NEOPLASMA accepted, ahead of print version;
Cite article as https://doi.org/10.4149/neo_2019_190318N244

Running title: Biomarkers of CDDP resistance in testicular germ cell tumors

Understanding the crosstalk of molecular factors and signaling pathways reveals novel biomarkers of cisplatin resistance in testicular germ cell tumors

J. ROSKA, D. JURKOVICOVA*

Biomedical Research Center, Cancer Research Institute, Department of Genetics, Slovak Academy of Sciences, Bratislava, Slovakia

***Correspondence**: dana.jurkovicova@savba.sk

Received March 18, 2019 / Accepted July 3, 2019

Testicular germ cell tumors (TGCTs) mostly affect young men, but fortunately belong to well curable solid tumors. Today, different treatment strategies are applied reaching excellent out-comes and introduction of alternative approach of patient active survilence or adjuvant chemotherapy after orchiectomy decreases number of unnecessary toxic treatments of young patients. Also for relapsing patients, salvage therapy offers high survival rates. However, small percentage of affected young men do not respond to conventional therapy regimen due to intrinsic or acquired therapy resistance. For precise watching of patients during active surveillance, for stratification of patients due to their prognosis, and detection of therapy resistance and early relaps before treatment initiation, reliable molecular biomarkers and diagnostic tools replacing conventional approaches are still needed. Complex understanding of disease development and progression as well as mechanisms of chemoresistance and their epigenetic or chronobiological regulation pre-requisite successfull search for such novel biomarkers. In this review, we aimed to highlight the importance of crosstalk of different regulatory mechanisms and their key players affecting treatment response, and focus on their potential as novel molecular biomarkers and/or druggable targets.

Key words: testicular germ cell tumor, cisplatin resistance, DNA damage and repair, epigenetics, hypoxia, chronobiology

Testicular germ cell tumors (TGCTs) are the most common form of malignant solid tumors in young adult men and their incidence is continuosly increasing. In Europe, the most affected individuals are young Caucasians, mostly from Scandinavian countries, and increasing trends are still observed [1]. About 23,000 new cases are predicted to occur in Europe by 2025 [2]. In the United States, the biggest increase in the incidence of TGCT was observed in Hispanic population [3]. Racial differences in predisposition to TGCT have been identified; white men show the highest risk, while African or Asian men have lower risk of disease development [4].

TGCTs account for ~98% of all testicular malignancies exerting wide histological heterogeneity, mostly attributed to pluripotency of the originating germ cell line [5]. TGCTs derived from arrested, improperly developing fetal gonocytes accumulate oncogenic mutations, become germ cell

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46 neoplasia *in situ* (GCNIS) in childhood and adolescence, and can turn to invasive TGCT in the
47 young adults [6]. Based on histological type, TGCTs are generally classified into seminoma (SE)
48 and non-seminomatous tumors (NSTs) [5, 7]. SE are homogenous and develop most frequently at
49 the age of 35-39 years, while NSTs at younger age of 25-29 years. NSTs are usually heterogeneous
50 due to dysregulated differentiation and generally more aggressive, containing different histological
51 tumor components. The undifferentiated embryonal carcinoma (EC) cells show pluripotential stem
52 cell character and can differentiate into either extra-embryonal tissues like choriocarcinoma (CC),
53 yolk sac tumor (YST) or somatic derivatives like teratoma (TE) [5, 8]. Differentiated EC cells lose
54 expression of the pluripotency factor OCT4, the unique embryonal transcription factor of TGCTs
55 [9]. The expression of OCT4 and other pluripotency markers, such as SOX2 and NANOG [10,11]
56 are strictly limited to the GCNIS and undifferentiated EC cells, while absent in differentiated
57 tissues like YST, CC and TE [12, 13]. Therefore, OCT4 is considered a suitable diagnostic marker
58 recognizing EC, seminoma and early pre-invasive GCNIS lesions [14].
59 Clinical diagnosis of TGCT is usually based on physical examination, testicular ultrasound and
60 determination of serum tumor markers such as alpha-feto-protein (AFP), human chorionic
61 gonadotropin (hCG) and lactate dehydrogenase (LDH). Tumor staging and histology are confirmed
62 by orchiectomy and initial treatment is designed. Most patients are diagnosed with localized disease
63 (>80% of SEs and >60% of NSTs) presenting clinical stage I (CSI), localized in testicle with no
64 evidence of distant metastases. After orchiectomy, active surveillance (AS), adjuvant chemotherapy
65 or radiotherapy and primary retroperitoneal lymphadenectomy managemenmt approach are usually
66 proposed [15, 16]. Today, AS, based on close watching of the patients and monitoring of tumor
67 markers [17], represents an accepted alternative to radiotherapy and valid management option for
68 patients with CSI seminoma as well as non-seminomas. This approach saves significant percentage
69 of young patients from acute and/or chronic toxicity related to adjuvant chemotherapy. Patients with
70 diseases progression can be effectively treated with chemotherapy. For relapsing patients, salvage
71 therapy offers excellent treatment outcomes [17].
72 In contrast to majority of other solid malignancies, TGCTs are highly sensitive to genotoxic
73 chemotherapy, especially to cisplatin (CDDP). Several efficient chemotherapy regimens have been
74 developed based on combination of CDDP with ifosfamide, etoposide, vinblastine, paclitaxel or
75 more recently gemcitabine [18]. The use of conventional chemotherapy and surgery provides an
76 outstanding five-year survival rate (95.3%), even for patients diagnosed with distant metastases
77 (73.7%) [19-21]. However, the relapse rate for patients with seminoma is around 15–20% and for
78 low-risk non-seminoma patients approximately 20%, which may increase to up to 50% when there
79 is lymphovascular invasion (LVI) in the primary tumor [15, 22]. These patients experience

