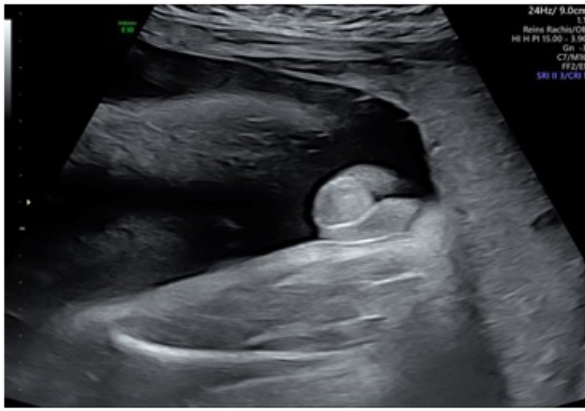
1a. Sagittal section centered on clitoral hypertrophy, 30 amenorrhea week

1b. 3D representation of the external genitalia, 30 amenorrhea week
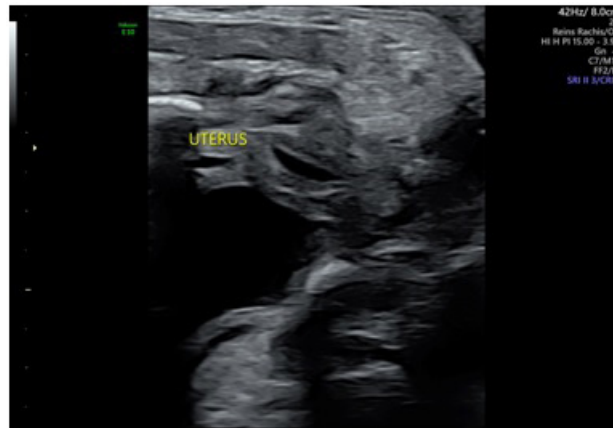
1c. Sagittal section centered on the uterus, 32 amenorrhea week
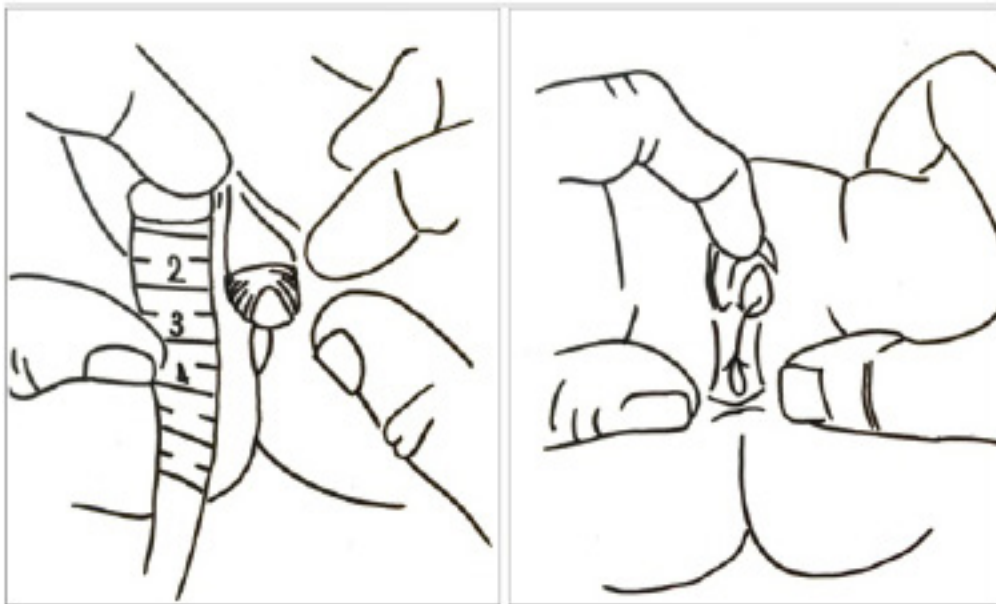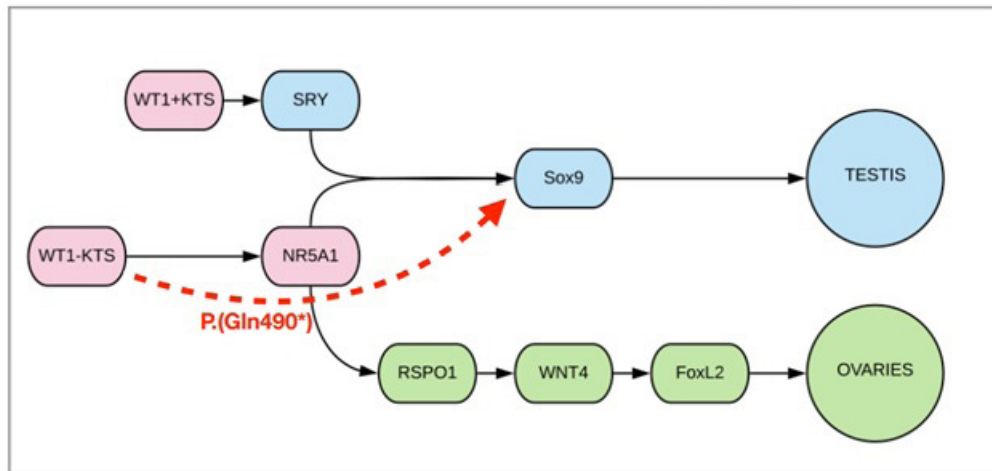
Figure 1: Ultrasound sonography

