

# Clinical value detection of testicular microlithiasis and recently discovered markers in testicular germ-cell tumors

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Testicular germ-cell tumors (TGCTs) are the most frequent malignant tumors in men 20–40 years of age and the most frequent cause of death in this age group. TGCTs consist of two major groups: seminomas and nonseminomatous germ-cell tumors (NSGCTs). NSGCTs can be further divided into embryonic carcinoma, teratoma, yolk sac tumor, and choriocarcinoma, which differ in therapy, prognosis, but all show characteristics of the primordial germ cells. New biomarkers potentially represent novel molecular targets for treatment. The role of microRNA is highlighted as molecular prognostic factor in TGCT. Testicular microlithiasis (TM) often detected during examination among men with TGCTs, cryptorchidism, infertility, testicular atrophy and dysgenesis. Patients with TM form a group of risk of development of TGCTs. Also at high risk are patients with infertility, bilateral TM, atrophic and undescended testes and history of TGCTs.

**Key words:** testicular germ cell tumors, immunohistochemical markers, testicular microlithiasis.

Testicular germ-cell tumors (TGCTs) are the most frequent solid malignant tumors in men 20–40 years of age and the most frequent cause of death from solid tumors in this age group. TGCTs comprise two major histological groups: seminomas and nonseminomatous germ-cell tumors (NSGCTs). NSGCTs can be further divided into embryonic carcinoma, teratoma, yolk sac tumor, and choriocarcinoma. Seminomas and NSGCTs significantly differ in clinical features, therapy, and prognosis, but both show characteristics of the primordial germ cells. New biomarkers – OCT3/4, SALL4, CD30, CD117, GATA3,  $\alpha$ -inhibin, SOX2, SOX17, HMGA1, Nek2, GPR30, Aurora-B, estrogen receptor  $\beta$ , Glypican 3 – represent novel molecular targets for antineoplastic strategies.

At the time of ultrasound testicular microlithiasis (TM) inside the parenchyma can be found in 0,6–9% of patients. Findings of TM in population of healthy men is rare, but TM often detected during examination among men with TGCTs, cryptorchidism, infertility, testicular atrophy and dysgenesis. The connection between TM and infertility is unclear, but probably relates to dysgenesis of the testes. TM is also associated with a risk of developing testicular cancer. Among patients with TGCTs TM can be detected in 6–46% of cases [1, 2].

When carrying out testicular biopsy in patients with TM, the frequency of detection of TIN is significantly higher, however, TM can't be regarded as an absolute indication for testicular biopsy. Detection by ultrasound of the TM itself can't be considered a precancerous condition, since TM quite often accompanies benign changes in the parenchyma, but patients with TM form a group of risk of development of TGCTs. Also at high risk are patients with infertility, bilateral TM, atrophic and undescended testes, and with history of TGCTs.

It is known that most patients with TGCTs have a decrease in the quality of sperm even before the start of treatment. The performance of the orchidectomy can additionally contribute to a worsening of fertility, especially with an inferiorly functioning opposite

testicle, for example, in atrophy of the latter. Treatment of TGCT can also contribute to the deterioration of sperm quality. In addition to poor spermatogenesis, patients with TGCTs also have Leydig's cell dysfunction even in the opposite testis, so the risk of developing hypogonadism increases during treatment. The determination of the level of testosterone, SHBG, LH and oestradiol before and during treatment makes it possible to indirectly judge the degree of impairment and the possible timing of the restoration of fertility.

Patients cured of TGCTs for long-term follow-up may have androgen deficiency caused by TGCTs or due to age-related changes and, therefore, need long-term follow-up and, possibly, substitution therapy [3]. It can be considered statistically proven to increase the degree of hypogonadism depending on the volume of treatment received – the risk of hypogonadism increases in patients who received more than 3 courses of chemotherapy or irradiation of retroperitoneal lymph nodes. In this case, the greatest clinical manifestations of hypogonadism are observed in the period from six to twelve months after treatment, but in some patients testosterone deficiency can be observed and more than two years after the end of therapy. Erectile dysfunction and loss of libido can also be observed [4].