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80 recurrence and can develop CDDP resistance resulting in unfavorable prognosis [23].
81 Approximately 5% of all TGCT patients and 10-20% of patients with disseminated disease are
82 chemoresistant to CDDP and do not achieve a durable complete remission [24, 25]. Patients who
83 fail to achieve remission after either high-dose chemotherapy or second-line salvage therapy have
84 an extremely poor prognosis, and the vast majority eventually die of the disease [26]. CDDP
85 chemotherapy can trigger therapy-induced resistance, late toxicity and associates with
86 complications and secondary side effects including infertility, cardiovascular disease,
87 hypogonadism, chronic neurotoxicity, hearing loss, renal function impairment, pulmonary fibrosis,
88 secondary neoplasms and psychosocial and mental problems [27].
89 To identify the optimal treatment regimen for individual TGCT patients, the currently available
90 clinical risk stratification systems are considered not sophisticated enough to truly distinguish
91 TGCT patients with excellent and poor treatment outcomes. According to statistics and
92 International Germ Cell Consensus Classification (IGCCC) (1997), 55% of patients are expected to
93 be chemotherapy-resistant and 45% of patients are expected to respond standard CDDP therapy
94 [28]. However, such stratification is not possible upfront at the time of diagnosis.
95 It is therefore crucial to extend present knowledge of TGCT biology to develop combined clinico-
96 biological risk stratification algorithms, improved panels of biomarkers and targets for the
97 development of novel therapeutic agents and regimens for TGCT patients, especially for precise
98 watching of patients during active survillence and stratification due to risk of relaps and therapy
99 resistance.
100 **DNA damge and response in cisplatin sensitivity**
101 Due to very good responsiveness to CDDP treatment even in the late phase disease [29], TGCTs
102 serve as an excellent model system for studying the molecular mechanisms associated with
103 chemosensitivity and resistance. However, novel diagnostic, prognostic and therapeutic approaches
104 are not extensively applied due to lack of novel biomarkers and druggable targets. Consequently,
105 patients refractory to standard chemotherapy lack the possibility to receive novel effective
106 treatments and their prognosis is unsatisfactory [30]. Understanding mechanisms responsible for
107 chemoresistance might disclose novel important biomarkers and potentially offer new therapeutical
108 targets.
109 Chemoresistance can arise from reduced CDDP uptake, increased eflux, its inactivation by
110 intracellular antioxidants, and increased DNA repair capacity [31]. An impressive CDDP response
111 rates have been linked to an intrinsic hypersensitivity to DNA damaging agents, as observed in
112 several human EC lines derived from TGCT [32] and strong correlation with the expression of
113 OCT4, SOX2 and NANOG pluripotency factors. OCT4 expressed in embryonic stem cells controls

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114 their survival and pluripotency by cooperating with different transcription factors such as SOX2
115 [33]. Loss of OCT4 expression leads to CDDP resistance development [34].
116 In general, somatic cancers´ resistance to genotoxic chemotherapy associates with accumulated
117 mutations in DNA damage response (DDR) pathways, mostly in the *TP53* gene [35]. In response to
118 chemotherapeutic drugs, the DDR is triggered by early phosphorylation-driven signaling cascades
119 followed by a delayed response and induction of CDK (cyclin-dependent kinase) inhibitors for
120 prolonged cell cycle arrest [36]. The cytotoxic effect of CDDP is triggered by intra- and inter-strand
121 platinum-DNA adducts and DNA-protein crosslinks that form on DNA, such as Pt-GpG adducts
122 [24], promoting the apoptotic pathways [37]. DDR mechanisms can repair DNA lesions *via*
123 different DNA repair pathways, represented by six major classes of repair factors: (1) structure-
124 specific nucleases, which recognize and incise specific DNA structures, (2) translesion DNA
125 synthesis, (TLS) polymerases, error-prone polymerases that are able to tolerate DNA damage in the
126 template DNA strand, (3) homologous recombination (HR), (4) mismatch repair (MMR), (5)
127 nucleotide excision repair (NER), and (6) DNA damage response and repair pathway that is
128 defective in patients suffering from devastating genetic disease, known as Fanconi anemia pathway
129 (FA) [38] (Figure 1). NER represents the main defensive barrier against DNA damage [39] and a
130 major repair system for chemo- as well as radiotherapy-induced DNA damage [40]. Therefore, the
131 status of NER is a critical indicator of the CDDP chemotherapy outcome.
132 **NER and cisplatin resistance biomarkers**
133 NER represents a complex repair process carried out by seven xeroderma pigmentosum (XP)
134 proteins (from XPA to XPG) and approximately two dozens of non-XP proteins [41]. NER works
135 *via* two pathways: global genome repair (GGR) involved in injury repair for any genomic sequence,
136 important to prevent carcinogenesis and transcription-coupled repair (TCR) [42] responsible for
137 repair of the DNA damage of actively transcribed chains, potentially associated with tumor
138 chemosensitivity.
139 Initial steps in NER involve DNA damage recognition and verification. Proteins of XPC
140 complementation group and its accessory subunits have been identified as protein complexes
141 involved in recognizing DNA lesions and consequent recruitment of other repair proteins [43]. XPA
142 protein group is involved in crosstalk of GGR and TCR pathways [44] and neither GGR nor TCR
143 are initiated in the absence of XPA. It has been shown that XPA binds to the damaged duplex DNA
144 and recruits a heterodimer endonuclease complex consisting of ERCC1 (excision repair cross-
145 complementation group 1) and XPF that cleaves the damaged strand contributing to the assembly of
146 downstream NER complexes [45]. ERCC1 subunit mediates interaction of XPA with this nuclease
147 complex, which is highly specific and essential for NER. Due to fact that no other proteins in the

