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Review

An up-date on newly discovered immunohistochemical biomarkers for the diagnosis of human testicular germ cell tumors

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Summary. Although testicular germ cell tumors (TGCTs) are relatively uncommon, they are particularly important, as they tend to affect children and young men, the most common tumor being in males aged from 20 to 40 years, and the incidence has been increasing in the last years.

TGCTs comprise two major histologic groups: seminomas and non-seminomas germ cell tumors (NSGCTs). NSGCTs can be further divided into embryonal, carcinoma, Teratoma, yolk sac tumor, and choriocarcinoma. Seminomas and NSGCTs present significant differences in clinical features, therapy, and prognosis, and both show characteristics of the Primordial Germ Cells (PGCs).

For proper diagnosis of the different histological subgroups, immunohistochemistry is required using different molecular markers, such as Aurora B, GPR30, Nek2, HMGA1, HMGA2, and others, and they could represent useful novel molecular targets for antineoplastic strategies. More insight into the pathogenesis of TGCTs is likely to contribute not only to better treatment of these tumors but also to a better understanding of stem cells and oncogenesis.

Key words: Testicular germ cells tumors, Seminomas, Aurora B, GPR30, PATZ1

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Introduction

Testicular germ cell tumors (TGCT), the most common malignant tumors in males among adolescent and young adults, represent a major cause of death attributable to cancer in this age group (Chaganti and Houldsworth, 2000; Oosterhius and Looijenga, 2005; Ulbright, 2005; Chieffi et al., 2009, 2012). TGCTs are histologically classified as seminomas and non-seminomas according to the international classification of oncological diseases. Both these tumors display an invasive phenotype and are believed to be derived from a common ancestor, carcinoma *in situ* (CIS), where the generation and expansion of tumor cells are limited to within the seminiferous tubules (Chaganti and Houldsworth, 2000; Oosterhius and Looijenga, 2005; Ulbright, 2005; Chieffi et al., 2009, 2012). Non-seminomas, including embryonal carcinoma and teratoma, contain stem cells as well as cells that have differentiated toward somatic lineages to various degrees, thus giving rise to a morphologically pleiotropic appearance (Oosterhius and Looijenga, 2005). In contrast, seminomas have a rather uniform appearance, at least at the histological level. Due to this apparently homogenous cell composition, seminomas are particularly suitable for investigations of tumor-associated alterations in gene expression. In addition, the cells that constitute seminomas resemble the primordial germ cells and/or the cells in the CIS. Thus, the gene expression profile in seminomas is interesting not only with regard to understanding their oncogenesis, but it also may be useful for research into primordial germ cells (PGCs) (Ulbright, 2005).

The incidence of TGCT varies between different countries and races, being greater in Scandinavia and Switzerland than Asia and Latin America, and in Caucasian Americans compared to African Americans. The incidence in western countries has been increasing over the last decades, probably because of an increased exposure to etiologic factors (Jones and Vasey, 2003). Remarkably, differences in incidence between adjacent countries such as Sweden and Finland are still largely unexplained, calling for further studies. Both clinical and epidemiological evidence strongly suggest that genetic and environmental factors play an important role in the genesis and development of TGCT. Several genes are implicated in the pathogenesis of TGCT, but the involvement of other genetic factors remains unknown (Skakkenbaek, 1972; Rajpert-De Meyts and Skakkenbaek, 2011). Susceptibility genes and environmental factors may deregulate normal differentiation processes of PGCs. In fact, TGCT have an invasive phenotype and are believed to be derived from a common ancestor, CIS, where the generation and expansion of tumor cells are limited to within the seminiferous tubules (Skakkenbaek et al., 1987). A number of environmental factors have been investigated to explain the possible links. Some evidence suggests association of increased TGCTs risk and maternal smoking during pregnancy, adult height, body mass index, diet rich in cheese, and others (Bonner et al., 2002; Dieckmann and Pichlmeier, 2002; Dieckmann et al., 2009). However, the biological mechanisms remain to be elucidated.

Hypothesized environmental agents involved in the development of TGCTs include pesticides (McGlynn et al., 2008) and nonsteroidal estrogens, such as diethylstilbestrol (DES) (Martin et al., 2008). It has been proposed that increased levels of estrogen exposure *in utero* increase the risk of TGCTs (Garner et al., 2008) and the exposure of women to the nonsteroidal estrogen DES during pregnancy increases the risk of TGCTs (Strohsnitter et al., 2001). However, other studies have not confirmed a role for estrogen in TGCT development (Dieckmann et al., 2001).