Figure 2 : Representation of the external genitalia

Figure 3 : Schematic overview of gonadal differentiation signalling pathway
the red arrow represents the hypothetical mechanism of diversion from the normal pathway by the *WT1* nonsense variant c.1468C>T p.(Gln490*)

5. Discussion

Here we identified a novel heterozygous de novo variant in *WT1*: c.1468C>T p. (Gln490*) in a 46,XX DSD child with diaphragmatic hernia. This nonsense variant was never reported in the literature and was absent from GnomAD. According to the ACMG guidelines [8], this variant can be considered as pathogenic (PVS1, PM2, PP3). A gain-of-function effect seems to be predicted as proved by *in vitro* and *in silico* studies of protein interaction and stability [7, 9].

WT1 gene [MIM*607102] (Wilm's tumor suppressor gene 1), located at chromosome band 11p13, is involved in various embryological process stages such as in early gonadal differentiation [3], kidney formation [4] and diaphragm development [5].

WT1 is a 50 kb gene encoding for a four-zinc finger DNA-binding protein with 10 exons and various isoforms with two majors due to the insertion of three amino acids (KTS) between fingers three and four, named as -KTS or +KTS isoforms [10].

Hammes *et al.* generated mice lacking those specific isoforms of *WT1*. Heterozygous mice with reduced +KTS protein levels developed a glomerular syndrome and presented a model for Frasier syndrome. Each type of homozygous mice died early at birth due to impaired renal development. Interestingly, mice lacking the +KTS isoform showed complete XY reversion by reduced SRY expression. Lack of -KTS isoforms resulted in a more severe developmental phenotype than loss of +KTS isoforms [11].

WT1 has a key role in gonad differentiation, by the early enabling activation and maintenance of *NR5A1* through the *WT1*(-KTS) isoform [3, 12]. At the same time, *WT1*(+KTS) activates *SRY* expression [13]. SF1 and SRY act as cofactors to activate the transcription of Sox9[14]. Activating this pathway leads to male gonadal differentiation. Comparatively, *WNT4* and *RSPO1* play a

role in the activation of female pathway in XX gonads. [15, 16]. In the now three different cases of 46,XX DSD with *WT1* variant affected the fourth zinc finger, we describe the activation of male gonadal pathway. (Figure 3)

Heterozygous *WT1* variants have either been reported in individuals with non-syndromic Wilms tumor [MIM#194070] or nephrotic syndrome type 4 [MIM#256370], and in association with syndromic forms in Frasier Syndrome [MIM#136680] (gonadal dysgenesis and focal segmental glomerulosclerosis) [15], Denys-Drash Syndrome [MIM#194080] (gonadal dysgenesis and diffuse mesangial sclerosis)[16] or Meacham syndrome [MIM#608978] (gonadal dysgenesis, cardiac malformation, and diaphragmatic defect with pulmonary hypoplasia) [17]. Moreover, the contiguous gene deletion at 11p13 is well-known as WAGR Syndrome[MIM#194072], causing the association of Wilms tumor, aniridia, genitourinary anomalies such as hypospadias or cryptorchidism for boys and vaginal, uterine or ovarian abnormalities for girls and mental retardation syndrome (aniridia due to *PAX6*, other features probably due to *WT1*) [5, 18]. All of these syndromes are linked with *WT1* decreased expression and lead to a male virilization defects even to complete sex reversal comparatively to no effect on female gonadal development.

During mammalian embryonic development, *WT1* is expressed in both pleural and abdominal mesothelium which contribute to the diaphragmatic formation [5]. The link between diaphragmatic hernias and *WT1* variants was already described [19]. Homozygous *WT1* null-mice who died early at birth had diaphragmatic hernias and urogenital defects [20]. The association of a gonadal dysgenesis with diaphragmatic hernia must therefore lead to a screening of *WT1*.

