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POTENTIAL BIOMARKERS FOR TESTICULAR CANCER IN DOGS – GROUNDWORK FOR INNOVATIVE SCREENING PROGRAMS: A REVIEW

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Abstract

Testicular cancer is a frequently encountered pathological entity, which is distinguished by wide morpho-histological variability. Only recent research efforts were able to highlight, throughout advanced diagnosis techniques, certain biochemical, immunological and hormonal characteristics. In men, testicular cancer is one of the most common malignant oncopathologies, the usually affected age group being 25-35 years. Statistically, the increasing prevalence in humans can be correlated with veterinary in-field reports, an increase in canine testicular cancers being incriminated. Practically, 90% of the tumours with genital localization, in dogs involve the testicular parenchyma, this being the second most common site of neoplastic evolution in intact males. The aim of this paper was to review scientific papers published, regarding potential biomarkers for testicular cancer in dogs such as the anti-Müllerian hormone, insulin-like factor 3, α-fetoprotein, lactate dehydrogenase, c-KIT, CD-30, placental alkaline phosphatase and cytokeratins expression. Furthermore, this paper intends to lay the foundation for further research in order to establish proper screening protocols for testicular cancer similar to those used in human medicine.

Key words*: testicular cancer biomarkers, dogs, anti-Müllerian hormone, insulin-like factor 3, α-fetoprotein.*

INTRODUCTION

Testicular neoplasia has become increasingly common in veterinary medicine (Foster, 2012). The dynamics of oncological cases was multimodally justified taking into account all predisposing factors including environmental changes and genetic traits (Grieco et al., 2008; Nødtvedt et al., 2011). Nine out of ten tumours with genital location in dogs affect the testicular parenchyma (Nødtvedt et al., 2011; Manuali et al., 2020). The high prevalence reported in both human and veterinary medicine backs up the importance of the research in gynaecological oncology field. Due to the availability of advanced diagnostic techniques and thanks to latest technological advances, the heterogenicity of the testicular tumours was studied and cited. Immunological, biochemical and hormonal depiction can contribute to future establishment of screening programs and novel diagnosis methods.

Testicular tumours can be divided into 2 main categories: stromal sex cord tumors and germ cell tumors. In the first category, we include sertolinoma and Leydig cell tumors, the latter being considered the most common testicular tumor in canids, rats and mice (Creasy et al., 2012; Kudo et al., 2019). As for the tumors originating from the germinal epithelium of the seminiferous tubules we include: seminomas, teratomas, embryonal carcinoma (EC) and yolk sac tumours. In addition, histological findings showed subsequent evolution of mixed tumours and also primary tumors without specificity for the testicular parenchyma (Yu et al., 2009).

A retrospective study on the prevalence of testicular tumors which gathered 476 cases in total showed a detection rate of 16.8% (80/476) for neoplasia in male dogs, 94.1% (80/85) being localized in the genital area. The calculated prevalence for each tumor subtype in this case was: 34.4% seminoma, 26% interstitial cell tumors, 22.9% mixed germ cell and stromal tumors and 16.6% tumors with Sertoli cells (Liao et al., 2009). Research in certain geographical areas has shown similar data. In central Italy, Umbria region, 1969 individuals developed some kind of tumors. Out of the total, 388 (6.42%) had testicular

localization, the histopathological diagnosis incriminating a more frequent evolution of interstitial cell tumors, 50% of the total, 194 cases, respectively. Most of the cases presented a unique tumoral subtype (82.5%), only 63 males being diagnosed with mixed cell tumors. (Manuali et al., 2020).

Nascimento et. al. (2020) in the mass study which gathered 3,323 biopsies from male dogs, during a 19 years period, revealed a prevalence of 11.2% for testicular neoplasia. Seminomas were most commonly involved (40% rate), Leydig cell tumors (29.1%), Sertoli cell tumors (27.7%) and only 3.2% rate for mixed germ cell-sex cord stromal tumors.