Familial predisposition to TGCTs, ethnic variations in incidence, and an association with certain chromosome abnormality syndromes strongly suggest that inherited factors also play a role in disease development. The familial predisposition is one of the strongest for any tumor type, since the increased relative risk of TGCT development associated with fathers and sons of TGCT patients is fourfold (Forman et al., 1992). However, gene(s) involved in familial TGCTs have not been identified so far (Oosterhius and Looijenga, 2005). Genome-wide linkage analysis of affected families has provided evidence for two susceptibility loci, one at Xq27 locus for undescended testis probably playing an indirect role, and another at 12q which results in hyperexpression of the product of the CCND2 gene (Lutke Holzik et al., 2004). It is probable that both genetic and environmental factors produce the high familial risk seen in TGCTs and that the interplay between these two factors, along with genetic heterogeneity, may make familial associated susceptibility loci difficult to determine.

Histopathology

It has been suggested that the initiating event in the pathogenesis of TGCT occurs during embryonal development (Chieffi and Chieffi, 2013). The most widely accepted model of postpuberal TGCT development proposes an initial tumorigenic event *in utero* and the development of a precursor lesion known as intratubular germ cell neoplasia undifferentiated (ITGCNU), also known as carcinoma *in situ* (CIS) (Skakkebaek, 1972). This is followed by a period of dormancy until after puberty when postpuberal TGCTs emerge. This prepubertal dormancy suggests that the TGCT development is hormone dependent.

Recently, it has been proposed that tumors originate from neoplastic cells that retain stem cell properties such as self-renewal (Wicha et al., 2006), and this novel hypothesis has fundamental implications for the pathogenesis of TGCTs. According to the stem cell hypothesis, tumors originate from tissue stem cells or from their immediate progeny. This cellular subcomponent drives tumorigenesis and aberrant differentiation, contributing to cellular heterogeneity of the tumor and also to the resistance to antineoplastic treatments.

ITGCNU cells are generally accepted as the common preinvasive precursor cells that give rise to postpuberal TGCT (Oosterhius and Looijenga, 2005). ITGCNU are almost invariably found in the periphery of overt postpuberal TGCTs and it is estimated that it is present in approximately 5% of the contralateral testis of patients with postpuberal TGCTs (Berthelsen et al., 1982). Preinvasive ITGCNU cells are supposed to be able to develop in different germinal and somatic tissues and are regarded as pluripotent or totipotent cells and therefore can be considered as TGCT stem cells. ITGCNU cells share morphological similarities with gonocytes and it has been proposed that ITGCNU cells could be remnants of undifferentiated embryonic/fetal cells (Nielsen et al., 1974; Skakkebaek et al., 1987).

Their fetal origin is also supported by immunohistochemical studies of proteins present in ITGCNU, also shown to be present in primordial germ cells (PGCs) and gonocytes. The identification of ITGCNU cells in prepubertal patients, who later developed TGCTs, indicated that the cells had originated prior to puberty (Muller et al., 1984).

Therefore, the ITGCNU cell represents an interesting variant of cancer stem cell since it originates before the tissue that it propagates in is fully differentiated and functional. The observation that two transcription factors, POU5F1 (OCT3/4) and NANOG, known to be associated with pluripotency in ES cells are expressed in ITGCNU has further contributed to assess

the embryonic origin of these cells. A link between ITGCNU cells and embryonic cells has been further supported by a substantial overlap between human ES cells and ITGCNU cell gene expression profiles, as shown by Almstrup and co-workers (Almstrup et al., 2004). All hystotypes could be present in postpuberal TGCTs, because of its totipontent profile, even seminoma can switch to nonseminoma hystotype through reprogramming phenomenon (Fig. 1) (Hoei-Hansen et al., 2005). The role of these factors will be discussed in more detail in the next sections.