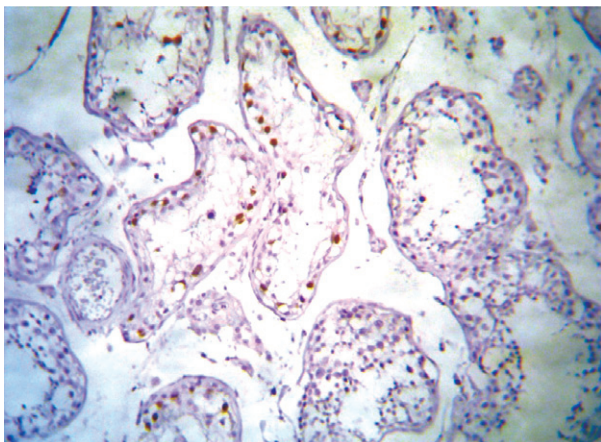
Three serum tumor markers (alpha fetoprotein, chorionic gonadotropin and lactate dehydrogenase) are currently used for prognostic purposes. AFP is a serum protein produced by the fetal yolk sac, liver, and gastrointestinal tract. The highest concentrations observed during 12–14 weeks of gestation and decline 1 year after birth. AFP is secreted by embryonic carcinoma and yolk sac tumor, but not by pure choriocarcinoma or pure seminoma. Elevated AFP can be seen after treatment in patients with liver disease, and several malignancies including hepatocellular carcinoma, lung, pancreatic, colon, and gastric cancers. During pregnancy, hCG is produced by the syncytiotrophoblastic cells of the placenta. In TGCTs, syncytiotrophoblastic cells are also responsible for production of hCG.

All patients with choriocarcinoma and 40–60% of patients with embryonic cell carcinoma have elevated hCG and 20% of patients with pure seminoma have elevated serum hCG. LDH is an enzyme found in all cells and represent a nonspecific marker for the burden of disease, and can be elevated in many malignancies and chronic disease (liver and heart failure, pancreatitis, hemolytic anemia and collagen disorders).

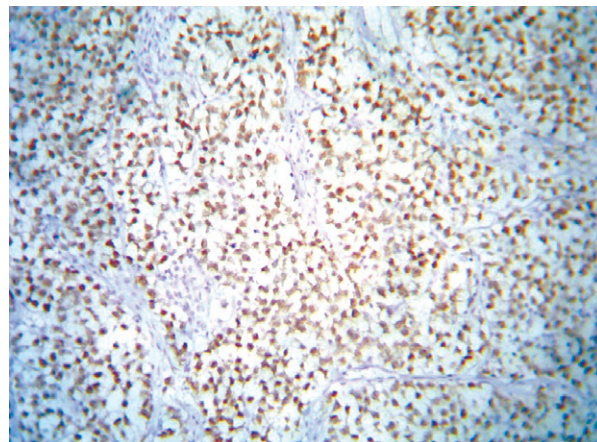
Nowadays the testing serum for tumor markers (AFP, hCG, LDH) is a standard diagnostic procedure in managing patients with germ cell testicular tumors, although highest prognostic value is seen in nonseminomatous malignancies. Historically, these serum markers were one of major tests to differentiate seminoma, non-seminomatous or mixed primary tumors. Elevation of «classic» tumor markers is usually seen in 60% of pts with germ cell testicular tumors. This justifies further search of newer molecular, genetic and immunohistochemical markers [5–7].

Review of literature yields new immunohistochemical markers, which help in diagnosis of different types of GCT, and present a potential targets for developing new pharmaceutical agents.

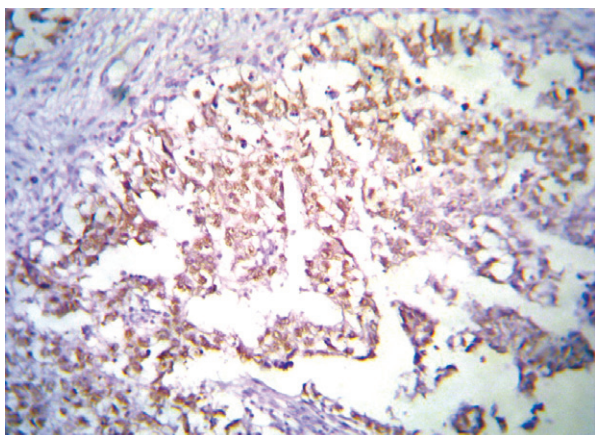
To name, CD117 (C-kit or KIT-marker of tyrosinekinase transmembrane receptors and stem cells growth marker) and D2-