The constant noted in both veterinary and human medicine studies is the relation between cryptorchidism and testicular neoplasms. The retained testes are prone to develop sertolinomas due to the higher temperature, which will lead to the destruction of all cells except Sertoli cells. This statement is backed up by several research papers, which focused on the depiction of testes tumors found in dog populations all over the world (Nascimento et. al. 2020; Gazin et al. 2022). Actually, the least diagnosed tumor in cryptorchid testes was the interstitial cell tumor, according to Gazin et al. (2022) and Liao et al. (2009).

According to some authors, the inguinal retention offers an intermediate thermal regime, between the abdominal and the intrascrotal one, predisposing to the development of seminomas (Ciaputa et al., 2012).

Studies on the molecularity and immunohistochemical features have highlighted the importance of discovering new markers for early diagnosis of testicular cancer. Cytokeratin, c-KIT, CD30, epithelial membrane antigen, α-inhibin, and (placental alkaline phosphatase) PLAP are some of the markers commonly researched or used, in human medicine, for the diagnosis of testicular tumors (Yu et al., 2009). Another example would be the evaluation of the expression of the Ki-67 gene to establish the character of malignancy, based on the relationship between Ki-67 and the number of mitotically active cells. In veterinary medicine, it could be considered a marker for metastatic evolution and it was used in order to assess the prognosis of different oncological cases. Ki-67 discriminated malignant from benign mammary gland tumors in bitches (Kudo et al., 2019).

Serum biomarkers such as α-fetoprotein (AFP), human chorionic gonadotrophin (hCG) or lactate dehydrogenase (LDH) are often included in staging and prognostic evaluation protocols, being used as screening factors for testicular neoplasms in men (Leão et al., 2020). Another possible biomarker could be the antimüllerian hormone (AMH) for which there is preliminary data on its applicability in the diagnosis of cryptorchidism (Walter, 2020). Also, establishing variations in insulin-like 3 (INSL 3) in patients with Leydig cell tumors could represent a non-invasive serum marker to be used in the diagnosis of testicular pathologies.

Adapting these markers for veterinary medicine can allow us to formulate the premises for innovative screening programs for testicular neoplasia in canids.

Therefore, the purpose of this work was to review the latest data extracted from research papers aiming to assess the potential biomarkers with a defined role in the early diagnosis and prognosis of testicular tumors. Thus, this research is based on the analysis of the current information related to the previously studied biomarkers. Topics such as the role of AMH for the diagnosis of sertolinomas and the serum INSL 3 levels in Leydig cell tumor cases will be subjects to debate. In addition, the differential diagnosis of non-seminomatous tumors based on AFP and LDH, the distinction between classic seminoma from spermatocytic seminomas based on C KIT and PLAP and the use of CD 30 as a high specificity biomarker will also be discussed in the present subchapters.

ANTIMULERIAN HORMONE AND SERTOLINOMAS

AMH is a glycoprotein produced by Sertoli cells in males and by granular follicular cells in females. Its primary role is to arrest the development of the Müllerian ducts in male embryogenesis, in females these ducts represent the origin of the external ovarian epithelium, salpinx, uterus, cervix and cranial portion of the vagina (Walter, 2020).

AMH secretory dynamics also differ between sexes. Ovarian granulosa cells will produce

small amounts of AMH until puberty. This will inhibit aromatase activity and will reduce the ability of androgens to convert to oestrogens. Having a well-established role in the ovarian follicle formation, its concentrations will be higher in women with polycystic ovaries and lower during menopause. (Dólleman et al., 2014; Hagen et al., 2014; Walter, 2020). In human medicine it is actively used to assess oocyte reserve (Sahmay et al., 2014), being also an important tool in the case of premature birth prevention (Stegmann et al., 2015). It was also used for the evaluation of ovarian function postoperatively, after chemotherapy (Lind et al., 2015), and may also assess the adverse effects of endocrine disorders, such as hypothyroidism, on the ovarian reserve (Kuroda et al., 2015). In men, AMH values were assessed for the diagnosis of testicular atrophy, Sertoli cell tumors or to evaluate possible sex development issues (Walter, 2020).

AMH being the specific protein most rapidly expressed by Sertoli cells, allows us to detect it even in foetuses or puppies up to 45 days of age (Banco et al., 2012). As sexual maturity is reached, serum values decrease. Therefore, high AMH levels in an adult can reveal the development of sertolinoma.