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148 cell can compensate the loss of XPA, the NER capacity can attractively be targeted *via* XPA.
149 Appart from ERCC1 and XPF, XPA interacts with many other NER proteins, and is considered as a
150 key scaffold for this repair pathway [46]. Another NER interacting partners of XPA include XPE,
151 TFIIH (transcription factor II H), RPA (replication protein A) and PCNA (proliferating cellular
152 nuclear antigen) (Figure 1). Besides the proteins directly involved in NER, XPA is also known to
153 interact with other involved proteins, including ATR (ataxia telangiectasia and Rad3-related) kinase
154 and PARP-1 (poly[ADP-ribose] polymerase 1). XPA is therefore considered one of the key rate-
155 limiting factors of the cellular NER capacity.
156 TGCT cell lines have been shown to express relatively low amounts of XPA protein. This low
157 expression was associated with CDDP hypersensitivity and reduced NER capacity [47]. On
158 contrary, overexpression of XPA protein has been shown in CDDP-resistant cancers, [48, 49],
159 where capacity of the tumor cells to repair DNA damage and avoid apoptosis is substantially
160 increased. Additionally, high responsiveness of TGCT cells to CDDP has also been associated with
161 increased induction of apoptosis and decreased efficiency of cell cycle arrest, probably caused by
162 altered p53 pathway [50]. Compared to other solid tumors, most of TGCTs express wild type p53,
163 usually in higher amounts than normal tissue [51].
164 **Hypoxia and cisplatin resistance biomarkers**
165 Solid tumors are usually partly hypoxic and increased levels of hypoxia are typically associated
166 with poor prognosis. Hypoxia in tumors promotes abnormal angiogenesis, desmoplasia and
167 inflammation, pre-selects cancer cells with more malignant phenotype, thus promoting tumor
168 progression and metastasis and triggers resistance to radiotherapy and chemotherapy [52]. The
169 cellular response to the drop of oxygen concentration leads to stabilization of the hypoxia-inducible
170 transcription factor (HIF) that regulates the gene expression by binding to the hypoxia response
171 element (HRE) in the promoter region of different genes [53].
172 Increased HIF protein expression is controlled through PI3K/PTEN/AKT and RAS/RAF/MAPK
173 signaling [54]. HIF controls multiple tumor promoting signaling pathways including growth factor
174 signaling, cancer cell invasion, epithelial–mesenchymal transition, metastasis and decreased
175 apoptosis and evasion from the immune system [52]. HIF directly inhibits apoptosis by decreasing
176 the expression of the pro-apoptotic Bcl-2 family proteins Bid and Bax [55] and induces the
177 expression of the apoptosis inhibitor survivin [56] and anti-apoptotic proteins Bcl-2 and Bcl-xl [57].
178 In this way, cancer cells escape apoptosis and decrease their drug responsiveness. Hypoxia also
179 modifies the cell surface proteins, which can shield the cells from immune system. HIF has been
180 shown to directly up-regulate programmed death-ligand 1 (PD-L1) that suppress T cell and CD47
181 and prevents phagocytosis by macrophages [58]. Hypoxia contributes to setting a dormancy

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phenotype through up-regulation of the main dormancy genes *NR2F1, DEC2*, and p27, which persist post-hypoxia helping the cancer cells to become therapy-resistant [59].

In TGCTs, HIF expression may be the master regulator of CDDP sensitivity. It stimulates glucose uptake and production of higher amounts of NADPH and glutation (GSH) [60], the two main defenders against oxidative stress. In chemosensitive TGCTs decreased HIF relates to low GSH level, reduced detoxification of CDDP and vulnerability to chemotherapy-induced oxidative stress. Increased HIF up-regulates multidrug resistance 1 (*MDR1*) gene expression [61] and transcriptionally represses cyclin D1 that induces G1 arrest contributing to cytotoxicity and resistance [62, 63].

HIF binds to HRE of XPA promoter and as a key regulator of its transcription increases XPA expression, that has been associated with CDDP resistance in several solid tumors [64, 65]. Inhibition of HIF using siRNA or PX-478 protein inhibitor decreased the expression of XPA resulting in an inability to repair CDDP-induced DNA damage and enhancement of chemosensitivity of various cancer cell lines and xenografts [66].

