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Gene 661 (2018) 22–33

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Review

Epigenetics and testicular germ cell tumors

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ARTICLE INFO

Keywords:
Testicular cancer
Mutation
Epimutation
Biomarker
Therapy

ABSTRACT

Malignant testicular germ cell tumors (TGCTs) are the most frequent testicular cancers in Caucasian males, developing at the most productive age of man. We are briefly reviewing TGCT-tumorigenesis with an emphasis on epigenetics. Epigenetic mechanisms such as DNA methylation and histone modifications together with RNA interference that all change gene expression are driving early spermatogenesis. Deregulation of normal development might lead to a testicular germ cell neoplasia *in situ* (GCNIS), from which TGCTs originate. The breakthrough epigenetic research, both in normal development and TGCT tumorigenesis, has been going on to find better biomarkers and therapy for this type of tumors.

1. Introduction

Malignant testicular germ cell tumors (TGCTs) are the most frequent testicular cancers in Caucasian males which make a total of 95% of all testicular tumors. A 70% increasing incidence in the last 20 years is probably due to combined action of (*epi*)genetic and (micro)environmental factors. They are clinically very important because they occur most often between 20 and 45 years, the most productive age of man (Elzinga-Tinke et al., 2015). Their incidence has doubled in the last 40 years with recorded annual growth of 3–6% among the Caucasian population. The incidence of these tumors is highest in northern Europe, such as in Denmark and Sweden, while it is relatively low in Africa. We have recently shown that Croatian population has an intermediate incidence rate compared to other European or European ancestry populations, but exerts a rapid and constant incidence increase instead (Sincic et al., 2012). Namely, while some authors suggest testicular neoplasms incidence rates may have leveled off or even shown signs of decline in certain populations (Holmes Jr et al., 2008; Znaor et al., 2014) temporal data for Croatian population showed no such effect. Instead, testicular neoplasms incidence was found steadily increasing and possibly with the steepest increase reported worldwide (Sincic et al., 2012). Indeed, model-based predictions in 40 countries, using population-based registry data, estimated that around one in 100 men would be diagnosed with testicular neoplasm annually in three highest risk countries of Europe among which is Croatia (Le Cornet et al., 2014). By the year 2026, Hispanics will have the highest rate of TGCT of any racial or ethnic group in the USA (Ghazarian et al., 2017).

Germ cell tumors of the testis (TGCT) are histologically divided into two main types of tumors, seminomas, and nonseminomas. Seminomas usually develop in the later stages of life, in the fourth and fifth decade, and are composed of a homogeneous population of neoplastic gonocytes. Nonseminomas occur among young men between the second and third decades of life and are more aggressive with heterogeneous histological features, including partially differentiated populations such as

Abbreviations: TGCTs, testicular germ cell tumors; GCNIS, germ cell neoplasia *in situ*; ESCs, embryonic stem cells; ECCs, embryonal carcinoma cells; PGCs, primordial germ cells; IGCNU, Intratubular germ cell neoplasia of the unclassified type; CIS, Carcinoma *in Situ*; hPGCs, human pluripotent germ cell; hEGCs, human embryonic germ cells; SNPs, single nucleotide polymorphisms; ER, estrogene receptor; CpG, cytosine ring-guanine dinucleotide complex; 5mC, 5-methylcytosine; MBDs, Methyl-CpG-a coupling protein; HAT, histone acetyltransferase; HDACs, histone deacetylases; miRNAs, microRNAs; *endo*-siRNAs, endogenous small interfering RNAs; piRNAs, PIWI-interacting RNAs; DNMT, DNA methyltransferase; TET, ten-eleven translocation protein; 5hmC, hydroxymethylated cytosine; EC, embryonal carcinoma; GCT, germ cell tumor; AFP, alpha-fetoprotein; HCG, human choriogonadotropin; YST, yolk-sac tumor; CHC, choriocarcinoma; PLAP, placental alkaline phosphatase; LDH, lactate dehydrogenase; CRISPR-Cas9, Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR associated protein 9

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https://doi.org/10.1016/j.gene.2018.03.072
Received 10 October 2017; Received in revised form 7 February 2018; Accepted 21 March 2018
Available online 29 March 2018

teratomas, yolk sac tumors and choriocarcinomas (Mikuz, 2015). Nonseminomas may contain a pluripotent component known as embryonal carcinoma (EC). EC cells are considered to be a malignant variant of embryonic stem cells (ES) because they share many morphological and biochemical features of the cells derived from the inner cell mass (Andrews et al., 2005) The first large-scale quantitative proteomic study of human embryonic stem cell-line (ESC-line) and embryonal carcinoma cell-line (ECC-line pointed to the possible regulators of these two related stem cells (Chaerkady et al., 2010). TGCT seminomas typically express SOX17 in their nuclei and are negative for SOX2, while embryonal carcinomas express SOX2 and are negative for SOX17. Both types of tumors express OCT3/4 and NANOG (Nonaka, 2009; Santagata et al., 2007).

Since spermatids and spermatozoa do not proliferate, TGCTs should arise from their precursors, cells that are mitotically active, such as primordial germ cells (PGCs) arising from the epiblast or gonocytes that have settled within the genital ridge (Osterhuis and Looijenga, 2005). According to the testicular dysgenesis syndrome (TDS) hypothesis, only cases with pre- or perinatal testicular dysgenesis may suffer from testicular cancer or infertility later in life (Meyts et al., 2013). It has been widely acknowledged that TGCTs, with the exeption of the spermatocytic tumor, arise from residual immature fetal germ cells within the adult seminiferous tubules. By the latest WHO classification, this alteration is called “Testicular germ cell neoplasia *in situ* (GCNIS)” (Moch et al., 2016) formerly called “Intratubular germ cell neoplasia of the unclassified type (IGCNU)” or “Carcinoma in Situ (CIS)” (Damjanov and Mikuz, 2013; Berney et al., 2016; Almstrup et al., 2011). We shall use the term GCNIS in the rest of the text, regardless of the expression used in original articles. For recognition of GCNIS immunohistochemical (IHC) staining of placental alkaline phosphatase (PLAP) (Fig. 1A and B), OCT4, AP2gamma, M2A/D240/PDPN or LIN28 are being used (Rajpert-De Meyts and Skakkebaek, 1994). In a recent clinical study on Disorders of sexual development (DSD) children it was proposed to differentiate between delayed germ cell maturation with lower or no neoplastic potential and infantile GCNIS with a high risk of malignant evolution using a triad: OCT 3/4 expression, quantification of germ cell atypia and ploidy in dysgenetic testes (Chemes et al., 2015).

The aim of this article is to briefly review TGCT tumorigenesis with an emphasis on aberrant epigenetic mechanisms, lately in focus of a breakthrough research that opens new possibilities in diagnostics and therapy of a wide variety of tumors (Feinberg et al., 2016).

2. Embryological frame of testicular germ cell tumors development

The primordial germ cells (PGCs) are the precursor germ cells arising from the pluripotent embryonic stem cells and can be identified in the human embryo already at the gestational age of five to six weeks (Donovan, 1994; McLaren, 2003). Led by the KIT ligand and its receptor and by the chemokine SDF1 and its receptor CXCR4, PGCs travel from the proximal epiblast through the mesentery to the genital ridge and become gonocytes (Donovan, 1994; Godin et al., 1991; Runyan et al., 2006). PGCs and gonocytes can be identified by the stem cell markers PLAP, NANOG, KIT, SOX2 (mouse) or SOX17 (human), AP2γ, SALL4, POU5F1 (OCT3/4) (Wylie, 1993; de Jong et al., 2008; Gashaw et al., 2007; Gaskell et al., 2004; Honecker et al., 2004; Kerr et al., 2008; Wong et al., 2008; Wu et al., 2006).