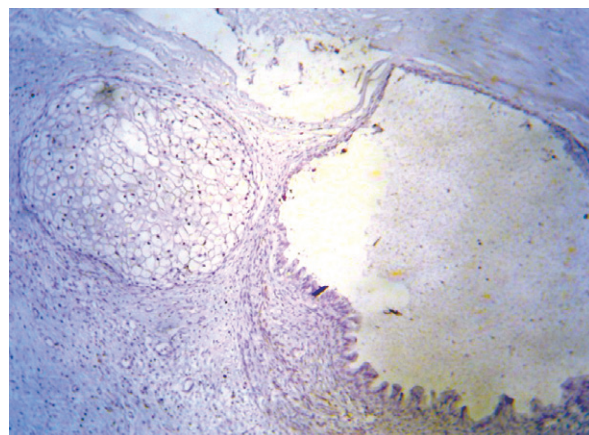
**Fig. 1. Positive expression of the OCT-3/4 in TIN (×400, author's photo)**



**Fig. 2. Positive expression of the OCT-3/4 in seminoma cells (×200, author's photo)**



**Fig. 3. Positive expression of the OCT-3/4 in embryonic carcinoma cells (×400, author's photo)**



**Fig. 4. Absence of OCT-3/4 marker expression in teratoma cells (×100, author's photo)**

40 (marker of lymphatic vessels endothelium, used to study lymphovascular invasion and lymphangiogenesis in tumors) can be used to differentiate atypical seminoma and embryonic carcinoma.

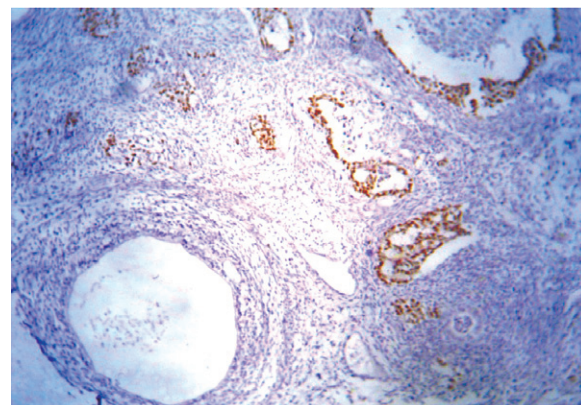
CD117 and D2-40 are being detected in cells of seminoma, and their expression is absent in embryonic carcinoma [8].

Study of the multifunctional nonhistone high mobility proteins (HMGA) (high mobility group, isoforms 1 and 2), which take part in transcription in non-seminomatous tumors demonstrate their higher expression compared to seminoma tumors. Hyperexpression of HMGA speaks for malignant phenotype, resistance to chemotherapy drugs, early and fast metastases and unfavorable prognosis [9–10].

NEK2 – belongs to centrosomal serine/treonin kinases engaged in correct split of chromosomes in G2/M stage of cell cycles (the gene is located on 20q13). Many factors affect the activity of these protein kinases, such as damage to DNA. Higher expression of this gene causes anomaly of centrosome and chromosome instability, which leads to abort of signal to apoptosis and preservation of genetically changed cells.

Aberrant expression of NEK2 is found in seminoma cellular nuclei and in cellular line TCam-2 and correlates with level of expression of stem cell markers (pluripotency) – PLZF та OCT4. It was discovered that NEK2 plays a role of modulating factor for alternative splicing, which is key event in regulation of gene expression and most frequently is damaged in cancer cell [11].

Alternative splicing is a process which allows generation of different mRNA transcripts from same gene, and different pro-



**Fig. 5. Positive expression of the OCT-3/4 in embryonic carcinoma cells, absence of expression in teratoma cells (lower left, ×100, author's photo)**

teins, respectively. This allows for diversity of final proteins considering limited amount of genes. Up to 94% of human genes adopt alternative splicing.

OCT3/4 – is one of transcription factors from POU family, controls mRNA synthesis through binding with specific site on DNA. Transcription factors may be oncogenic and oncosuppressive, their mutation or changes in their regulation may start the cancerogenesis. Study on cell lines demonstrated that OCT3/4

was a key factor in a process of self-renewal of nondifferentiated embryonic stem cells, thus maintaining pluripotency potential. OCT3/4 can be used as a marker of non-differentiated cells.