Seminoma consists of transformed germ cells, which closely resemble the PGC/gonocyte, apparently blocked in their differentiation. Nonseminoma could be constituted by cells with typical pluripotency of PGC/gonocyte. In particular, embryonal carcinoma reflect undifferentiated stem cells, Teratoma represent somatic differentiation, while choriocarcinoma and YST extraembryonal differentiation. Genetic studies have shown that postpubertal testis tumors are often aneuploid with a consistent chromosomal abnormality composed of a gain of short arm of chromosome 12, usually in the form of an isochromosome, i(12p). In contrast tumors arising in prepubertal gonads are typically unassociated with 12p amplification and tend to be diploid. The most consistent structural chromosomal abnormality is an isochromosome 12p. Tumors lacking i(12p) have other structural abnormalities of 12p, among them the amplification of 12p11.2-p12.1. Gain of 12p sequences may be related to invasive growth (Chaganti and Houldsworth, 2000) suggesting that cyclin D2 (mapped to 12p13) is the most likely candidate gene of pathogenetic relevance.

Newly discovered biomarkers detected by immunohistochemistry in TGCT subtypes

Many novel biomarkers have been described in literature that can help to discriminate the different TGCTs, and they represent new potential molecular therapeutic targets. These biomarkes which are helpful for immunohistochemistry analysis are summarized in table 1 in order to clearly define each TGCT histotypes.

HMGA1 and HMGA2 represent a useful diagnostic markers (Chieffi et al., 2002; Franco et al., 2008). In fact, it has been demonstrated that the two isoforms are differently expressed with respect to the state of differentiation of TGCTs (Chieffi et al., 2002; Franco et al., 2008). Indeed, HMGA1 is able to bind proteins involved in transcriptional regulation, such as RNF4

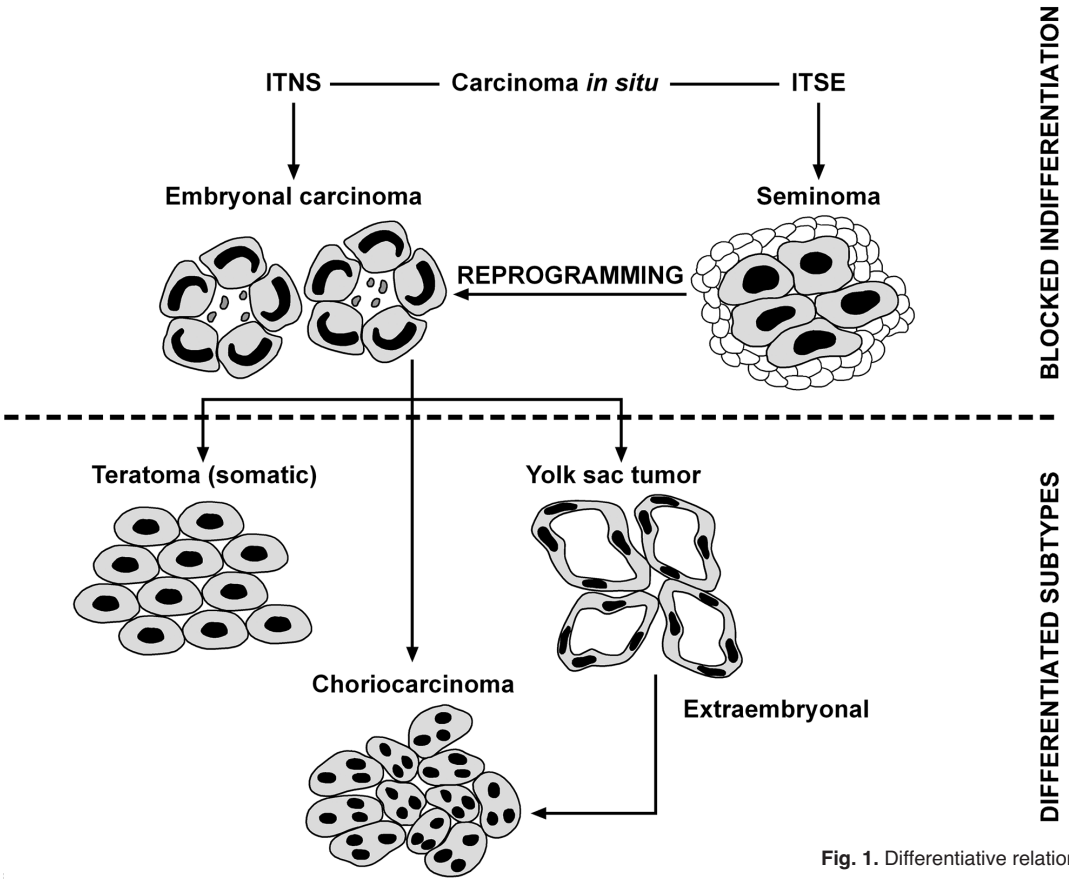

Fig. 1. Differentiative relations between TGCT histotypes.