In the presence of the Y chromosome, gonadal stem cells express the transcription factor SRY targeting *SOX9* gene which leads to the growth of Sertoli cells (Vigueras-Villasenor et al., 2015). Sertoli cells create the microenvironment required for differentiation of gonocytes to prospermatogonia and spermatogonia. During the process of differentiation, some genes become expressed (*e.g.*, *MAGE4A*), and some are silenced (Gashaw et al., 2007; Gaskell et al., 2004; Wong et al., 2008; Vigueras-Villasenor et al., 2015; Cao et al., 2009). At that time, malignant transformation within the seminiferous tubules can occur. GCNIS (Figs. 1A, B, 2A) is considered to be a precursor lesion of most germ cell tumors, a predecessor of seminoma (Figs. 1C, D and 2C) and nonseminoma. If GCNIS retains its phenotype and its cells continue to divide similarly as spermatogonia or fetal spermatocytes, a seminoma tumor will arise. If, however, GCNIS cells are transformed into embryonic cells, the nonseminoma tumors of reproductive cells arise. It is of a fundamental biological interest to understand why and how different tumors can seemingly arise from the cells of the same type.

Nonseminomas can be divided into several types of tumors: embryonal carcinoma (Figs. 2D, 3A and B), teratoma (Fig. 3C), yolk sac tumor (Fig. 3D) and choriocarcinoma (Sheikine et al., 2012). Sometimes mixed forms can be found (Fig. 2B). Embryonal carcinoma is the most common nonseminoma tumor and occurs in 87% of cases (Bosl and Motzer, 1997). The only testicular germ cell tumor (TGCT) not

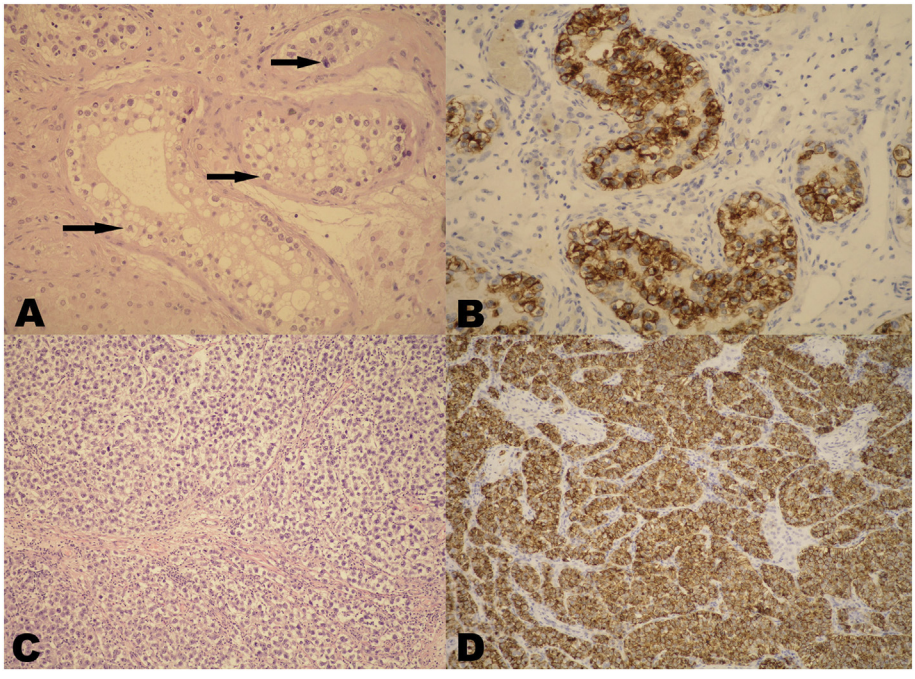

Fig. 1. Histology of testicular germ cell neoplasia *in situ* and seminoma. A) Seminiferous tubules with germ cell neoplasia *in situ* (GCNIS) (arrow); HEx200. B) Immunostaining showing positive cells of germ cell neoplasia *in situ*; anti-PLAP, DAB, counterstained with hematoxylinx200. C) Seminoma; HEx100. D) Seminoma. Positive immunostaining for PLAP; anti-PLAP, DAB, counterstained with hematoxylinx100.

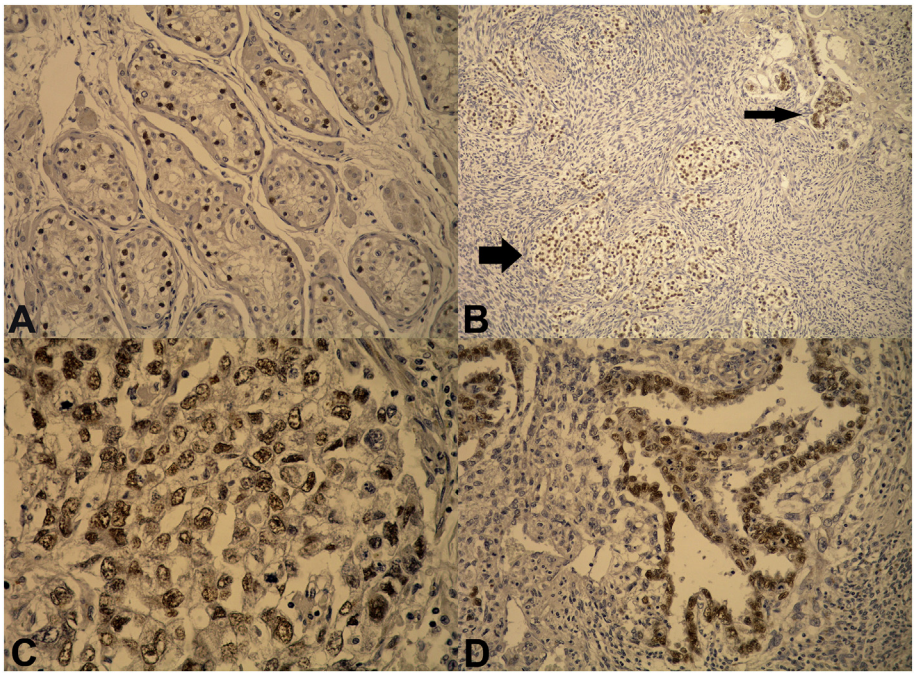

Fig. 2. Expression of pluripotency and stemness marker OCT3/4. A) Seminiferous tubules with positive cells of the germ cell neoplasia *in situ* (GCNIS); anti-OCT3/4, DAB, counterstained with hematoxylinx200. B) Mixed form consisting of seminoma (thick arrow) and embryonal carcinoma (EC) (thin arrow); anti-OCT3/4, DAB, counterstained with hematoxylinX100. C) Seminoma; anti-OCT3/4, DAB, counterstained with hematoxylinx400. D) Embryonal carcinoma; anti-OCT3/4, DAB, counterstained with hematoxylinx200.

associated with GCNIS but possibly with spermatogonial stem cells is the spermatocytic seminoma that has recently changed its name to the spermatocytic tumor (Moch et al., 2016; Waheeb and Hofmann, 2011; Osterhuis and Looijenga, 2005). A widely accepted theory of GCNIS tumorigenesis is that it starts *in utero*, under the influence of important predisposing factors, such as an increased level of the maternal estrogen or in the presence of environmental toxins. Disruption of germ cell development leads to the arrest of fetal stem cells. It seems that the influence of hormones potentiates the proliferation of dormant GCNIS cells during adolescence and young adulthood. Progression and the development of invasive forms of TGTCs are what can follow (Depue et al., 1983; Swerdlow et al., 1987).