Epigenetic biomarkers

Compared to other solid tumors TGCTs, in fact, lack relevant and reliable biomarkers. Several driver mutations in *KIT, KRAS*, and *NRAS* genes were suggested, but discovered in only a minority of patients. Due to quiet mutational landscape [67], TGCTs are considered rather polygenic. However, these tumors share unique epigenetic landscape and complex microRNA regulation. Understanding epigenetic regulation in TGCTs seems to provide novel biomarkers with significant potential for better management of this malignity.

DNA methylation

In premordial germ cells, the genome is typically highly methylated, but when developing TGCT, the genome-wide methylation becomes ebolished, leading to epigenetic re-programming and initiation of tumorigenesis [68]. The epigenetic profile of TGCTs is characterised by genome-wide demethylation [69]. However, seminomas and non-seminomas exert significantly different promoter methylation profiles, as well as different genetic alterations, environmental component and the familial risk, reflecting specific clinical features including CDDP resistance in TGCT patients [70-72].

Especially familial susceptibility of TGCTs has been associated with promoter methylation in identified TGCT risk genes [73]. However, aberrant promoter hypermethylation of TGCT candidate suppressor genes or tumor-related genes is not that frequent as in other human cancers [74]. While in seminomas minimal or no methylation occurs, in non-seminomas and highly differentiated non-seminomas specific gene promoters are typicaly hypermethylated. Testicular carcinoma *in situ* cells

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216 express features resembling embryonic stem cells. Genes of pluripotency transcription factors, such
217 as *POU5F1/OCT-3/4, NANOG, T1A-2, MYCL1, GDF3, LIN28-A, DPPA4, DPPA5, KIT, AP-2γ* [75,
218 69] and promoters of supressor genes including *BRCA1, TP53, RASSF1A, CALCA* or *MGMT* are
219 usually highly methylated [76].
220 A tumor suppressor RASSF1A (RAS association domain-containing protein 1A), has been found as
221 one of the main supressors with variable promoter methylation in TGCTs. This gene participates on
222 cell-cycle control, microtubule stabilization, cellular adhesion, motility and apoptosis. CpG
223 methylation of the *RASSF1A* has been observed in many cancres [77] and depletion of this gene has
224 been associated with higher risk of chromosome rearrangements, accelerated mitotic progression
225 and enhanced cellular motility [78]. The *RASSF1A* promoter hypermethylation has been found in
226 both seminomas and non-seminomas being considered as the first epigenetic event of TGCT
227 tumorigenesis [68].
228 DNA methylation profiles can serve as potential molecular biomarkers for prognosis prediction and
229 treatment outcomes monitoring of TGCT patients. Using candidate gene approach Martinelli et al.
230 [68] assessed a set of potential DNA methylation biomarkers able to discriminate accurately the
231 clinical outcome of TGCT patients. A high frequency of *MGMT* (O-6-methylguanine-DNA
232 methyltransferase) and *CALCA* (calcitonin related polypeptide alpha) methylation were associated
233 with non-seminomatous tumors while *CALCA* methylation was associated with refractory disease.
234 Moreover, promoter methylation of both genes has been identified as predictive for poor clinical
235 outcome for TGCT patients [68].
236 Concordantly, Costa et al. [79] have reported that promoter methylation of
237 *CRIPTO/HOXA9/SCGB3A1* panel and *RASSF1A* best discriminate between controls and non-
238 seminomatous or seminomas tumors, and *HOXA9/RASSF1A* panel display the best discriminative
239 performance between non-seminomas and seminomas. Significant differences in *CRIPTO, MGMT*
240 and *RASSF1A* methylation levels were depicted between pure forms and matched mixed
241 components of seminomas and embryonal carcinoma. *HOXA9, RASSF1A* and *SCGB3A1* promoter
242 methylation are significantly associated with tumor stage proving that methylation patterns may
243 significantly contribute to identification of more clinically aggressive tumors.
244 **MicroRNAs**
245 The wide histological diversity of TGCTs and their individual sesitivity to chemotherapy treatments
246 are significantly affected also by post-transcriptional regulatory network of microRNAs (miRNAs).
247 Strictly regulated expression of miRNAs is important for various biological processes from
248 embryonic development to cell proliferation [80], cell differentiation [81], apoptosis and
249 metabolism [82], or tumorigenesis [83]. MiRNAs are essential for spermatogenesis and play an