The use of immuno-enzymatic kits, in patients with Sertoli tumor both preoperatively and postoperatively, highlighted the dynamics of this hormone. Preoperatively, the recorded AMH values were much higher than those obtained from dogs without testicular pathologies, compared to those of the same post-orchiectomy patients (Ano et al., 2014).

A study, which involved 20 dogs with testicular masses, tried to establish the reference values for AMH, by comparison to a control group. Thus, they set the level of 10 ng/ml as the physiological maximum for intact dogs without testicular pathology, stating that patients with sertolinoma or mixed tumors had AMH values > 22 mg/ml (Holst et al., 2015).

Advanced immunohistochemical research highlighted the potential of AMH in the diagnosis of several testicular pathologies. By collecting samples from foetuses, new-borns, puppies aged between 43 and 180 days, 6 adult dogs and 24 dogs with sertolinoma, it was possible to highlight the degree of expression of AMH genes according to age, development stage, and presence or absence of testicular masses. Thus, AMH was expressed in the cytoplasm of Sertoli cells in both foetuses and new-borns, the percentage of labelled cells being 71-100%. Individuals up to 120 days were also intensely positive, with AMH expression becoming absent in dogs between 120 and 180 days. The adults included in the study did not express AMH in Sertoli cells, unless they had Sertoli cells tumors. Since the results for AMH expression are extremely variable between age groups, the relation between intense production of this glycoprotein and certain stages of cell differentiation can be considered. However, the potential of AMH in the diagnosis of Sertoli cell tumors has been formulated, detailed research on larger groups of individuals remains necessary (Banco et al., 2012).

INSL 3 IN LEYDIG CELL TUMORS

INSL 3, a peptide from the relaxin family, is one of the most innovative markers described in both human and veterinary medicine (Rossato et al., 2011). INSL 3's potentially high specificity for the diagnosis of Leydig cell tumors is motivated by the origin of this molecule. This peptide is produced exclusively by Leydig cells within the testes. This fact supports the premises for high value screening protocols focused around INSL 3 serum concentrations. The applicability of INSL 3 has also been stated for early diagnosis of canine cryptorchidism (Hannan et al., 2015), but due to its characteristic physiology, further research is considered necessary. However, the diagnostic value of this hormone has not been yet fully elucidated.

INSL 3 has been characterized as a reliable indicator of Leydig cell functionality. Unlike testosterone, INSL 3 is produced constantly without proving a pulsating release pattern. It is not influenced by other hormonal factors and it is not a subject of the hypothalamic-pituitary-gonadal axis regulation. Small differences between the expression capacity of INSL 3 in the testicular tissue and its serum values were cited, due to its independence related to the modulating mechanisms already mentioned. Practically, INSL 3, once produced by active

Leydig cells, will be directly released into the tissues and bloodstream (Ivell et al., 2013).
Currently, there is a lack of data on the direct association of INSL 3 values and testicular tumor pathology. Gene expression patterns for INSL 3 have been studied throughout immunohistochemistry in human medicine. In a study that included individuals with benign and malignant forms of Leydig cell tumors, it was shown that INSL 3 was expressed in all tissue samples collected from diagnosed leydigomas. However, no differences in peptide expression rates were observed in malignant samples compared to those with benign tumors. The authors note the potential of this marker and point out the need to establish correlations between serum values of INSL 3 and various testicular pathologies (Rossato et al., 2011).