(Pero et al. 2001, 2003) and PATZ1, which have been shown to be delocalized and over-expressed in human testicular seminomas (Fedele et al., 2008). More recently, we have shown that PATZ1 interacts with ERß in normal germ cells, while down regulation of ERß associates with transcriptional coregulator PATZ1 delocalization in human testicular seminomas (Fig. 2) (Esposito et al., 2011, 2012).

Another marker that could help to discriminate the different TGCT histotypes is Aurora-B expression; it has been detected in all CIS, seminomas and embryonal carcinomas analysed but not in teratomas and yolk sac carcinomas (Chieffi et al., 2004; Esposito et al., 2009; Portella et al., 2011).

NEK2 is a serine/threonine kinase that promotes centrosome splitting and ensures correct chromosome segregation during the G2/M phase of the cell cycle, through phosphorylation of specific substrates. Aberrant

Table 1. Immunohistochemical markers identified in TGCT subtypes.

	OCT3/4	SOX2	SOX17	HMGA1	HMGA2	PATZ1	GPR30	CCDC6	Nek2	Aur. B
Seminoma	+	-	+	+	-	+©	+	-	+	+
Embr. carc.	+	+	-	+	+	+©	+	-	±	+
Teratoma	-	±	±	-	-	+©	±	-	-	-
Yolk sac	-	-	±	-	+	+©	+	-	-	-

Notes: +, expressed; +© cytoplasmic localization; -, not expressed; ±, variable expression.