Experiments dealing with transplantation of the mice embryo parts from which PGCs originate (*e.g.*, epiblast, early primitive-streak embryo) to ectopic sites *in vivo* resulted in the development of teratoma and teratocarcinoma. Teratocarcinoma contained derivatives of three germ layers together with malignant embryonal carcinoma cells (ECCs) (Solter et al., 1970; Bulic-Jakus et al., 2016). Already in 1981, N. Škreb assumed that these could be “tumors without mutation” (Skreb, 1981). In other words, only the changes in gene expression could lead normal gastrulating embryos to acquire a malignant phenotype. So, for decades epigenetic cues arising from the ectopic environment were thought to be the only cause for the development of malignant cells within the differentiated tissue derivatives (Solter et al., 1970; Bulic-Jakus et al., 2016; Skreb, 1981). Indeed, “recent investigations of a variety of pediatric cancers have surprisingly identified tumor types with few or no

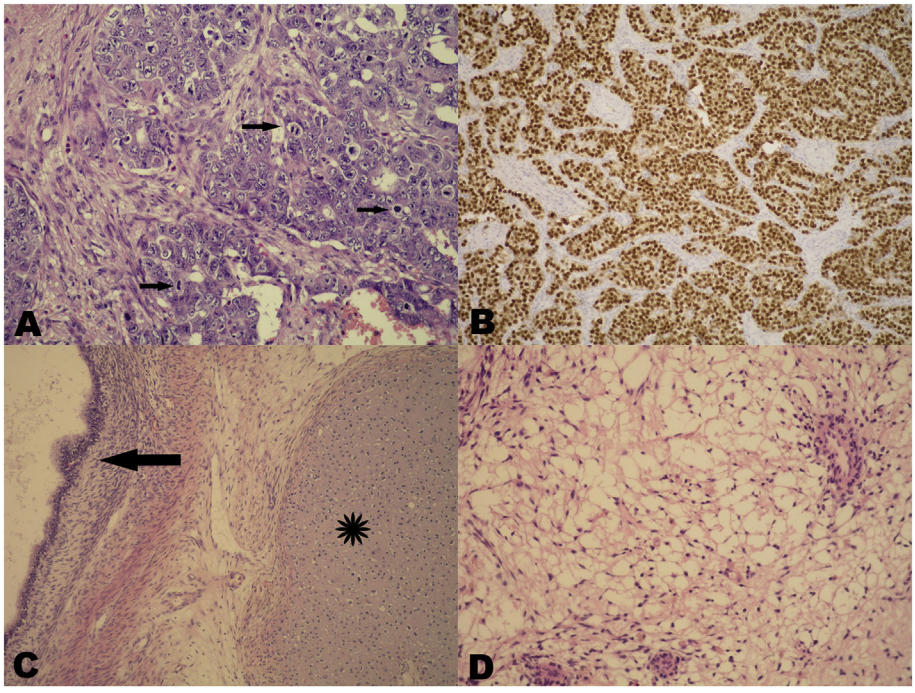

Fig. 3. Histology of testicular embryonal carcinoma, teratoma and yolk sac tumor. A) Embryonal carcinoma with numerous mitotic figures (arrow); HEx200. B) Immunostaining showing positive EC cells; anti-OCT3/4, DAB, counterstained with hematoxylinx100. C) Teratoma with cartilage (asterisk) and cystic structures lined by pseudostratified epithelial cells; HEx100. D) Yolk sac tumor; HEx200.

mutations, suggesting that epigenetic derangements can themselves drive these cancers" (Feinberg et al., 2016).

Moreover, above mentioned results of ectopic transplantation in the mouse speak for the embryonic origin of teratocarcinoma, even at an earlier stage of development - before the formation of genital ridges (Bulic-Jakus et al., 2016). Human pluripotent germ cell lines (hPGCs) were derived from the genital ridges and were characterized *in vitro* but failed to produce teratomas after transplantation to the immunocompromised mice *in vivo* in contrast to mouse embryonic stem cells (Shamblott et al., 1998; Shamblott et al., 2001). It was stated that in the absence of the definitive chimeric experiment, the *in vivo* pluripotency of human embryonic germ cells remains unresolved. Considering a possible therapeutic effect of human embryonic germ cells (hEGCs) derived *in vitro* from PGCs, the lack of teratoma formation should be desirable for therapy in human recipients (Turnpenny et al., 2006).

3. Risk factors

The exact etiology of the TGCTs is still unknown, but the geographic and racial differences in its prevalence suggest that the causes may lie in the environment or that the disease is genetic in nature. For now, risk factors include cryptorchidism, gonadal dysgenesis, TGCT in the contralateral testicle, infertility, family history, testicular microlithiasis and environmental factors. Persons with cryptorchidism suffer from testicular cancer ten times more often than those with the normal *descensus* testis. Surgical orchidopexy does not diminish the risk (Mikuz, 2015). Testicular microlithiasis is a condition characterized by deposits of calcium which is associated with the possible occurrence of testicular malignancy, especially its familial form (Korde et al., 2008; Greene et al., 2010). Environmental factors such as the prenatal exposure to diethylstilbestrol were shown to increase the relative risk of TGCT from 2.8 to 5.3% (Berney et al., 2016).

However, accordingly to the recent systematic review, not so many epidemiological studies investigated the impact of parental occupational or environmental exposure, and results were inconsistent. According to the authors, many occupational exposures during adulthood cannot be clearly associated with TGCT etiology, which might also be in line with the current hypothesis of prenatal and/or early-life origin of TGCT (Béranger et al., 2013). However, the question of parental exposure is not likely to be disregarded because *e.g.*, an investigation of parental exposure to heavy metals/welding fumes and TGCT in offspring has recently highlighted a possible association of TGCTs and high paternal chromium exposure (Togawa et al., 2016).

On the other hand, family history of TGCTs is one of the strongest and most consistent risk factors for these cancers. Patient's brothers have eight to ten times higher relative risk compared to the general population, fathers four times and sons six times (Greene et al., 2010; Béranger et al., 2013; Dong and Hemminki, 2001; Hemminki and Li, 2004).

Therefore it seems that both, the inherited susceptibility and environmental factors, may be involved in the development of the disease. The genvironmental hypothesis assumes an interaction between environmental and (*epi*)genetic parameters influencing developmental processes during early spermatogenesis (proliferation, differentiation, apoptosis) through aberrations in signaling pathways (Looijenga et al., 2013).

4. Genetic hypotheses

Somatic mutations that may lead to the development of germ cell tumors include the deletion of genes, chromosomal duplication, and loss of heterozygosity (Depue et al., 1983). Mutations in single genes are not common for TGCTs. Nonseminomas are typically hypotriploid and seminomas usually hypertriploid (Gilbert et al., 2011). Isochromosome 12p is the most common alteration, which can be found in 80% of cases in seminomas and nonseminomas. However, the existence of this alteration is not necessarily required for the development of the tumor (Looijenga et al., 2003).

Although point mutations of genes are rare, *KIT*, *TP53*, *KRAS/BRAF* and *NRAS*, that are also involved in the pathogenesis of other cancers, were found to be mutated in TGCTs (Gonzalez-Exposito et al., 2016). The most commonly mutated gene in TGCTs is the proto-oncogene *KIT*, located on chromosome 4q11-q12. KIT is the stem cell growth factor, a tyrosine kinase receptor that is phosphorylated upon binding to its ligand (KITLG) and has a crucial role in the survival, proliferation, and migration of the germ cells (Besmer et al., 1986; Flanagan et al., 1991; Yarden et al., 1987). Spermatogonia express KIT at a low level, while it is well expressed in all GCNIS, most seminomas, and some nonseminomas (Biermann et al., 2012). It is assumed that the production of KIT by malignant cells is a temporary autocrine and paracrine stimulation of tumor cell growth. *KIT* gene is mutated in 19% of seminomas and 2% of non-seminoma tumors. Two studies have shown a significantly higher percentage of *KIT* gene mutations in patients with bilateral TGCT, 93% *versus* 63.6% (Rajpert-De Meyts and Skakkebaek, 1994; Kemmer et al., 2004; Madani et al., 2003).

