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Newly discovered immunohistochemical markers and micro-RNAs detected 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 histological 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 – OCT3/4, SOX2, SOX17, HMGA1, Nek2, GPR30 – represent novel molecular targets for antineoplastic strategies. The role of micro-RNA is highlighted as molecular prognostic factor in patients with TGCT.

Key words: *immunohistochemical markers, micro-RNA, germ–cell tumors.*

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. Many new biomarkers – OCT3/4, SOX2, SOX17, HMGA1, Nek2, GPR30, Aurora-B, estrogen receptor β-represent novel molecular targets for antineoplastic strategies.

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 syncythiotrophoblastic cells of the placenta. In TGCTs, syncyhtiotrophoblastic 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, nonseminomatous or mixed primary tumors [1–5].

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 immunihistohemical markers [6].

Review of literature yields new immunhistochemical 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 D2-40 (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 [7].

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 [8–11].

NEK2 – belongs to centrosomal serine/treoninkinases 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 proteinkinases, 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 [12]. 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 [13]. (Alternative splicing is a process which allows generation of different mRNA transcripts from same gene, and different proteins, 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 pluropotency potential. OCT3/4 can be used as a marker or 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 trophoectoderm. The main function of OCT3/4 is restraining stem cells from differentiation. As per Looijenga L.H. et al. [14], 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 pluripotent 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. Having examined more than 100 tumors of different location, no OCT3/4 expression was observed in malignancies of non-germ cell origin. These tumors demonstrated no difference in OCT3/4 expression depending on sensitivity/resistance to chemotherapy.

A. Gillis et al. (2011) besides OCT3/4 test for expression of the following genes - **NANOG, SOX2 та LIN28** – as they are main regulators of pluripotency in the cells of germ cell tumors [15]. Immunohistochemical tests demonstrate expression of LIN28 in primordial germ cells, gonocytes, pre-spermatogonias 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 micro-RNA 371–373 in managing germ cell tumors of testis [16–21]. Micro-RNA (or miRNA) is a small molecule RNA (18–25 nucleotides), which may repress translation of iRNA on ribosomes and regulate gene expression. Micro-RNAs were shown to play a role in embryogenesis, cell differentiation, apoptosis and tumorigenesis, repression of function of many genes [22–24]. One miRNA may regulate the function of many genes at the same time, including oncosuppressors and oncogenes [25, 26]. The role of micro-RNAs in cancer stem cells and in development of resistance to chemotherapy is being thoroughly studied. Micro-RNAs 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 micro-RNA 371-3 cluster was demonstrated. To date, serum levels of micro-RNA-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 micro-RNA-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 micro-RNA-367-3p. It is worthy to note that in Stage I disease after removal of primary tumor micro-RNA-371a-3p significantly dropped in serum. This fact signifies micro-RNA-371a-3p as a potential new diagnostic tool for either active surveillance or monitoring residual tumor after chemotherapy [6].

Nowadays there is a limited experience using micro-RNA in urooncological practice. There were more than 40 micro-RNAs 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 micro-RNAs [27].

M. Spiekermann et al. (2015) state that three micro-RNAs can be considered as potential markers in treatment of germ cell tumors of testis – micro-RNA371a-3p, 372 and micro-RNA-373-3p [19]. Studies of other authors support these data [28–30]. A. Gillis et al. (2013) studied 80 germ cell tumors and discovered elevation of micro-RNA-371/372/373/367 [30]. In Stage I tumor after orchidectomy the level of micro-RNA fell to normal. Authors found a correlation between level of micro-RNA and stage of the process. Overall, comparing to traditional markers, micro-RNA 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%).

R. Palmer et al. (2010) studied 615 different micro-RNAs from germ cell tumors in children, adults and cell lines [29]. Authors found elevation in expression of micro-RNA clusters 371–373 and 302 ($p<0.00005$) disregard histological buildup of primary tumor, age of patients and gonadal/extragonadal location of the tumor.

C. Ruf et al. (2014) based on monovariate analysis determined 35 different micro-RNAs presence of which is indicative of metastases (lymphogenic or dormant) in patients with seminoma [31]. In multivariate analysis the metastases were accurately predicted by two micro-RNAs in peripheral blood.

I. Syring et al. (2015) tested different micro-RNAs 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 [20].

Authors established an elevation of micro-RNAs 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 buildup, 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. Further studies should provide answers to a number of questions, such as whether germ cell tumor cells indeed produce those micro-RNAs? For example, serum concentration of micro-RNA from testicular vein anticipated to be higher than in vena ulnaris. Can micro-RNA test aid in detecting TIN? Does elevated micro-RNA correlate with stage of the tumor? Can micro-RNA 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 micro-RNA 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. Recent studies point at micro-RNAs 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.

2. Immunohistochemical studies detecting new markers (OCT3/4, SOX2, SOX17, HMGA1 and HMGA 2, Aurora-B, PATZ1, GPR30) has given further advantages in discriminating between subgroups of testicular tumors, and yield potential novel molecular targets for antineoplastic strategies.

Современные иммуногистохимические маркеры и микро-РНК при герминогенных опухолях яичка

А.В. Сакало, П.Г. Яковлев

Герминогенные опухоли яичка чаще встречаются в возрасте 20–40 лет и являются одной из основных причин смерти от онкологических заболеваний в указанной возрастной группе. В статье рассмотрены новые иммуногистохимические маркеры: OCT3/4, SOX2, SOX17, HMGA1, Nek2, GPR30. Также освещена роль исследования микро-РНК в качестве молекулярного прогностического маркера герминогенных опухолей яичка.

Ключевые слова: *иммуногистохимические маркеры, микро-РНК, опухоли яичка.*

Сучасні імуногістохімічні маркери та мікро-РНК при герміногенних пухлинах яєчка

А.В. Сакало, П.Г. Яковлєв

Герміногенні пухлини яєчка найчастіше зустрічаються у віці 20–40 років та є однією з основних причин смертності серед чоловіків молодого віку. У статті розглянуті імуногістохімічні маркери OCT3/4, SOX2, SOX17, HMGA1, Nek2, GPR30. Також висвітлено значення дослідження мікро-РНК у якості молекулярного фактора прогнозування перебігу захворювання на герміногенні пухлини яєчка.

Ключові слова: *імуногістохімічні маркери, мікро-РНК, пухлини яєчка.*

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