The expression of its gene is finely regulated, because even slight changes (up- or down-regulation) cause the differentiation of cells. Normally OCT3/4 is being activated in oocyte and stays activated until its implantation. Knockdown of OCT3/4 gene causes differentiation of cells, which proves the role of this factor in maintenance of self-renewal of embryonic stem cells. It is known, that mice embryos with low level of OCT3/4 protein do not build up the cellular population and differentiate into trophoblast. The main function of OCT3/4 is restraining stem cells from differentiation.

As per Looijenga L.H. et al. [12], beside some types of germ-cell tumors (seminoma, germinoma, dysgerminoma), the embryonic carcinoma cells hold pluripotency potential (ability to differentiate). They are considered as stem cell component in non-germ cell tumors. The cells of seminoma, TIN, germinoma and dysgerminoma hold phenotype of early germ-cell tumors, the pluripotency potential of these tumors can also be activated. Teratoma, yolk sack tumor, spermatocytic seminoma (as well as ovarian dermoid cysts) are composed of differentiated cells and host no stem cells. TIN, seminoma and embryonic cell carcinoma, as well as germinoma and dysgerminoma, are marked with higher OCT3/4 expression (Fig. 1–5).

Gillis A. et al. (2011) besides OCT3/4 test for expression of the following genes - NANOG, SOX2 and LIN28 – as they are main regulators of pluripotency in the cells of germ cell tumors [13]. Immunohistochemical tests demonstrate expression of LIN28 in primordial germ cells, gonocytes, pre-spermatogonia and TIN cells as well as in seminoma, embryonic cell carcinoma and yolk sack tumor, which correlates with high malignant potential of these germ cell tumors. LIN28 was not detected in teratoma and spermatocytic seminoma. Study on cell lines with knockdown of LIN28 gene demonstrated its role in maintaining non-differentiated status of seminoma and embryonic cell carcinoma along with OCT3/4 and NANOG.

Recent studies demonstrated prognostic value of microRNA 371-373 in managing germ cell tumors of testis [14–16]. MicroRNA (or miRNA) is a small molecule RNA (18–25 nucleotides), which may repress translation of mRNA on ribosomes and regulate gene expression. MicroRNAs were shown to play a role in embryogenesis, cell differentiation, apoptosis and tumorigenesis, repression of function of many genes. One miRNA may regulate the function of many genes at the same time, including oncosuppressors and oncogenes.

The role of microRNAs in cancer stem cells and in development of resistance to chemotherapy is being thoroughly studied. MicroRNAs hold a potential to be used as tumor markers in oncological practice due to their stability in liquid systems and sharp elevation in many malignancies. For germ cell testicular tumors the diagnostic value of microRNA 371-3 cluster was demonstrated. To date, serum levels of microRNA-367-3p, 371a-3p, 372-3p and 373-3p in patients with germ cell testicular tumors are high than in healthy men, specificity and sensitivity of microRNA-371a-3p is 84.7% and 99%, respectively, which is better compared to hCG and AFP.

There is also a correlation between stage of the tumor, build-up of the primary tumor (seminoma vs. non-seminoma) and level of microRNA-367-3p. It is worthy to note that in Stage I disease after removal of primary tumor microRNA-371a-3p significantly dropped in serum. This fact signifies microRNA-371a-3p as a potential new diagnostic tool for either active surveillance or monitoring residual tumor after chemotherapy [17].

Nowadays there is a limited experience using microRNA in urooncological practice. There were more than 40 microRNAs discovered as potential markers for monitoring urological malignancies of different location, as well as tools for developing new treatment strategies, which would be based on selective modeling of microRNAs [18].

Spiekermann M. et al. (2015) state that three microRNAs can be considered as potential markers in treatment of germ cell tumors of testis – microRNA371a-3p, 372 and microRNA-373-3p [15]. Studies of other authors support these data. Gillis A. et al. (2013) studied 80 germ cell tumors and discovered elevation of microRNA-371/372/373/367 [19]. In Stage I tumor after orchidectomy the level of microRNA fell to normal. Authors found a correlation between level of microRNA and stage of the process. Overall, comparing to traditional markers, microRNA appeared to be more sensitive – 98% (sensitivity of AFP/hCG is 36%/57%, sensitivity for seminoma/non-seminoma: AFP – 3%/45%, and hCG – 62%/66%).

Palmer R. et al. (2010) studied 615 different microRNAs from germ cell tumors in children, adults and cell lines [20]. Authors found elevation in microRNA clusters 371-373 and 302 ( $p < 0.00005$ ) disregard histological buildup of primary tumor, age of patients and gonadal/extragenital location of the tumor.