In TGCT, predisposing single nucleotide polymorphisms (SNPs) such as 5q31, 9p24 and 12q21 were confirmed in several independent studies. 12q21 locus has a significantly lower incidence in African-Americans compared to the white population, which could potentially explain a significantly lower incidence of TGCTs in African-Americans (Gajendran et al., 2005; McGlynn et al., 2005). Products of genes located in these loci participate as inhibitors of mitogens and as promoters of apoptosis. All these genomic variants are believed to participate in the KITLG/KIT signaling pathway (Yan et al., 2000) which is very sensitive to oncogenic stimuli. Although these SNPs are biologically important, they took part in only 15% of the family risk (Turnbull and Rahman, 2011). It has been shown that the development of the GCNIS may involve aberrant activation of the KITLG/KIT pathway and excessive expression of embryonic transcription factors such as the NANOG and POU5F1, suppressing apoptosis, increasing cell proliferation and accumulating mutations in the gonocytes. In addition to the genes whose mutations lead to a change of the cell cycle, mutations in genes encoding for the estrogen receptors have been found. It has been shown that *ER alpha* gene polymorphism is associated with azoospermia and the risk for seminoma and metastases (Brokken et al., 2012; Romerius et al., 2011). Two recent meta-analyses of GWAS studies, published in 2017, identified multiple new SNP-loci associated with TGCTs. In the Testicular Cancer Consortium study some of previously reported markers could not be identified, possibly also due to the residual population substructure. Racial differences were again noted to parallel population-specific TGCT risk. The 12 new markers that were identified increased estimation of heritability to 25% for brothers and 37% for sons (Wang et al., 2017). The second study of Litchfield et al. suggests also a polygenic model of TGCT susceptibility with transcriptional dysregulation, developmental arrest of primordial germ cells, chromosomal instability through defective microtubule function and upregulation of KIT–MAPK signaling (Litchfield et al., 2017).

A difference in structural genetic alterations for genes involved in the malignant tumor phenotype occurrence between seminomas and nonseminomas has recently been observed (Vladusic et al., 2014). The first report on exome sequencing of seminomas detected somatic mutations in almost a hundred new genes, several of which may present driver mutations. Although seminoma mutation rates were found to be five times higher than previously thought, they were lower than in other common cancers (Cutcutache et al., 2015).

5. Epigenetic breakthrough

Epigenetics is a rapidly developing discipline in biology that studies mechanisms leading to changes in the gene expression that are not caused by a change in the sequence of the DNA molecule *per se*.

Epigenetic mechanisms play a major role in cellular processes such as cell differentiation, apoptosis, and DNA repair which are compromised in tumorigenesis.

5.1. Epigenetics and cancer

Most epigenetic signatures are established during differentiation and are held stable through multiple cycles of division, allowing the cells to have a different function while containing the same genetic information. Heritability of gene expression patterns is mediated by epigenetic modifications, which include the methylation of cytosine bases in the DNA, posttranslational modifications of histone proteins and reorganization of the chromatin. Failure to maintain proper hereditary epigenetic code caused by epimutations can result in inappropriate activation or inhibition of a variety of signaling pathways leading to diseases such as the carcinoma (Sincic and Herceg, 2011). Epimutation, such as an aberrant promoter methylation, can result in the silencing of tumor suppressor genes. It may act in combination with harmful genetic mutation as a second hit required for initiation of cancer in agreement with the Alfred Knudson's "two-hit" hypothesis (Ellinger et al., 2009; Manton et al., 2005). Epimutations can lead to tumorigenesis also by the activation of oncogenes. Endogenous conditions may induce methylation of promoter regions of certain genes through estrogen or androgen inhibitors (Brait et al., 2008).

Methylation of the DNA molecule was first described, and it is the most explored among epigenetic modifications (Sincic and Herceg, 2011; Serman et al., 2006). It represents a covalent addition of methyl groups to cytosine in a cytosine ring-guanine dinucleotide complex (CpG). Methylation is catalyzed by DNA methyltransferase (DNMTs), which converts a cytosine to 5-methylcytosine (5mC). DNA methylation marks are usually located near or within the promoter region of the gene. DNA methylation can cause gene silencing, directly interfere with the DNA binding of specific transcription factors or by binding of the Methyl-CpG-a coupling protein (MBDs) that inhibit expression of genes by chromatin remodeling (Jones et al., 1998; Wade, 2001). Another frequent epigenetic modification is the posttranslational modification of histone proteins. Histones are basic proteins possessing the flexible N-terminal tail which is protruding from the nucleosome and represent the main target for modifications such as the acetylation and methylation. Acetylation is controlled by the balanced activity of histone acetyltransferase (HAT) that is adding an acetyl group at the N-terminus of lysine, while histone deacetylases (HDACs) have the opposite role. Transcriptional regulation is quite direct, acetylation of histones results in chromatin unfolding and gene transcription (Iizuka and Smith, 2003). Histone methylation also regulates gene expression. An addition of the methyl group to the N-terminal lysine is associated with either the transcriptional activity or non-activity, depending on which amino acid is modified (Santos-Rosa and Caldas, 2005). Some other posttranslational histone modifications can also change gene activity (Bannister and Kouzarides, 2011). The third mechanism involved in regulation of gene activity is the mechanism of RNA interference aimed at the destruction of specific mRNA molecules at the posttranscriptional level. It is executed by the noncoding RNAs such as microRNA, siRNA that have lately been investigated in the context of cancerogenesis and possible clinical application (biomarkers, novel therapeutics) (Hirsl et al., 2014; Hayes et al., 2014). Small RNAs, including microRNAs (miRNAs), endogenous small interfering RNAs (*endo*-siRNAs) and PIWI-interacting RNAs (piRNAs) that are involved in the control of male gamete differentiation may also participate in TGTCs tumorigenesis (Meikar et al., 2013).

Recently, even the genes that are mutated in cancer have been classified accordingly to their role in cancer epigenetics. "Epigenetic mediators" that are corresponding to the tumor progenitor genes, rarely mutated or not mutated, increase pluripotency or survival (*e.g.*, *OCT4*, *NANOG*, *LIN28*, *SOX2*, *KLF4*). "Epigenetic modifiers" of the mediators are frequently mutated in cancer (*e.g.*, *SMARCA4*, *PBRM1*, *ARID1A*, *ARID2*, *ARID1B*, *DNMT3A*, *TET2*, *MLL1/2/3*, *NSD1/2*, *SETD2*, *EZH2*, *BRD4*). "Epigenetic modulators", upstream of the modifiers, are responsive to changes in the cellular environment and are often linked to the nuclear architecture. Modulators, mutated or not, activate or repress the epigenetic machinery in cancer (*e.g.*, *IDH1/2*, *KRAS*, *APC*, *TP53*, *STAT1/3*, *YAP1*, *CTCF*) (Feinberg et al., 2016). Therefore, apart from investigations of gene mutations in TGCTs, it is important to thoroughly investigate changes in gene expression caused by epigenetic mechanisms, which are lately found in most, if not all malignant neoplasms. Because certain epigenetic marks that compose cell-specific epigenetic signatures (Hernandez-Vargas et al., 2009) are necessary for the maturation of a germ cell, it is believed that precisely their aberrations play a major role in the development of germ cell tumors.

5.2. Epigenetics of early development and TGCTs

Migrating PGCs (primordial germ cells) and gonocytes situated in the male gonad at the gestation week six are undifferentiated embryonic cells which have gone through the process of epigenetic reprogramming which includes erasure and re-establishment of DNA methylation and exchange of histone modifications (Kristensen et al., 2013). DNA demethylation erases the original epigenetic state in gonocytes, among which the genomic imprints, that are necessary for the development of two types of germ cells accordingly to the sex of the child (Meikar et al., 2013). As has been just said, germ cells, during the early stages of their development, undergo a phase of generalized DNA demethylation. This very demethylation can be either passive or active. The passive form is due to the lack of DNMT reduction that is in charge of the process of demethylation. As for active demethylation process, two possible mechanisms are proposed. The first initiator of the process may be the ten-eleven translocation (TET) protein that converts 5mC in the hydroxymethylated cytosine (5hmC) because expression of TET1 and TET2 coincides with the rapid disappearance of 5mC. Another possible mechanism can be a direct deamination of the 5mC to T, mediated by AID/APOBECI. Such deamination induces a mismatch T:G in the DNA molecule (He et al., 2011; Hajkova et al., 2010; Ito et al., 2011; Morgan et al., 2004; Iizuka and Smith, 2003; Santos-Rosa and Caldas, 2005; Bannister and Kouzarides, 2011; Hirsl et al., 2014).