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250 important role during mitotic, meiotic, and post-meiotic stages of spermatogenesis [84]. TGCTs
251 display miRNA profiles similar to embryonic stem cells [85] and their dysregulated expression
252 relates to cancer development and progression [69].
253 Usually, terminally differentiated histological subtypes express high levels of the most
254 discriminating miRNAs compared to poorly differentiated tumor subpopulations, such as seminoma
255 or EC [86]. In seminoma, the most different expression profiles have been identified for miRNAs
256 controling expression of pluripotency maintaining genes *OCT4, NANOG* and *SOX2*. The expression
257 of miR-302 cluster or key oncomiRs miR-21 and miR-155 were found strongly up-regulated [86]
258 and expression of tumor supressor miRNAs as miR-133a, miR-145, miR-146 or miR-199a were
259 found down-regulated in TGCTs [86, 87]. In human embryonic stem cells (ESCs) miR-145
260 suppressed the expression of *OCT4*, and partially repressed the expression of *SOX2* promoting their
261 differentiation [88]. Additionally, *NANOG, SOX2* and *OCT4* are regulated by miR-134, miR-296
262 and miR-470 in ESCs [89]. *Vice-versa*, the pluripotency factors NANOG, OCT4 and SOX2 are able
263 to regulate the expression of miRNA genes *via* direct binding to miRNA promoters, *e.g.* OCT4 to
264 miR-302 cluster [90].
265 During the male germ cell development, the expression of tumor suppressor let-7 miRNA family
266 increases along with miR-125a and miR-9 families [91]. These miRNAs regulate the expression of
267 *LIN28*, a key controller of stem cell pluripotency implicated in the formation of testicular TE [92].
268 Similarly, miR-30 family [92] or miR-181 [93] have been reported to down-regulate LIN28 in
269 ESCs and cancer cells. All these miRNAs have been identified under-expressed in malignant
270 TGCTs [94].
271 It is a typical feature that most germ cell tumours contain wild type p53 and over-express miR-371,
272 miR-372 and miR-373 (miR-371–373 clusters) [95, 86]. The miR-371–373 cluster is involved in
273 cellular senescence induced by oncogenic stress triggering malignant transformation of the cells
274 [86]. MiR-371-373 clusters and members of miR-302 family (miRNA-302a, miRNA-302b,
275 miRNA-302c) have recently been proposed as markers for TGCTs [85]. These clusters are highly
276 TGCT-specific, especially miR-371a-3p that is strongly up-regulated in patients with SE, EC, YS
277 and mixed non-seminomas. During the differentiation from EC to TE and post-orchiectomy, the
278 expression of these miRNAs significantly declines. Similarly, miR-17-5p and miR-154 are
279 expressed at higher levels in EC but are down-regulated upon differentiation to TE [86].
280 Gillis and co-workers [86] found miR-301 predominantly expressed in more differentiated tissues,
281 such as spermatocytic seminomas, YST and TE, but absent in embryonic stem cells and EC. On
282 contrary, miR-375 was highly expressed in TE, YST and mixed tumors, but not in SE or EC.
283 Overexpression of these miRNAs was found to down-regulate expression of histone-lysine N-

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284 methyltransferase Suv39-H1 and LATS2 (large tumour suppressor homolog 2), both involved in the
285 Ras oncogene pathway. LATS2 deletion causes cell proliferation and oncogenic transformation,
286 while its over-expression was shown to prevent Ras-mediated transformation of the cells. miR-371-
287 373 cluster was found to directly bind to the LATS2 3′UTR to control its translation and its over-
288 expression allowed cells to proliferate regardless of negative signals originating from p53 and the
289 cell cycle inhibitor p21 [95]. MiR-373 has been identified as a cell migration and metastasis-
290 promoting factor in breast cancer cells [96]. The same role of this miRNA can be expected in TGCT
291 metastasis. The up-regulation of miR371–373 cluster was detected together with other miRNA
292 molecules in several CDDP-resistant germ cell tumour cell lines and is expected to play a role in
293 inhibition of cell death and promotion of differentiation in response to CDDP [97]. Other miRNAs
294 were found significantly up-regulated in seminoma, including miR-221, miR-222, miR-372 and
295 miR-374 [98], while others have been observed down-regulated *e.g.* miR-30a, miR-34a, miR-106a,
296 miR-136, miR-382 or miR-217 [98, 99].
297 In solid tumors, numerous miRNAs are differentially regulated by hypoxia. For example, miR-210,
298 miR-155, miR-372, miR-373, miR-21 and miR-10b, known to have responsive element HRE in
299 their promotor region, were found to be up-regulated [100], whereas miR-20b, miR-200b or miR-
300 199a were found down-regulated [101]. On the other hand, miR-20b, miR-199a, miR-210 and miR-
301 424, directly target *HIF* gene and control its expression [102]. MiR-210 is up-regulated by hypoxia
302 in most of solid tumors and targets genes involved in cell cycle regulation, cell survival,
303 differentiation, angiogenesis, metabolism and cancer cell immune evasion [103]. This miRNA is
304 considered a master hypoxamiR and a new biomarker of metastatic potential and chemoresistance
305 in different solid tumors [104-106].
306 Downregulation of DDR genes by binding miRNAs to their 3´UTR sensitizes cancer cells to
307 chemotherapeutic agents. For example, miR-182, miR-1255b, miR-193b, and miR-148b were found
308 to regulate important HR proteins like BRCA1, BRCA2 and RAD51 [107]. HIF-induced miR-210
309 regulates RAD52, miR-96 regulates RAD51 and sensitizes cancer cells to CDDP and PARP
310 inhibitors [108]. Similarly, in TLS, inhibition of DNA polymerase REV1 by miR-96 increases
311 sensitivity of cancer cells to PARP inhibitors and CDDP treatment [33]. Crosby et al. [33] used
312 HeLa, MCF-7, and mouse embryonic fibroblasts to analyze the role of miRNAs in DNA repair
313 under hypoxic conditions. They observed up-regulated miR-210 and miR-373 in hypoxic cells in
314 HIF-dependent manner. Incresed expression of miR-210 was able to suppress levels of RAD52, a
315 key factor in HR, whereas miR-373 overexpression down-regulated both, RAD52 and RAD23B, a
316 component of the XPC/RAD23B complex involved in the NER machinery. Both miRNAs are