At the moment, the child does not have gonadoblastoma but his

gonads need close monitoring because some DSDs are associated with a hight risk of cancer. Rathered together, the prevalence of germ cell tumour (GCT) in DSDs is 12% [21]. Known risk factors for GCT cancer are: cryptorchidism (RR x 2.9), presence of streak gonads, familial predisposition, presence of the Gonadoblastoma-Y-locus region on the Y chromosome and more specifically the *TSPY* gene [22, 23]. On out of 292 DSD XY studied, 15% developed GCT, of which 51% were malignant. Average age at diagnosis is between 14 to 21 years [24]. Incomplete testicular differentiation with delayed maturation or blockage of germ cells is also associated with an excess risk of GCT. This can be assessed by immunohistochemistry with the presence of the OCT 3/4 protein [21, 22]. Frasier syndrome seems to have the highest risk of degeneration, with a 67% of GCT risk apparition. They appear around the age of 12[15]. In both 46,XX Denys-Drash or Frasier syndromes, no GCT was reported. In Gomes case, the presence of bilateral testes with seminiferous tubules containing predominantly Sertoli cells and rare germ cells was confirmed; no gonadoblastoma was encountered [7].

Two tools can be assessed for the risk of degeneration. On one hypothesis, testicular germ cell-derived tumours (TGCTs) in humans have a highly different gene expression profile and a specific epigenetics regulation from normal germ cells residing in adult testes [25]. Kristensen *et al* [26] proposed the exploration of DNA methylation profiles as a predictive risk of GCT. Voorhoeve *et al* [27] showed that some microRNAs were overexpressed in all Germ Cell Cancer including carcinoma *in situ*. The detection of these microRNAs, especially miR-371-3 and miR-302, could be used as biomarkers in the screening and monitoring of GCT [28-30], as serum sensibility detection is high, at 98%[30]. This analysis could be then interesting to perform in our family.

6. Conclusions

This case report confirms the implication of *WT1* gene with the third description of a predicted gain-of-function variant in a 46, XX DSD child and expands its phenotype with the association of diaphragmatic hernia.

References:

1. Cools M, Nordenström A, Robeva R, Hall J, Westerveld P, Fluck C, et al. Caring for individuals with a difference of sex development (DSD): A Consensus Statement. Nat Rev Endocrinol. 2018; 14: 415-29.

2. Knarston I, Ayers K, Sinclair A. Molecular mechanisms associated with 46,XX disorders of sex development. Clin Sci. 2016; 130: 421-32.

3. Kim J, Prawitt D, Bardeesy N, Torban E, Vicaner C, Goodyer P, et al. The Wilms' Tumor Suppressor Gene (wt1) Product Regulates Dax-1 Gene Expression during Gonadal Differentiation . Mol Cell Biol. 1999; 19: 2289-99.

4. Zirn B, Wittmann S, Gessler M. Novel familial WT1 read-through mutation associated with wilms tumor and slow progressive nephropathy. Am J Kidney Dis. 2005; 45: 1100-4.

5. Scott DA, Cooper ML, Stankiewicz P, Patel A, Potocki L, Cheung SW. Congenital diaphragmatic hernia in WAGR syndrome. Am J Med Genet. 2005; 134 A: 430-3.

6. Parker KL, Schedl A, Schimmer BP. Gene Interactions in Gonadal Development. Annu Rev Physiol. 1999; 61: 417-33.

7. Gomes NL, de Paula LCP, Silva JM, Silva TE, Lerario AM, Nishi MY, et al. A 46,XX testicular disorder of sex development caused by a Wilms' tumour Factor-1 (WT1) pathogenic variant. Clin Genet. 2019; 95: 172-6.

8. Richards S, Aziz N, Bale S , Bick D, Das S, Gastier-Foster J, Grody WW, et al. Standards and Guidelines for the Interpretation of Sequence Variants: A Joint Consensus Recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology Sue. Genet Med. 2015; 17: 405-24.

9. Eozenou C, Gonen N, Touzon MS, Jorgensen A, Yatsenko AS, Fusee L, et al. Testis formation in XX individuals resulting from novel pathogenic variants in Wilms' tumor 1 (WT1) gene. Proc Natl Acad Sci U S A. 2020; 117: 13680-8.

10. Hastie ND. Wilms' tumour 1 (WT1) in development, homeostasis and disease. Dev. 2017; 144: 2862-72.

11. Hammes A, Guo JK, Lutsch G, Leheste JR, Landrock D, Ziegler U, et al. Two splice variants of the wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell. 2001; 106: 319-29.