POTENTIAL MARKERS FOR NON-SEMINOMATOUS TUMORS

In human medicine, the markers intensively used in the diagnosis and screening of testicular tumors are represented by: hCG, AFP and lactate dehydrogenase. They are considered to have satisfactory specificity and sensitivity, which is why they are often used not only to identify an ongoing neoplastic process, but also to monitor therapeutic success. The 3 stated markers are expressed in 60-80% of non-seminomatous tumors. The techniques involved also have another advantage related to their lack of invasivity (Pedrazzoli et al., 2021).
LDH is a glycolytic enzyme present in all tissues, but mostly in the muscles, brain and liver. Its widespread distribution in the body causes modified LDH values to produce many false positive results (Liao et al., 2009). LDH levels change in many conditions such as: pulmonary thromboembolism, muscle damage, myocardial infarction, thalassemia or haemolysis. These aspects underline the importance of interpreting the obtained results in a holistic manner, taking into account all clinical aspects and other objective parameters. However, according to the meta-analysis provided by the literature, elevated LDH values occur in 40-60% of cases of testicular tumors (Liao et al. 2009; Pedrazzoli et al., 2021).
Unlike hCG, which cannot be extrapolated to veterinary medicine for this purpose, AFP has the potential to be included in diagnosis protocols for testicular tumors (Pedrazzoli et al., 2021). AFP has been previously used in canids to detect multiple liver diseases and hepatic carcinoma (Yamada et al., 1999). AFP is an oncofoetal protein expressed in the embryonic sac, gastrointestinal tract and liver. Its role in the human body is not fully understood. However, reported data shows that in non-seminomatous tumors, it becomes detectable serologically, while in seminoma cases it is not produced (Pedrazzoli et al., 2021). Statistically, 60-70% of non-seminomatous tumors in men were correlated with serum detection of AFP (Pedrazzoli et al., 2021).
Although they do not excel in terms of specificity or sensitivity, they can be useful tools in the diagnosis of testicular tumors, the main advantage being their lack of invasivity.

C KIT AND PLAP – HIGHLY SENSITIVE MARKER USED FOR DIFFERENTIATING CLASSIC AND SPERMATOCYTIC SEMINOMA

The immunohistochemical technique is frequently used in human medicine in order to differentiate tumoral subtypes, as it is also a viable technique for testicular tumors diagnosis. Various established markers have recently been proposed for insertion in novel diagnosis protocols in the veterinary field. c-KIT is a proto-oncogene responsible for coding the tyrosine kinase receptor KIT, a transmembrane receptor. The before mentioned receptor is found in many different cell types, including germ cells, Purkinje cells from the cerebellum, precursors of the hematopoietic cells and melanocytes (Webster et al. 2006; Lennartsson et al. 2012; Gil da Costa et al., 2015).
The primordial germ cells will express KIT, followed by the progressive migration and proliferation until they reach the embryonic testicular tissue where they interact with the Sertoli cells expressing stem cell factor (SCF), which will condition their differentiation until gonocyte stage. The KIT – SCF interaction is essential for cellular differentiation and maturation processes (Grieco et al. 2010).
Right before birth, gonocytes will evolve to the pre-spermatogonia stage. Subsequently, during

puberty and adult life, KIT – SCF interactions will directly induce progressive differentiation towards the spermatogonia stage.
In males, KIT is present in Leydig cells as well, possibly playing a modulator role in the testosterone synthesis (Grieco et al., 2010). Unlike other markers present in the early stages of spermatogenesis, such as PLAP, which is only detected until prespermatogonia stage, KIT's expression is maintained for a longer period of time, along the different maturing stages (Rajpert-De Meyts et al, 2003; Grieco et al., 2010). Both markers are used for the immunohistochemical analysis of seminoma in males, c-KIT and PLAP's simoultaneous immunoreactivity being characteristic for this kind of testicular tumor. (Stoop et al., 2008).
Both markers, c-KIT and PLAP are often included in human medicine protocols in order to differentiate classic seminoma from spermatocytic seminoma. Grieco et al. (2010) showed possible similarities with human medicine research results, namely that the simultaneously expression of KIT and PLAP concurs with classic seminoma diagnosis. It was also highlighted that both markers have high specificity, KIT not being expressed by the Sertoli cells in neither cases, being identified exclusively in Leydig cells and spermatogonia (Grieco et al., 2010).
Although they have depicted the dynamics of the mechanism through which PLAP and KIT are expressed, the latter being indentified even after the primary stage of differentiation of the germ cell, at which point PLAP diminishes, Hohšteter et al. (2014) backed a different hypothesis from that of Bush et al. (2011) and Thorvaldsen et al. (2012). In the study conducted on 52 canids he was able to demonstrate, by determining the expression of KIT and PLAP through immunohistochemistry, that the majority of seminoma cases in dogs are classical seminoma, resembling the incidence reported in men, opposing Thorvaldsen and Bush's statement on the predominance spermatocytic seminoma.
Yu et al. (2009) tested the applicability of multiple tumoral markers, including PLAP and c-KIT on a number of 35 individuals diagnosed with seminoma and sertolinoma. In this case, it was concluded that c-KIT remains a highly sensitive marker for seminoma diagnosis, PLAP being unable to highlight the development of this specific tumoral type. On the other hand, in some sertolinoma cases included in the study PLAP showed increased reactivity.
Markers such as PLAP and c-KIT offer unexploited potential, possibly being able to identify the tumor type and consequently indicate the proper diagnosis and therapeutic protocol. Unfortunately, the insufficient data in the veterinary medicine field does not allow the precise establishment of protocols that include advanced immunohistochemical determinations centered around these two factors.
Even so, the possible implications in the oncologic diagnostic remain under debate, more information being needed in order to accurately assess the applicability, sensibility and specificity of the two factors.