Because in gonocytes the original epigenetic signature becomes erased, it is possible that an unusual combination of activated and inactivated genes in gonocytes transforms them into GCNIS. GCNIS develops into seminoma or embryonal carcinoma. Embryonal carcinoma, being totipotent, can further differentiate into teratomas (comprising cells of all three germ layers), yolk-sac tumors and choriocarcinomas (both comprising extraembryonic tissues). Importantly, it was shown that TCam-2 seminoma cell line upon xenotransplantation might transit into EC which is accompanied by considerable remodeling of the methylome. A reprogramming of a seminoma to an embryonal carcinoma increases the risk of a poor outcome and requires adjustment of the treatment strategy (Nettersheim et al., 2015).

In comparison to the normal spermatogonia, GCNIS genome remains unmethylated in the adult testis (Nielsen et al., 1974). Morphological and immunohistochemical studies show that GCNIS cells resemble fetal germ cells. GCNIS cells express the transcription factors POU5F1 (OCT3/4), NANOG, TIA-2, MYCL1, GDF3, DPPA4, KIT and TFAP2C, associated with the pluripotency of the embryonic stem cells (Almstrup et al., 2010; Sperger et al., 2003; He et al., 2011). Octamer-binding transcription factor 4 (OCT4) and SOX2 are transcription factors that play a key role in maintaining the pluripotency of ES cells. OCT4 protein, encoded by the gene *POU5F1*, is critical in the self-regeneration of undifferentiated embryonic stem cells. Therefore, OCT4 is often used as a marker of undifferentiated cells. Expression of *POU5F1* must be precisely regulated because any error will lead to changes in differentiation. Very low levels of DNA methylation are observed in cells of the GCNIS, but the activity of DNA methyltransferase1 was demonstrated. Although 5MC and 5hmC were detected in primordial

germ cells, GCNIS contain very low levels of 5MC and 5hmC in the absence of TET protein expression. Studies have shown that GCNIS cells express proteins that facilitate demethylation of DNA, such as the AID/APOBECI and BER. The same demethylation proteins are expressed in fetal germ cells but at significantly lower levels (Kristensen et al., 2014). It is believed that the pluripotency genes are associated with hypomethylation. This facilitates the increased proliferation of the GCNIS cells that are subjected to an excessive hormone activity in the post-puberty testis. All these components undoubtedly contribute to the progression of invasive cancer. The most commonly used marker for detecting GCNIS is the Placental-like alkaline phosphatase (PLAP) (Manivel et al., 1987).

Generalized DNA hypomethylation is a feature of fetal germ cells, but after birth, it changes to hypermethylation in male germ cells. GCNIS and seminomas have lowest levels of DNA methylation with relaxed chromatin structure associated with the high transcriptional activity (Lind et al., 2007). Smiraglia et al. have proposed a model where seminomas arise from GCNIS cells that emerged from the primordial germ cells, which have undergone a process of global demethylation, while nonseminomas are resulting from GCNIS cells that have passed through the *de novo* methylation (Smiraglia et al., 2002).

Indeed, undifferentiated GCTs (seminomas, GCNIS, and gonadoblastomas) are hypomethylated, whereas more differentiated GCTs (teratomas, yolk sac tumors, and choriocarcinomas) show a higher degree of methylation. Embryonal carcinomas show an intermediate pattern (Wermann et al., 2010). Different extents of methylation in different subtypes of the TGCTs favor the developmental model of male germ cell tumors origin (Sheikine et al., 2012). Change of the methylation status is linked to a gene that encodes the DNA methyltransferase 3 beta (DNMT3B). It is usually expressed in pluripotent embryonic cells and induces *de novo* methylation at that stage of development. Through DNA methylation, differentiation of embryonal carcinoma in different subtypes may also be regulated (Sheikine et al., 2012).

So far it was shown that a blocked PGC/gonocyte possesses a biallelic expression of imprinted genes (van Gurp et al., 1994; Verkerk et al., 1997), demonstrating the erased pattern of genomic imprinting. A recent study of genome-wide DNA methylation profiles of various germ cell tumors subtypes related them to specific stages of early developing embryonic germ cells. Somatic imprinting in TGCTs (belonging to the type II germ cell tumors) that was discovered might indicate a cell of origin after global demethylation but before erasure of imprint. This is earlier than previously described but agrees with the totipotent/embryonic stem cell-like potential of TGCTs (van Gurp et al., 1994; Verkerk et al., 1997; Rijlaarsdam et al., 2015).

Global DNA hypomethylation is characterized by a global loss of 5-methylcytosine (5mC) which contributes to malignant transformation by activation of oncogenes and latent retrotransposons, such as *LINE-1* (long interspersed nuclear element 1). *LINE-1* retrotransposons are highly active in TGCTs and can cause insertional mutagenesis, transcriptional dysregulation, DNA breaks and an increased level of recombination. It is believed that such changes in the *LINE-1* retrotransposons contribute to genomic instability and malignant transformation (Dobrovic and Kristensen, 2009). In a study from 2010 Mirabello and associates showed that *LINE-1* methylation level can be inherited from the parents and that hypomethylation is associated with risk of testicular cancer (Mirabello et al., 2010). Correlation between decreased levels of *LINE-1* methylation and TGCTs is stronger in patients with seminoma and patients with bilateral tumors (Mirabello et al., 2010).

Histone modifications H3K9me2 and H3K27me3 that are both associated with a restrictive chromatin structure were expressed in low levels in GCNIS cells in contrast to H3K4me1, H3K4me2/3, H3K9ac and a histone variant H2A.Z that are associated with relaxed and permissive chromatin structure. At the same time, RNA polymerase II was very active, and cells had a high proliferation rate (Almstrup et al., 2010). A recent study on 44 independent TGCT risk loci confirmed a significant enrichment of enhancer or promoter associated histone marks H3K4me1, H3K4me3, H3K9ac in TGCT cell line NTERA 2 that was tissue specific in comparison to 41 other cell lines. This was followed by analysis of expression quantitative trait loci (eQTL) and *in situ*-chromosome conformation capture in TGCT cells that confirmed physical interactions of SNPs and candidate causal genes (Litchfield et al., 2017). Seminomas show high levels of selected repressive modifications, exemplified by H3K9me2 and H3K27me3. On the other hand, nonseminomas show high methylation levels (Manivel et al., 1987; Netto et al., 2008), but the embryonal carcinoma, retains a very open and fetal-like histone profile (Almstrup et al., 2010).

5.3. Aberrant epigenetic marks in TGCTs

Seminomas show almost no CpG island methylation, while nonseminomas show the methylation of the same islands at the level of other solid tumors. Seminomas are more hypomethylated genome-wide than nonseminoma tumors and hypermethylation in specific promoter regions in nonseminomas, in contrast to seminomas was found. Also, studies of X chromosome showed a weak or a lack of methylation in seminoma, and higher levels of methylation in nonseminomas, especially in the differentiated types (Peltomaki, 1991; Looijenga et al., 1997). CpG island hypermethylation results in a change in chromatin structure and lowers the transcription. However, certain studies (Cheung et al., 2011) show that regardless of the different levels of methylation in different genes, only 20% of genes showed an association of the hypermethylation and gene activity suppression. DNA methylation is involved in genomic imprinting and X chromosome inactivation (Wilkins, 2005). There are three types of DNA methyltransferase. DNMT1 maintains methylation during DNA replication and thus contributes to the stability of gene expression of parental cells in the daughter cells. In the process of germ cells differentiation, DNMT3A and DNMT3B transfer methyl groups to cytosine residues. Analyses have recorded an increased level of the DNMT3A expression in seminoma, while the level of DNMT1 and DNMT3B remains equal. Other studies confirm an increased level of DNMT3B in non-seminomas (Okada et al., 2003). DNMT3A in healthy tissues presents a mixture of methylated and unmethylated CpG islands in intron 25, while most of the CpG islands in intron 25 are demethylated in samples of TGCTs (Chen et al., 2014).