12. Wilhelm D, Englert C. The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. Genes Dev. 2002; 16: 1839-51.

13. Hossain A, Saunders GF. The Human Sex-determining Gene SRY Is a Direct Target of WT1. J Biol Chem 2001; 276: 16817-23.

14. Sekido R, Lovell-badge R. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer.Epub ahead of print 2008; 453: 930-4.

15. Ezaki J, Hashimoto K, Asano T, Kanda S, Akioka Y, Hattori M, et al. Gonadal tumor in Frasier syndrome: A review and classification. Cancer Prev Res. 2015; 8: 271-6.

16. Roca N, Munõz M, Cruz A, Vilalta R, Lara E, Ariceta G. Long-term outcome in a case series of Denys-Drash syndrome. Clin Kidney J. 2019; 12: 836-9.

17. Suri M, Kelehan P, O'Neill D, Vadeyar S, Grant J, Ahmed SF, et al. WT1 mutations in Meacham syndrome suggest a coelomic mesothelial origin of the cardiac and diaphragmatic malformations. Am J Med Genet Part A. 2007; 143: 2312-20.

18. Le Caignec C, Delnatte C, Vermeesch JR, Boceno M, Joubert M, Lavenant F, et al. Complete sex reversal in a WAGR syndrome patient. Am J Med Genet A. 2007; 143: 2692-5.

19. Kardon G, Ackerman KG, McCulley DJ, Shen Y, Wynn J, Shang L,

et al. Congenital diaphragmatic hernias: From genes to mechanisms to therapies. DMM Dis Model Mech. 2017; 10: 955-70.

20. Carmona R, Canete A, Cano E, Ariza L, Rojas A, Munoz-Chapuli R. Conditional deletion of WT1 in the septum transversum mesenchyme causes congenital diaphragmatic hernia in mice. Elife. 2016; 5: e16009.

21. Cools M, Drop SLS, Wolffenbuttel KP, Oosterhuis JW, Looijenga LHJ. Germ cell tumors in the intersex gonad: Old paths, new directions, moving frontiers. Endocr Rev. 2006; 27: 468-84.

22. Cools M, Looijenga LHJ, Wolffenbuttel KP, T'Sjoen G. Managing the risk of germ cell tumourigenesis in disorders of sex development patients. Underst Differ Disord Sex Dev. 2014; 27: 185-96.

23. Kido T, Lau YFC. The Y-located gonadoblastoma gene TSPY amplifies its own expression through a positive feedback loop in prostate cancer cells. Biochem Biophys Res Commun 2014; 446: 206–211.

24. Huang H, Wang C, Tian Q. Gonadal tumour risk in 292 phenotypic female patients with disorders of sex development containing Y chromosome or Y-derived sequence. Clin Endocrinol (Oxf). 2017; 86: 621-7.

25. Van Der Zwan YG, Stoop H, Rossello F, White SJ, Looijenga LHJ. Role of epigenetics in the etiology of germ cell cancer. Int J Dev Biol. 2013; 57: 299-308.

26. Kristensen DG, Skakkebæk NE, Rajpert-De Meyts E, Almstrup K. Epigenetic features of testicular germ cell tumours in relation to epigenetic characteristics of foetal germ cells. Int J Dev Biol. 2013; 57: 309-17.

27. Voorhoeve PM, le Sage C, Schrier M, Gillis AJM, Stoop H, Nagel R, et al. A Genetic Screen Implicates miRNA-372 and miRNA-373 As Oncogenes in Testicular Germ Cell Tumors. Cell. 2006; 124: 1169-81.

28. Murray MJ, Halsall DJ, Hook CE, Williams DM, Nicholson JC, Coleman N. Identification of microRNAs from the miR-371-373 and miR-302 clusters as potential serum biomarkers of malignant germ cell tumors. Am J Clin Pathol. 2011; 135: 119-25.

29. Dieckmann KP, Spiekermann M, Balks T, Flor I, Loning T, Bullerdiek J, et al. MicroRNAs miR-371-3 in serum as diagnostic tools in the management of testicular germ cell tumours. Br J Cancer. 2012; 107: 1754-60.

30. Gillis AJM, Rijlaarsdam MA, Eini R, Dorssers LCJ, Biermann K, Murray MJ, et al. Targeted serum miRNA (TSmiR) test for diagnosis and follow-up of (testicular) germ cell cancer patients: A proof ofprinciple. Mol Oncol. 2013; 7: 1083-92.