CD 30 - HIGH SPECIFICITY MARKER

CD 30 is a glycoprotein integrated in the tumor necrosis factor superfamily. Its main site for expression is considered to be the surface cells of EC. One of the proposed applications for this marker was to differentiate seminomas from EC (Leroy et al., 2002). However, CD 30's potential asks for more research based on other possible physio-immunological aspects that can indicate other medical involvements.
CD 30's expression has been highlighted in a series of cells with certain malignant characteristics such as cells extracted from highly anaplastic lymphomas or Hodgkin's lymphoma (van der Weyden et al., 2017).
The expression dynamics of this possible valuable immunohistochemical marker is explained by the defined reactivity sites. Some certain activated B and T lymphocytes are the main actors in CD 30 expression. Thus, it could be shown that for certain cases of lymphoma, their expression in the incriminated cells becomes defining for diagnosis (van der Weyden et al., 2017).
Gopalan et al. (2009), during their stem cell markers research have proved that CD 30 could be a useful tool for testicular mixed germ cell tumor diagnosis. Their findings showed that 98% of the EC were positive for CD 30 expression, with some staining variations that need to be further investigated.

Unlike other markers, CD 30 can be used also as an indicator for therapeutic success. This fact has been showed by Albany et al. (2018) during their clinical trial on human patients with CD 30 expressing germ cell tumors and stromal cord tumors.

CD 30 has also been considered a good prognostic factor. Human patients with CD 30 ‑ expressing EC have worse progression ‑ free survival rates and overall survival rates than those with CD 30 ‑ negative tumors (Albany et al., 2018).

Considering the premises based on CD30 characteristic overexpression in some tumoral subtypes, it can be stated that the incriminated component can work as both a diagnosis and therapeutic target (van der Weyden et al., 2017).

Veterinary research efforts have not focused so much on the study of this biomarker. One of the few studies on testicular tumors that included immunohistochemical testing of CD 30 showed its poor rate of expression in both classical and spermatocytic seminoma. Backing up Leroy's suggestions, Yu et al. (2009) pointed again the role in differential diagnosis between seminomas and EC, stating that CD 30 was not expressed by any tissue sample originating from either classic or spermatocytic seminoma.

In fact, the link between CD 30 and EC, already being established in the literature, allowed the authors to suggest that the low expression of CD 30 in a study group demonstrates the low incidence of this tumor type. Hohšteter et al. (2014), obtained low or absent reactivity for CD 30 in dogs. He motivated his findings by not identifying any EC within the individuals included. Judging by its high specificity, the low positivity encountered in some seminomas, was explained by the possible abnormal transformation of the neoplastic cells into EC. This cellular drift has been previously described in human medicine (Hitmair et al., 1996).

According to Yu et al. (2009) CD 30 was not identified in Sertoli cell tumors either, which is consistent with the idea that CD 30 is highly specific for EC.

CONCLUSIONS

Given the many possible applications in the diagnosis, prognosis and even monitoring of the therapeutic success in testicular tumors, the value of these markers needs to be emphasized. Extrapolating these markers from human medicine may help establish new screening protocols for animal testicular oncopathologies. Corroborating the ascending trend and the high values of prevalence noted for testicular neoplasms in veterinary medicine, we can state the importance of the continuous research efforts focused on this subject.

Therefore, this area requires extensive research with a consequent increase in data that veterinary medicine has on tumor markers and their possible implications.

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