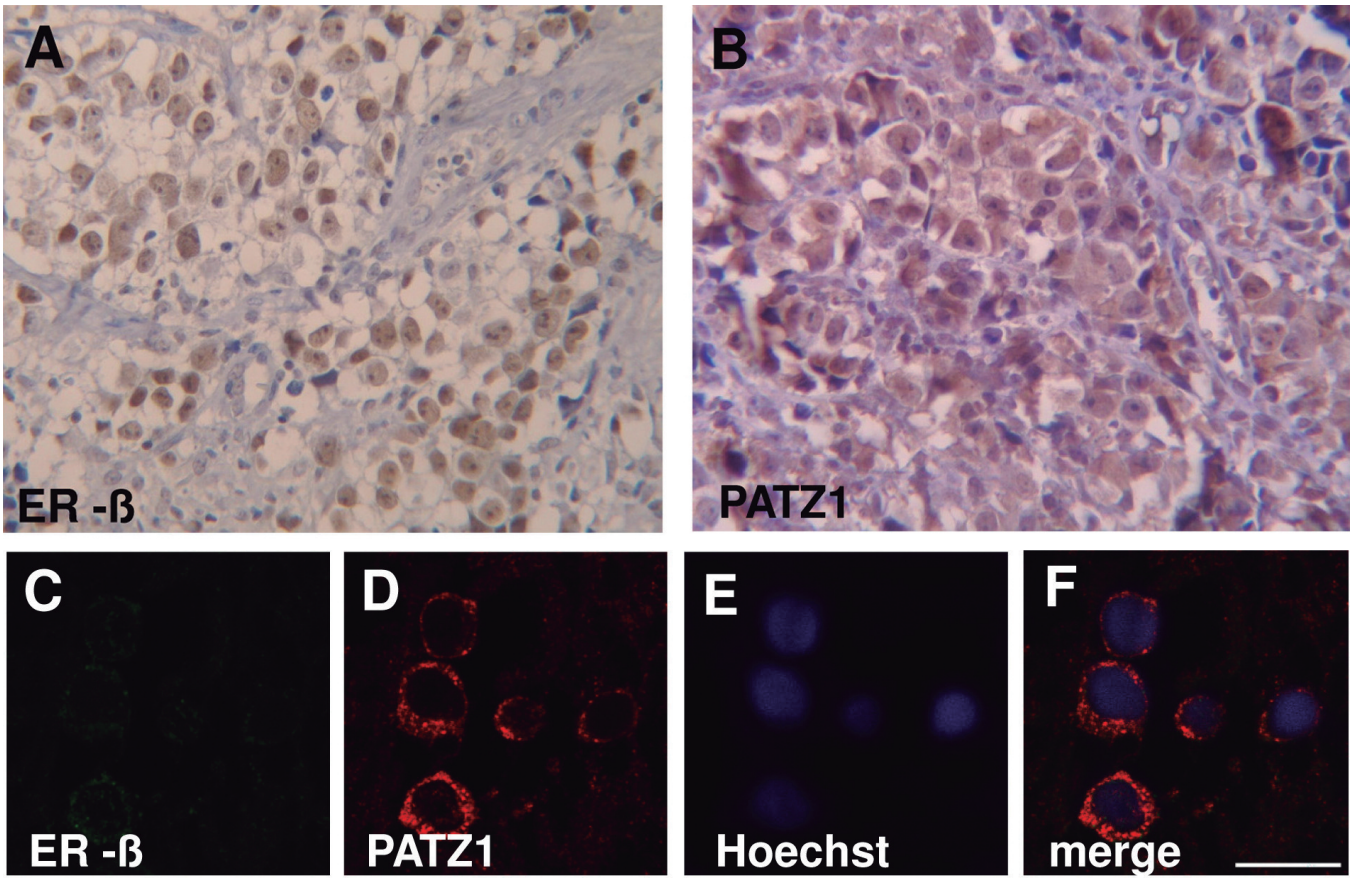

Fig. 2. Immunohistochemistry and immunofluorescence analyses of ERß and PATZ1 protein expression in human testicular seminomas. Seminoma in which an absence of immunopositivity of ERß **(A)** was observed in association with an intense and diffuse PATZ1 cytoplasmic immunosignal **(B)** by immunohistochemistry. Confocal microscopic images displaying an absence of immunopositivity of ERß **(C)** and PATZ1 cytoplasmic immunoreactivities **(D)**, Hoechst staining **(E)** and merge image of C and D **(F)**, in the same seminoma case. A, B, x 40; Scale bar: C-F, 10 μm.

expression and activity of NEK2 is present in neoplastic cells of seminomas. In addition, nuclear localization and the upregulation of Nek2 protein was also observed in the TCam-2 seminoma cell line, and correlates with expression of the stemness markers PLZF and OCT4 (Di Agostino et al., 2004; Barbagallo et al., 2009). OCT3/4 is a well-characterized marker for PGCs, and of CIS, seminoma, and embryonal carcinoma (Ledford, 2007). It has been demonstrated that OCT3/4 is also expressed in normal adult stem cells and non-germ cell-derived cancers (Ledford, 2007; Atlasi et al., 2008). OCT3/4 is a transcription factor of the family of octamer-binding proteins (also known as the POU homeodomain proteins) and is regarded as one of the key regulators of pluripotency (Atlasi et al., 2008). In addition to OCT3/4, several other embryonic stem-cell-specific proteins are important for maintaining the so-called ''stemness'' of pluripotent cells, such as NANOG and SOX2 (Avilion et al., 2003; Yamaguchi et al., 2005; de Jong and Looijenga, 2006).

NANOG protein was detected in gonocytes within the developing testis. In addition, NANOG is highly and specifically expressed in CIS, embryonal carcinoma, and seminomas, but not in teratoma, and YSTs revealing a molecular and developmental link between TGCTs and the embryonic cells from which they arise (Hart et al., 2005).

SOX2 is a member of the SOX protein family, transcription factors that regulate development from the early embryonal stage to differentiated lineages of specialized cells. SOX proteins are known to cooperate with POU proteins; in particular, the interaction between SOX2 and OCT3/4 has been well demonstrated. SOX2 is not detected in human germ cells regardless of their developmental age, in contrast to data in mouse embryos (de Jong et al., 2008). SOX2 is expressed in embryonal carcinoma, but it is not present in seminomas, YSTs, and normal spermatogenesis (de Jong et al., 2008). SOX17 maps to the chromosomal region 8p23, which is gained in seminoma. This indicates that SOX17 is a candidate SOX protein for cooperation with OCT3/4 in CIS and seminoma. These data also demonstrate that SOX17 is a good marker to discriminate CIS and seminoma from embryonal carcinoma. Of interest is that SOX17 distinguishes embryonic from adult hematopoietic stem cells (Kim et al., 2007). Current research focuses on the processes that may regulate the differential expression of SOX2 versus SOX17 and on the role of these SOX proteins in the different histologies of the TGCT subtypes involved.