Ruf C. et al. (2014) based on monovariate analysis determined 35 different microRNAs presence of which is indicative of metastases (lymphogenic or dormant) in patients with seminoma [21]. In multivariate analysis the metastases were accurately predicted by two microRNAs in peripheral blood.

Syring I. et al. (2015) tested different microRNAs in serum (miR-302a-3p, 302b-3p, 302c-3p, 367-3p, 371a-3p, 372-3p and 373-3p) in 30 patients with germ cell tumors [17].

Authors established an elevation of microRNAs in serum (miR-367-3p, 371a-3p, 372-3p and 373-3p) in patients with tumor, sensitivity/specificity of microRNA371a-3p was determined as 84.7%/99% (which exceed those for AFP/hCG); the level of miR-367-3p is higher in tumors of non-seminoma build-up, compared to seminoma, and level of miR-371a-3p in Stage I disease decreased after removal of tumor.

Abovementioned data provide a ground for considering microRNA371a-3p, 372 and 373-3p as new potential marker for germ cell tumors of testis [22, 23]. Further studies should provide answers to a number of questions, such as whether germ cell tumor cells indeed produce those microRNAs? For example, serum concentration of microRNA from testicular vein anticipated to be higher than in vena ulnaris. Can microRNA test aid in detecting TIN? Does elevated microRNA correlate with stage of the tumor? Can microRNA be tested and detected in other biological fluids, such as urine, ejaculate, pleural exudate, and would that have a diagnostic value? From clinical stand point we consider important the decline of microRNA to normal after removal of the primary Stage I tumor. Specificity of the test should be validated by means of testing microRNA in control group (cancer patients with other malignancy, or patients with benign processes in scrotum).

## CONCLUSIONS

1. Testicular microlithiasis often detected during examination among men with TGCTs, cryptorchidism, infertility, testicular atrophy and dysgenesis. Patients with TM form a group of risk of development of TGCTs. Also at high risk are patients with infertility, bilateral TM, atrophic and undescended testes, and with history of TGCTs.
2. Recent studies characterize microRNAs 371a-3p, 372 and 373-3p as new potential markers of germ cell tumors of testis. When compared to traditional markers (AFP, hCG, LDG) they yield higher sensitivity and specificity.
3. Immunohistochemical studies detecting new markers has given further advantages in discriminating between subgroups of testicular tumors, and yield potential novel molecular targets for antineoplastic strategies.
4. According to the immunohistochemical study with OCT 3/4, the pluripotency potential retains in seminoma and embryonic cancer cells. The teratoma cells as a more differentiated tumor do not possess pluripotency.

**Клінічне значення тестикулярного мікролітіазу та визначення нових маркерів при герміногенних пухлинах яєчка**

**А.В. Сакало**

Герміногенні пухлини яєчка (ГПЯ) найчастіше зустрічаються у віковій групі 20–40 років, вони є однією з основних причин смертності серед чоловіків молодого віку. Розглянуто деякі нові імуногістохімічні маркери, вивченню яких приділяється увага в публікаціях останніх років, та які є потенційними мішенями для розроблення нових таргетних препаратів. Висвітлено значення мікроРНК як молекулярного фактора прогнозування перебігу захворювання на ГПЯ. Визначено, що виявлення тестикулярного мікролітіазу може поєднуватися з порушенням фертильності та іншими ознаками дисгенезії яєчок і супроводжуватися наявністю TIN у паренхімі.

**Ключові слова:** герміногенні пухлини яєчка, імуногістохімічні маркери, мікролітіаз яєчок.

**Клиническое значение тестикулярного микролитиаза и определение новых маркеров при герминогенных опухолях яичка**

**А.В. Сакало**

Герминогенные опухоли яичка (ГПЯ) диагностируют чаще в возрасте 20–40 лет, они являются одной из основных причин смерти от онкологических заболеваний в указанной возрастной группе. Рассмотрены новые маркеры, которые потенциально могут быть использованы как мишени для таргетной терапии. Затронута роль микроРНК в качестве молекулярного прогностического маркера при ГПЯ. Отмечено, что обнаружение тестикулярного микролитиаза (особенно двухстороннего) при проведении УЗИ может сопровождаться другими признаками дисгенезии яичек, нарушением фертильности и наличием TIN.

**Ключевые слова:** герминогенные опухоли яичка, иммуногистохимические маркеры, микролитиаз яичек.

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