It is believed that an important role in TGCT tumorigenesis has the epigenetic change in tumor suppressor genes such as *RASSF1A*. *RASSF1A* methylation was detected in 40% seminomas and 83% nonseminoma TGCT components (Honorio et al., 2003).

Testisin (PRSS21), a Glycosyl-phosphatidylinositol-linked serine protease, found in premeiotic spermatocytes, promotes malignant transformation *in vivo* and *in vitro* (Tang et al., 2005). Human *Testisin* gene is located on chromosome 16 and consists of six exons and five introns. It contains a 5′ CpG island and the 5′ CpG rich region. Within the gene, there are many sites containing CpG dinucleotides that can potentially be methylated. Downstream, within the field of transcription, the gene contains CpG dinucleotides, which in the case of methylation can bind Methyl CpG binding protein (MECP), such as MeCP2 and MeCP1 involved in transcription repression (Manton et al., 2005). A strong association was found between hypermethylation of the 5′ region of the gene and loss of *Testisin* mRNA expression in tumor cells. In a recent genome-wide profiling *PRSS21* was hypermethylated in all GCTs except spermatocytic tumor (Rijlaarsdam et al., 2015).

Research of Cheung and collaborators from 2011 (Cheung et al., 2011) identified the Carcinoma embryonic antigen-like protein Podocalyxin-like protein 1 (PODXL1), the anti-adhesive protein expressed in aggressive tumors, to serve as a target of miR-199a-5p, one of two mature miRNA species derived from miR-199a. PODXL1 is overexpressed in malignant testicular tumors, and its cellular depletion results in suppression of cancer invasion. DNA methylation-linked dysregulation of a conserved miR-199a is caused by aberrant methylation

in an intronic region of DNM3 at 1q24.3. MiR-199a is more methylated in seminoma than in non-seminoma. Hypermethylation of the *DNM3* intron leads to miR-199a repression. Therefore, epigenetic alteration in *DNM3* intron leads to dysregulation of miR-199a and PODXL1, as critical factors in tumor malignancy (Cheung et al., 2011). As another direct target of miR-199a-5p, *V-maf musculoaponeurotic fibrosarcoma oncogene homolog B* (avian) (*MAFB*) was postulated. Expression of the MAFB was the strongest in the cancerous tissue of the testis. An antiproliferative role of miR-199a was realized through repression of MAFB in TGCT. Together with the antiinvasive effect of the PODXLl, miR-199a acts as a tumor suppressor in TGCT (Gu et al., 2013). DNA (cytosine-5)-methyltransferase 3A (DNMT3A), the *de novo* methyltransferase, was identified as a direct target of the other mature miRNA derivative miR-199a-3p. Overexpression of miR-199a-3p restored the expression of *APC* and *MGMT* tumor-suppressor genes in Ntera 2 (NT2), cells from an established pluripotent human testicular embryonal carcinoma cell line by affecting DNA methylation of their promoter regions (Chen et al., 2014).

Numerous analyses have shown a similar association between the combined level of methylation of the promoter region and the risk of developing seminoma and non-seminoma tumors. In a study from 2012 by Mirabello and collaborators, this connection was discovered and proved only for a lower level of *KITLG* promoter methylation in the development of seminoma and high *BAK1* promoter methylation. The lower level of *DND1* promoter methylation was associated with an increased risk of seminoma (Mirabello et al., 2012). Increased methylation of the promoter region of the gene *PDE11*, and *SPRY4*, *BAK1* and reduced promoter methylation of *KITLG* in primary cells is associated with an increased risk for the development of familial TGCT (Mirabello et al., 2012). The promoter region of *PDE11* contains 7CpG sites, and their methylation levels are elevated in all cases of TGCT (Muhlhauser et al., 1995).

Seminoma and EC showed a hypomethylated upstream region of *OCT3/4* whereas differentiated non-seminomas, teratoma and yolk sac tumor, which lack OCT3/4 expression, were found to be hypermethylated in the upstream region (De Jong et al., 2007).

NANOG is an important transcription factor, a key regulator of self-renewal and maintenance of pluripotency in undifferentiated embryonic stem cells (Muhlhauser et al., 1995; Chambers et al., 2003). NANOG expression was not observed in the healthy adult testis (Hart et al., 2005), while it was highly expressed in seminoma and embryonal carcinoma. NANOG expression is very low in teratomas, yolk sac tumors, choriocarcinomas and mixed nonseminomas. All this speaks in favor that the CpG methylation in NRR (NANOG regulatory regions) correlates with NANOG expression in germ cells (Nettersheim et al., 2011). NRR hypermethylation in sperm and adult healthy testis can be a way of epigenetic repression of NANOG expression to control pluripotency program and prevent the malignancy of germ cells (Nettersheim et al., 2011).

Some signaling pathways, such as PIWI/piRNA play a critical role in the development of male germ cells. Therefore their role in the development of testicular germ cell tumors was investigated. It seems that GCNIS cells and TGCTs do not express PIWI/piRNA pathway genes in contrast to adjacent normal tissue of the testis (Gainetdinov et al., 2018). Studies have shown that the methylation of the 5′ promoter region CpG islands leads to silencing *PIWIL1*, *PIWIL2*, *PIWIL4*, and *TRDI1* gene in primary testicular tumors (Ferreira et al., 2014). The specific hypermethylation of CpG islands in those genes is associated with piRNA which leads to transcriptional inactivation in testicular cancer. The most important is that the epigenetic inactivation of PIWI-class proteins and related TDRD1proteins in tumorigenesis occurs in the context of reduced expression of piRNA and DNA hypomethylation of *LINE 1*. Interestingly, epigenetic PIWI protein changes also occur in male infertility, which associates infertility to testicular cancer (Hotaling and Walsh, 2009; Peng et al., 2009).

According to a study from 2012, similar methylation pattern in seminomas, nonseminomas and normal cells of the testes were found in the following genes: *ARF*, *S100A2*, *SSBP2*, *ER-alpha*, and *ER-beta*. Interestingly, *SSBP2* and *ER-alpha* had a higher degree of methylation in healthy testicular cells than in the tumor cells. *MGMT*, *VEGF*, *ER-beta* and *FKBP4* were methylated in nonseminoma tumors, in contrast to seminoma. *APC* and *hMLH1* were methylated in both tumor types, *APC* with higher frequency and level of methylation, while *hMLH1* only with higher frequency (Brait et al., 2012).

By a recent genome-wide methylation study of GCT tumors (including female tumor types), *APC* and *SOX17* genes were found to be hypomethylated in all germ cell tumors, including embryonal carcinoma (EC) and seminoma (SE). *AR* (androgen receptor) was completely deprived of methylation in all male tumors and *SOX2* was hypomethylated in EC and SE tumor samples in contrast to the teratoma samples which showed higher levels of methylation. For *XIST*, seminoma showed a trend towards less methylation as compared to the strongly methylated profile of the non-seminomatous tumors (Rijlaarsdam et al., 2015). Some of these findings are in contrast to previous research (*e.g.*, *APC*), and therefore authors suggest further validation. In 2004, detection of *XIST* unmethylated fragment in plasma was even proposed for diagnostics of TGCTs in males because in TGCT cases unmethylated *XIST* DNA signals were significantly higher than in peripheral blood lymphocytes derived from healthy men, with the highest levels in advanced disease (Kawakami et al., 2004; Looijenga and Oosterhuis, 2004).