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317 complementary to seed sequences in 3´UTR of *RAD52* and *RAD23B* genes. Use of antagomirs for
318 miR-210 and miR-373 reverted hypoxia-induced RAD52 and RAD23B down-regulation [33].
319 Friboulet et al. [109] analyzed the role of ERCC1, a NER pathway protein involved in recognition
320 and removal of DNA platinum adducts and in repair of stalled DNA replication forks in non-
321 seminoma patients. ERCC1-positive tumors showed lower rate of genomic lesions than ERCC1-
322 negatives. In ERCC1-positive cancers, down-regulation of miR-375 was observed. Down-
323 regulation of miR-375 was previously described in hepatocellular carcinoma (HCC) and gastric
324 cancer where over-expression of miR-375 was able to inhibit cell proliferation [110]. This miRNA
325 was also predicted to target other genes involved in DNA repair, such as *TP53, USP1, APEX1,*
326 *TYMS, MLH3, PARP4, NTHL1, ERCC3*, and *XRCC6BP1* [110]. Similarly, miR-192 is involved in
327 DDR genes expression; it down-regulates ERCC3 and ERCC4 and its over-expression significantly
328 inhibits cellular NER [111].
329 miR-770-5p is another miRNA involved in CDDP resistance [112]. In ovarian cancer, the level of
330 miR-770-5p expression was low in CDDP-resistant patients, but its overexpression in resistant cell
331 lines increased sensitivity to CDDP. MiR-770-5p targets ERCC2 involved in NER and may
332 function as an anti-oncogene promoting chemosensitivity by downregulation ERCC2 [112].
333 The current clinical practice mostly requires reliable and specific tools for disease progression and
334 treatment outcome monitoring. In this regard, miRNAs as a biomarkers show a great promise. For
335 instance, miR-371 as a biomarker for TGCT demonstrated a sensitivity of 84.7 % and specificity of
336 99 %, in contrast with the serum markers AFP and hCG, that were found false negative in 50 % of
337 cases [113]. Upregulation of miR-371-373 and miR-302 clusters in both, the tissue and serum, were
338 detected in the TGCT patients but their expression dropped significantly after orchiectomy [86,
339 114]. Dieckmann's [114] group further revealed the existence of a concentration gradient for these
340 miRNAs around the tumour, being higher in the serum of the testicular vein than in peripheral
341 serum.
342 Gillis et al. [115] developed a robust protocol for analysing miR-371-373 and miR-302 clusters in
343 serum, bringing the Target Serum miRNA test (TSmiRNA). The combination of conventional
344 serum markers AFP, hCG and LDH evaluation and TSmiRNA provided an adequate and accurate
345 classification of all testicular cancer samples. [116, 117]. Recently, Mego et al. [118] showed very
346 promissing translation results that provided evidence, that plasma levels of miR-371a-3p correlate
347 with several disease characteristics including sites of metastases, serum tumor markers, IGCCCG
348 prognostic group, and favorable response to chemotherapy measured just prior to the start of
349 therapy. This group also managed to show that prognostic value of plasma miR-371a-3p in

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350 chemotherapy naïve TGCTs patients starting first line of chemotherapy, as well as prognostic value
351 of plasma miR-371a-3p changes during the treatment.

352 **Chronobiology**

353 To respect complex regulatory network, additional mechanism affecting precise control of DDR
354 and other signaling pathaways of the cell response (and potentially their contribution to CDDP
355 resistance) should be considered. Such control relates to the circadian clock regulation.
356 Approximately 40% of all proteins within the cell are subjected to circadian control [38], although
357 in tissue specific manner [119].

358 Each cell is equipped with positive regulators CLOCK and BMAL1, transcriptional factors, which
359 regulate expression of genes in mammals by interacting with E-box (CACGTG) in their promoter
360 [120]. Heterodimer CLOCK/BMAL1 targets different repressor proteins, including negative
361 regulators PERIOD (PER1, 2, 3) and CRYPTOCHROME (CRY1, 2), which in turn inhibit the
362 transcriptional activity of CLOCK/BMAL1 transcription factor [121].

363 Under normal physiological conditions, DDR processes are synchronized. However, the process of
364 DDR can run independently from circadian rhythm [122]. From the broad spectrum of DNA
365 damage responses, NER is the only one showing circadian rhythmicity. The rhythmicity of NER
366 relates to the rhythmic expression of XPA, as observed *e.g.* in brain [123], skin keratinocytes [124],
367 kidneys and liver [125]. However, rhythmic expression of XPA has not been described in testes
368 until now [126].