Although the physiologic responses to estrogens are mainly mediated by the ERα and ERß (Chieffi et al., 2000; Vicini et al., 2006), in the last few years, GPR30 has been shown to mediate estrogen signaling in a wide variety of cell types. GPR30 is an intracellular 7-transmembrane G protein-coupled estrogen receptor (GPR30) that functions alongside the traditional estrogen receptors (ERα and ERß) to regulate physiological responsiveness to estrogen. It has been shown that GPR30 is overexpressed in seminomas and in the derived human seminoma TCam-2 cell line. The design of specific GPR30 inhibitors could be a useful molecular target to block neoplastic germ cells with a high proliferative rate, suggesting its potential therapeutic role for the treatment of TGCTs (Chieffi, 2007; Franco et al., 2011; Chieffi and Chieffi, 2013).

Sariola and co-workers have shown that targeted overexpression of glial cell line-derived neurotrophic factor (GDNF) in undifferentiated spermatogonia promotes malignant testicular tumors, which express germ-cell markers. The tumors are invasive and contain aneuploid cells, but no distant metastases have been found. By several histological, molecular, and histochemical characteristics, the GDNF-induced tumors mimic classic seminomas in men, representing a useful experimental model for testicular germ-cell tumors (Meng et al., 2001). In addition, recently it has been shown that GDNF promotes invasive behaviour, an effect dependent on pericellular protease activity, possibly through the activity of matrix metalloproteinases. GFRA1 over-expression in CIS and seminoma cells, along with the functional analyses in TCam-2 cells, suggests an involvement of the GDNF pathway in the progression of testicular germ cell cancer (Ferranti et al., 2012).

DNA damage response has been clearly described as an anti-cancer barrier in early human tumorigenesis. Moreover, interestingly, TGCTs have been reported to lack the DNA Damage Response (DDR) pathway activation. CCDC6 is a pro-apoptotic phosphoprotein substrate of the Ataxia Telangectasia Mutated (ATM) able to sustain DNA damage checkpoint in response to genotoxic stress and is commonly rearranged in malignancies upon fusion with different partners. Recently, it has been shown that the loss of CCDC6 expression is the most consistent feature among the TGCTs and in the TCam-2 seminoma cell line (Staibano et al., 2013).

Micro-RNAs in TGCTs

In recent years, the role of miRNAs in carcinogenesis of human testicular cancer and germ cell development has emerged (Bernstein et al., 2003). It was demonstrated that knockout mice for Dicer suffered from an early decrease in germ cell number and an impaired ability to differentiate, indicating that Dicer1 and miRNAs are important for both survival and proper differentiation of male germ cells (Maatouk et al., 2008). Subsequently, it was demonstrated that miRNAs 372 and 373 can overcome cell cycle arrest mediated by p53 (Voorhoeve et al., 2006). In contrast, TGCT cell lines with mutated p53 or expressing low levels of p53 were shown to be negative for these miRNAs and it can be assumed that miRNAs 372 and 373 can bypass the p53 checkpoint allowing the growth of TGCT. Another interesting link to the importance of miRNAs for germ cells and GCTs came from research on the Dead end

gene (DND1). Until recently, DND1 was known to regulate germ-cell viability and to suppress the formation of germ cell tumors. Recently Kedde et al. (2007) demonstrated that DND1 counteracts miRNA-mediated destabilization of mRNAs by binding to mRNAs and prohibiting the association of miRNAs with their target sites. This underlines the important role of miRNAs and regulation of miRNA expression in germ cell development. Linger et al. (2008) focused on the role of DND1 in humans and analyzed the presence of DND1 mutations in 263 human TGCTs. Further research into the functional mechanisms of miRNAs and the role of DND1 in TGCT are likely to give more interesting clues.

Conclusions

It has been well established that genetic and environmental factors play an important role in the genesis and development of TGCTs, but the identity of genes involved and the nature of the environmental component remain largely undetermined. The interactions between pathogenetic factors cause the deregulation of the normal differentiation processes of PGCs leading to tumor development. Diagnosis is usually based on identification of histological subgroups. In recent years, immunohistochemistry with a panel of suitable markers, including OCT3/4, SOX2, SOX17, HMGA1, GPR30, Aurora B, Nek2, and others has given further advantages to discriminate between subgroups. Recent developments such as the discovery or the role of miRNAs in oncogenesis also revealed highly interesting features of TGCTs. Specific miRNAs were shown to be involved in bypassing the WT p53 pathway, which is another characteristic of TGCTs.

Better knowledge of of the molecular mechanisms underlying the development of TGCTs may provide new tools to specifically target neoplastic cells and could contribute to overcome acquired and intrinsic chemotherapy resistance.

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