A recent genome-wide analysis of testicular ECs identified methylation changes in several previously unknown genes. Among the genes that were hypermethylated in their promoters and consequently of a downregulated expression, five were sex-linked genes, including X-linked genes *STAG2*, *SPANXD/E* and *MIR1184*, and Y-linked genes *RBMY1A1/1B/1D* and *FAM197Y2P* that may provide insight of cross-talk between normal germ cell development and carcinogenesis (Cheung et al., 2016).

The gene encoding PRAME, one of cancer/testis antigens, was found to be hypomethylated at its promotor in seminoma and hypermethylated in EC. Moreover, increased methylation during the *in vivo* reprogramming of a seminoma cell line to EC was detected. Additionally, PRAME expression was upregulated in EC cell lines after treatment with histone deacetylase inhibitors (HDIs) that was seemingly able to override the repressive methylation mark. Knock-down of PRAME expression led to downregulation of pluripotency and PGC-related genes (*LIN28*, *PRDM14*, and *ZSCAN10*) and upregulation of somatic (endodermal, mesodermal) and germ cell differentiation markers (Nettersheim et al., 2016).

A group of genes associated with the germ cell state and/or pluripotency (*PRDM14*, *TDRD12*, *DDX43*, *MNS1*, *RBMXL2*, and *Klf4*) were methylated and silenced in non-seminoma cell lines as shown by a genome-wide methylation analysis (Noor et al., 2016).

Killian et al. in 2016 performed a lymphoid-compensated genome-wide DNA methylation analysis of TGCTs to obtain results without the obscuring effect of lymphoid tissue within the tumors. In TGCTs they found a PGC-like state characterized by the erasure of genomic-imprint and demethylation of *DPPA3* (STELLA), recurrent hypermethylation of cancer-associated targets, and subtype-dependent pluripotent, germline, or somatic methylation. The specific pluripotential methyl-CpH signature (H stands for anything but G) was discovered in EC and was lost during differentiation (Killian et al., 2016).

6. Clinical implication of TGCT epigenetics

6.1. Biomarkers

Although the serum biomarkers alpha-fetoprotein (AFP) and human choriogonadotropin (HCG) assist malignant GCT diagnosis, AFP is produced by yolk-sac tumor (YST) components and HCG predominantly by choriocarcinoma (CHC); consequently, neither marker is raised in all

cases of malignant GCT nor do both show elevations in non-malignant conditions (Murray and Nicholson, 2011). Therefore, novel preferably noninvasively obtained biomarkers are necessary for diagnostics of TGCTs.

Levels of methylation in the genomic DNA proved to be useful biomarkers for risk of a particular type of tumor and can be used in the risk assessment and molecular epidemiology (Mikeska and Craig, 2014). Recently, DNA methylation profiles for lung, breast, colon, and liver differentiated cancerous tissue from normal tissue with > 95% accuracy as well as almost all breast and colorectal cancer metastases to the liver (Hao et al., 2017). Overall, technical prerequisites seem to be met today for clinical diagnostics using methylation markers as has been recently published (Bock et al., 2016).

Some of the epigenetic marks have been earlier proposed for diagnostics of TGCTs, but have not been employed in the clinical practice because of some unsolved questions and doubts in the technical quality (*e.g.*, unmethylated *XIST*) (Kawakami et al., 2004; Looijenga and Oosterhuis, 2004). Previously mentioned Eco R1 locus in intron 25 of *DNMT3A*, could potentially serve as a useful epigenetic marker for TGCT (Meikar et al., 2013). Recently, it was shown that *CALCA* and *MGMT* are frequently methylated in non-SEs and are associated with poor clinical outcomes in TGCT patients (Bock et al., 2016).

Pediatric malignant GCTs are biologically different from their adult counterparts at a genomic and protein-coding transcriptome level, but they both display very similar microRNA expression profiles. Elevated serum levels of miR-371–373 and miR-302/367 microRNAs at the time of malignant GCT diagnosis, with levels falling after treatment may be exploited for diagnostic and/or therapeutic purposes (Martinelli et al., 2017). Actually a panel of four circulating microRNAs from these two clusters (miR-371a-3p, miR-372-3p, miR-373-3p and miR-367-3p) has been proposed as highly sensitive and specific for the diagnosis of malignant tumors, seminoma and embryonal carcinoma. Such diagnostics and follow-up may reduce reliance on serial CT scanning (Murray et al., 2015). In a most recent investigation miR-371a-3p that accurately correlated with disease activity outperforming AFP, bHCG, and LDH, was proposed for validation in a large-scale prospective human study (Murray et al., 2016). However, it should be noted that the expression of miRs in teratoma was as in normal testicular tissue (Murray et al., 2015; Murray et al., 2016). Therefore one may presume that growth of teratoma cannot be followed with miRNAs also in the rare “Growing teratoma syndrome”. In this syndrome benign teratomas develop at various extragonadal sites, after the systemic chemotherapy for the treatment of nonseminoma of the testis with normalization of the relevant tumor markers (Dieckmann et al., 2017).

Another promising noninvasive method for GCNIS screening seems to be the detection of specific TGCT miRNAs in semen because specific miRNAs associated with infertility have already been found in semen (Elzinga-Tinke et al., 2015).

6.2. Therapy

Although TGCTs are highly treatable, thousands of men still die from testicular cancer every year, and many challenges remain (Chieffi, 2016). TGCTs are highly sensitive to chemotherapy based on cisplatin, with the exception of teratoma (Kelland, 2007). The cure rate would be 95% if the treatments were initiated in the early stages of disease (Raghavan, 2003; Baylin and Chen, 2005). Seminoma morphology and phenotype resembles PGCs/gonocytes and is sensitive to radiation and chemotherapy based on platinum salts. Seminomas are mostly limited to one testicle and have an excellent prognosis because it is possible to cure > 90% of patients.

The resistance of TGCT to chemotherapy was associated with karyotype abnormalities, single gene mutations and epigenetic regulation of gene expression. Cisplatin acts *via* covalent binding to the DNA molecule which is being recognized by proteins participating in the process of DNA repair, leading to cell cycle arrest and apoptosis (Sheikine et al., 2012; Cavallo et al., 2013).

Studies have identified TP53 mutation and duplication of genes that may play a role in resistance to chemotherapy, as well as the existence of microsatellite instability. The dysregulation of the LIN28/let-7 axis in all malignant GCTs suggests a pathway that may be a target for the development of novel therapeutic agents (Murray et al., 2015).

A correlation of the methylation status with tumor chemoresistance was found. In general, undifferentiated tumors, often hypomethylated, seem to be much more sensitive to chemotherapy than well-differentiated tumors. *In vitro*, demethylation of resistant seminoma cell lines led to increased expression of cell pluripotency markers NANOG and POU5F1 and decreased resistance to cisplatin (Wermann et al., 2010). *RASSFIA* and *HIC1* promoter hypermethylation were associated with resistance to cisplatin-based therapy of NSGCT tumors *in vivo* (Koul et al., 2004). *RASSF1A* gene acts as a negative cell growth regulator (Chen et al., 2003), while *HIC1* encodes a transcription factor that acts as a tumor suppressor (Koul et al., 2004). On the other hand, *O-6-methylguanine-DNA methyltransferase* (*MGMT*), a DNA repair enzyme gene, and RARB gene (retinoic acid receptor gene) hypermethylation was associated with high sensitivity to cisplatin-based therapy of NSGCT tumors *in vivo*. Corresponding cell lines failed to respond to demethylating or histone deacetylase inhibiting agents in activating gene expression suggesting that irreversible changes occurred in pathways that control gene transcription (Koul et al., 2004).

Five different EC cell-lines, including two that were cisplatin-resistant, were highly sensitive to inhibition of cell growth and viability with low doses of the DNA methylation inhibitor 5-aza-29-deoxycytidine (5-aza-CdR) associated with significantly higher levels of DNMT3B (Beyrouthy et al., 2009). Therefore, in addition to cisplatin, a lower dose of 5-aza-CdR was proposed for treatment of TGCTs. The rationale for using 5-aza-CdR was that it causes the demethylation and reexpression of the tumor suppressor genes (Juttermann et al., 1994). The second mechanism involves apoptosis due to direct or indirect 5-aza-CdR-mediated DNA damage (Juttermann et al., 1994; Palii et al., 2008). Indeed, in EC cell lines treated with 5-aza-CdR, ATM activation, H2AX phosphorylation, increased expression of P21, and the induction of genes already known to be methylated in TGCTs (*MGMT*, *RASSF1A*, and *HOXA9*) was associated with decreased proliferation and survival (Beyrouthy et al., 2009).