369 The circadian clock controlls the steady state level of XPA [123] by HERC2 [126] and SIRT1
370 [127]. The transcriptional activity of the circadian clock induces a daily rhythm of *XPA* gene
371 expression, whereas HERC2 functions as an E3 ubiquitin ligase for XPA degradation in a
372 proteasome-dependent manner. The half-life of XPA protein is approximately 4 h in the absence of
373 DNA damage, but much longer in the presence of DNA damage [126]. In response to DNA
374 damage, XPA is phosphorylated by ATR kinase, which stabilizes XPA by preventing its association
375 with HERC2 [126]. Thus, ATR activity in response to DNA damage can be utilized to a certain
376 extent as a surrogate marker for NER activity. SIRT1, a NAD^+-dependent histone deacetylase, also
377 plays a critical role in NER pathway control.

378 Concerning circadian rhythms in oncological diseases, plentiful opposing, and tissue specific results
379 can be found. Some studies report, that tumors do indeed show rythmic circadian gene expression
380 [128], while others indicate that cancerous tissue either lacks rhythmicity or expression of clock
381 genes is compromised [129, 130]. Recent studies have declared absence of circadian rhythm in
382 TGCTs, although some authors proposed that cyclic expression of clock genes in testes is
383 suspended due to cellular differentiation during spermatogenesis [126,131].

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384 Because TGCTs are very good model for studying mechanism of CDDP resistance and DNA
385 damage response, understanding the absence of circadian rhythms in this tissue becomes of an
386 increased interest. Lu et al. [132] explained post-translational circadian rhythm disruption *via*
387 cancer/testis antigen (CTA) PIWIL2, which is able to repress circadian rhythms both in tumor cells
388 and in testis. PIWIL2 suppresses glycogen synthase kinase 3 (GSK3β)-induced phosphorylation to
389 regulate the stability and activity of circadian proteins. Besides protecting BMAL1 and CLOCK
390 from degradation, authors suggest that PIWIL2 can also bind to certain E-box sequences (PER2 and
391 Rev-Erbα promoters), to negatively regulate the transcriptional activation of main clock proteins.
392 Michael et al. [133] supported the hypothesis of CTA disrupting the circadian rhythms in testes and
393 in tumors. They identified the PAS domain containing protein 1 (PASD1), evolutionarily connected
394 to CLOCK protein, that can interact with the CLOCK/BMAL1 heterodimer and suppress circadian
395 rhythmicity.
396 To support the complexity of this regulation, several studies point out the effect of miRNA on
397 circadian clock. So far, miR-211 (a PERK inducible miRNA) has been shown involved in
398 circadiane clock control regulating BMAL1/CLOCK activity [134].
399 The presence of circadian rhythms in tumors, especially in TGCTs, needs further elucidation. Even
400 in lack of rhythmicity, the core clock proteins still affect tumor development [135]. New
401 information about circadian rhythms in TGCTs is needed to clarify its role and existence or the
402 reason of its complete lack. Importnatly, complex understanding of chronobiology could
403 significantly support future clinical implications and development of chronochemotherapy regimens
404 [136] that could potentially deminish consequences of chemoresistance in TGCT and other cancers.
405
406 **Conclusion**
407 Today, conventional clinical management of testicular germ cell tumors is largely based on the
408 monitoring of serum tumor markers, unfortunately showing limited sensitivity and specificity [137].
409 Due to limited informative value of these markers, as well as increasing requirement of individual,
410 personalized approach to the patient, there is an urgent need of discovery of novel reliable
411 biomarkers, mostly for diagnostics of advanced TGCT stages, precise stratification of patients prone
412 to relaps, and prediction of chemotherapy response. An increasing number of studies provide
413 cumulative evidence of specific epigenetic regulators as relevant oncogenic biomarkers of TGCTs
414 [138, 139]. Deep and complex understanding of molecular mechanisms, regulatory pathways and
415 their crosstalk involved in therapy response remains crucial for efficient treatment management of
416 TGCT patients, especially those that are CDDP-resistant. Particular attention has to be paid to the
417 role of DNA damage and repair genes, pluripotency factors, effect of hypoxia and their epigenetic

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418 and circadian controllers as they represent a pool of novel promising biomarkers or potential
419 druggable targets (Figure 2).
420

421 **Acknowledgments:** This work was supported by the Grant Agency of Slovak Republic under
422 Grants no. VEGA 2/0108/17 and 2/0053/19, by Ministry of Education, Science, Research and Sport
423 of Slovak Republic under Grant no. MVTS 34097104 and by Slovak Research and Development
424 Agency under Grant no. APVV-17-0384.
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868 **Figure legends**

869

870 **Figure 1. Activation of DNA damage repair pathways during cell cycle and schema of NER.**
871 At the top, predominant repair processes in each phase of the cell cycle are shown. Based on type of
872 damage during the cycle, different repair pathways are involved in DNA damage repair. NER is the
873 main repair pathway associated with CDDP-induced DNA damage in TGCTs, and key steps of
874 NER are illustrated below. Abbreviations: BER – base excision repair; ERCC1 – excision repair
875 crosscomplementation group 1; HR – homologous recombination; MMR – mismatch repair; NER –
876 nucleotide excision repair; NHEJ – non-homologous end joining; PCNA – proliferating cell nuclear
877 antigen; RAD23B - excision repair protein RAD23 homolog B, RPA – replication protein A; TFIIH
878 – transcription factor II H; TLS – translesion synthesis; TS – template switch; XP A-G – xeroderma
879 pigmentosum group A-G.

880

881 **Figure 2. The crosstalk of molecular factors and signaling pathways involved in CDDP**
882 **resistance in TGCTs.** Cancer cell can gain chemoresistance towards CDDP *via* several different
883 mechanisms: reduced cellular uptake and/or increased efflux of CDDP, increased CDDP
884 detoxification by glutathione (GSH), regulation of the cell cycle arrest prolonging time for DNA
885 damage response mechanisms to repair and restore CDDP-induced adducts (NER prominently), or
886 inhibition of apoptosis. Loss of pluripotency of the cell, hypoxia – typical for solid tumors, and
887 mutations in *TP53* gene are another crucial factors contributing to chemoresistance development.
888 All of these mechanisms are under control of transcription factors (*e.g.* HIF), epigenetic modulators
889 (DNA methylation and miRNAs) and potentially, circadian regulation *via* clock controlled genes.
890 To understand CDDP and other anti-cancer drug therapy resistance it is important to understand
891 orchestration and crosstalk of multiple regulatory pathways. The key factors of these signaling
892 pathways could represent a pool of potential novel diagnostic and prognostic biomarkers (in grey
893 boxes). Abbreviations: CDDP – cisplatin; DDR – DNA damage response; HIF – hypoxia-inducible
894 factor; NER – nucleotide excision repair; TGCT – testicular germ cell tumor.

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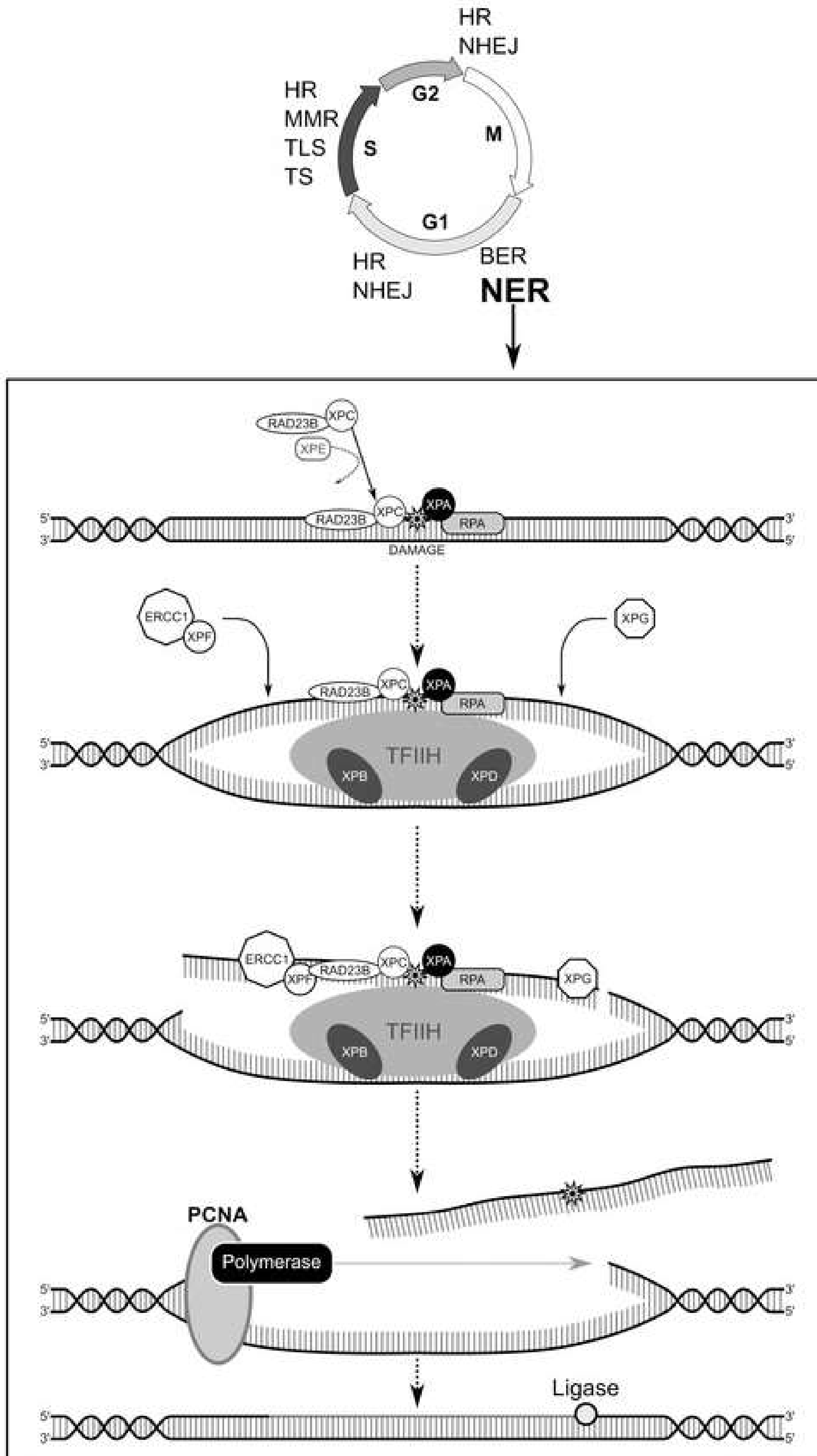

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