By the genome-wide transcriptional and promoter methylation analyses, it was discovered that hypersensitivity of NT2/D1 testicular cancer derived embryonal carcinoma (EC) cell lines cells to low-dose 5-aza-deoxycytidine involved the activation of p53 targets, repression of pluripotency genes, and activation of genes repressed by DNA methylation. Therefore, low-dose 5-aza-deoxycytidine therapy has been proposed to treat those tumors that are sustained by cells with embryonic stem-like properties (Biswal et al., 2012). Moreover, in a teratocarcinoma model obtained by transplantation of the mouse gastrulating embryo to an ectopic site under the kidney capsule, the DNA demethylating agent 5-azacytidine diminished the growth of tumors after treatment *in vivo* (Sincic et al., 2009).

Recently, in a preclinical model using a xenograft model of cisplatine resistant tumors, DNA methylation inhibitor guadecitabine completely abolished progression and induced complete regression of embryonal carcinoma. This effect has been the consequence of induction of P53 targets and immune signatures and repression of pluripotency genes (Albany et al., 2017) Indeed, the phase 1 clinical trial (ClinicalTrials.gov, Identifier: NCT02429466) in patients with relapsed refractory germ cell tumors with SGI-110 (guadecitabine) in combination with cisplatin is underway (*Study of the Hypomethylating Drug SGI-110 Plus Cisplatin in Relapsed Refractory Germ Cell Tumors*, n.d.). Guadecitabine, that represents the new generation of DNA-hypomethylating agents, was so far successfully used in the treatment of the myelodisplastic syndrome and acute myeloid leukaemia in a stage1 clinical trial, because it was well-tolerated, easily administered, and biologically and clinically active (Issa et al., 2015).

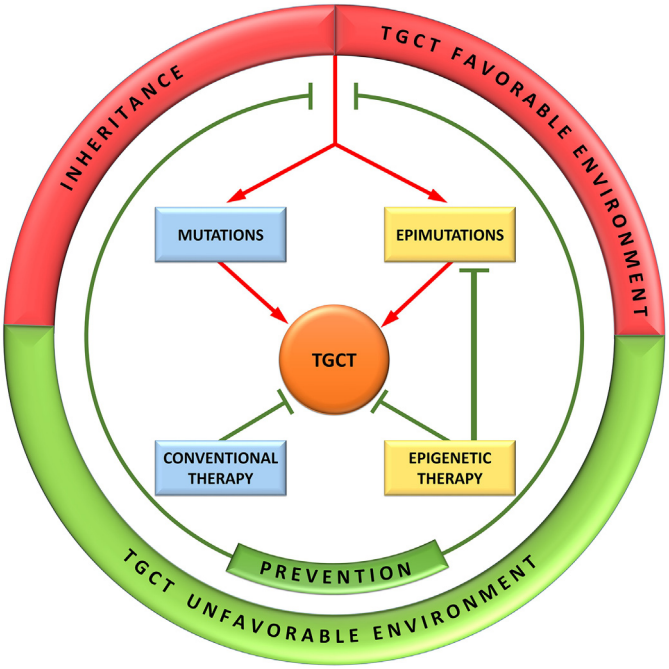

Fig. 4. Biological view of testicular germ cell tumor (TGCT) causes and its therapy. Heritable mutations and also epimutations that can be inherited at least in their daughter cells, originate from the favorable macro or micro-environment for the development of TGCT. The favorable environment might be "polluted" by various known or unknown factors such as mutagens or factors that epigenetically disrupt gene expression. Therefore, preventive measures are those that must be advocated. This is especially important in the case of mutations that are usually fixed and proven to be vertically inherited by the offspring. On the other hand, epimutations are reversible and targeted epigenetic therapy might both revert epimutations as the cause of malignant transformation and treat the tumor by restoring an adequate gene expression.

Overexpression of miR-199a-3p restored the expression of *APC* and *MGMT* tumor-suppressor genes in established pluripotent human testicular embryonal carcinoma cell line Ntera2 by affecting DNA methylation of their promoter regions. Therefore synthetic miR-199a-3p oligonucleotides as effective hypomethylating compounds have recently also been proposed in the treatment of TGCT (Chen et al., 2014).

In the end, it must be stressed that because epimutations may be reversed (Hernandez-Vargas et al., 2009) it seems that properly targeted epigenetic therapy could produce an unfavorable environment at least for the relapse in some problematic therapeutic outcomes where TGCTs cannot be totally destroyed by conventional therapies (Fig. 4). Theoretically more resistant elements might rise after a treatment of TGCT with an epigenetic drug, similarly as was discussed for the myelodysplastic syndrome relapse. In that case better diagnostics (*e.g.*, by NGS discovery of patient's resistance loci), new generations of epigenetic drugs, combination of epigenetic drugs (*e.g.*, DNA hypomethylating and HDACi), or other treatment options might be proposed and employed (Carraway, 2016; Enrica Marchi et al., 2015).

7. Conclusion

TGCT's present both an interesting biological problem (Bulic-Jakus et al., 2016; Bulić-Jakuš et al., 2006) and an important medical issue in various human populations (Znaor et al., 2014). To find appropriate biomarkers (Masterson et al., 2014; Boccellino et al., 2017) and design better therapies for TGCTs (van Agthoven et al., 2017), it is of paramount importance to understand the origin of the germ cell tumors in which epigenetics has a decisive role (van der Zwan et al., 2015; Okamoto, 2012). For example, epigenetic reprogramming in PGCs, although extensively investigated in mammals with a battery of contemporary techniques, has still been compared to a true blank slate (Messerschmidt et al., 2014). However, recent investigations of the methylome seem to have obtained important data on the origin of human germ cell tumors, among which TGCTs (Rijlaarsdam et al., 2015). It has been recently stated that epigenetic mediators, formerly also called tumor progenitor genes, generally become epigenetically disrupted at the earliest stages of malignancies, even before mutations. Because they influence phenotypic plasticity during the entire neoplastic process, they should constitute prime targets for both prevention and therapeutic interventions (Feinberg et al., 2016). Some of those genes being important also for TGCT-tumorigenesis, it is possible that they may become main therapeutic targets in TGCTs. The ongoing integrated analysis of The Cancer Genome ATLAS for GCT is underway that is to shed light on these issues (*The Cancer Genome Atlas. NIH, NHGRI, Cancers Selected for Study*, n.d.). Considering new therapeutic approaches for TGCT, a successful targeted DNA methylation *via* CRISPR-Cas9 system has recently been published (Vojta et al., 2016) that might in future be used for epigenetic silencing of aberrantly demethylated epigenetic mediators (*e.g.*, *OCT*). On the other hand, a targeted demethylation for reexpression of epigenetic modulators (*e.g.*, *p53*) that were impinged on by the favorable environment for the development of TGCTs may be proposed (Liu et al., 2016). Although targeted epigenome changes in cancer are in line with the contemporary aspirations towards precision medicine, epigenetic drugs that target the whole epigenome are already available. Such "genomic medicines" that may "lessen the need for precision approaches" are still being developed and some are used in clinical trials for treatment of various malignancies among which TGCT (Jones et al., 2016; Issa et al., 2015).

Acknowledgements

This publication was supported by the European Union through the European Regional Development Fund, Operational Programme Competitiveness and Cohesion, under grant agreement No. KK.01.1.1.01.0008, Reproductive and Regenerative Medicine - Exploring New Platforms and Potentials.

Conflicts of interest

None.

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