Central European Journal of Urology

ORIGINAL PAPER

UROLOGICAL ONCOLOGY

Association of metallothionein expression and clinical response to cisplatin based chemotherapy in testicular germ cell tumors

Emre Tuzel[1], Kutsal Yorukoglu[2], Esra Ozkara[2], Ziya Kirkali[3]

[1]*Baskent University, Department of Urology, Izmir, Turkey*
[2]*Dokuz Eylül University School of Medicine, Department of Pathology, Izmir, Turkey*
[3]*Dokuz Eylül University School of Medicine, Department of Urology, Izmir, Turkey*

Citation: Tuzel E, Yorukoglu K, Ozkara E, Kirkali Z. Association of metallothionein expression and clinical response to cisplatin based chemotherapy in testicular germ cell tumors. Cent European J Urol. 2015; 68: 45-50.

Article history
Submitted: Oct. 1, 2014
Accepted: Dec. 13, 2014
Published on-line: March 13, 2015

Corresponding author
Emre Tuzel
Zubeyde Hanim Arastirma ve Uygulama Merkezi
6471/5 sk. No. 7
BÜ Mavisehir plk. Binasi
35550 Izmir, Turkey
phone: +90 23 224 11 000 1022
emretuzel@gmail.com

Introduction The protective roles of metallothioneins (MT) against metal toxicity suggest that MT may have a functional role in cisplatin resistance. The aim of this study was to investigate the expression of MT in specimens of germ cell tumors and compare it with clinical sensitivity to cisplatin based chemotherapy.
Material and methods Tissue blocks of primary GCT specimens obtained from 39 patients were examined immunohistochemically for MT expression. Staining intensity was evaluated according to the percentage of MT positive cells and graded as [-], [+] and [++]. The staining characteristics were compared with the clinical response to chemotherapy.
Results Of the 39 tumors, 3 evidenced no MT expression while 26 and 10 specimens showed [+] and [++] staining, respectively. Although seminomas tend to stain weaker than non-seminomas, the difference of staining between them was not significant (p = 0.19). Of the 39 patients, 23 underwent cisplatin based chemotherapy. Of those, 6 progressed and 17 achieved complete remission. Of the non-responders, 5 showed [+] and 1 showed [++] staining. Six of the responders showed [+], 10 had [++] and 1 showed no staining. No association was found between MT staining and chemo-sensitivity (p = 0.53).
Conclusions MT expression in primary germ cell tumors did not differ between responding and non-responding patients and therefore may not be useful in predicting response to chemotherapy.

Key Words: testicular germ cell tumors ‹› metallothionein ‹› cisplatin ‹› immunohistochemistry ‹› chemoresistance

INTRODUCTION

Testicular germ cell tumors (GCT) are particularly interesting from a clinical perspective because of their exquisite sensitivity to cisplatin based chemotherapy. Approximately 80% of patients with advanced disease can be cured [1]. Despite the clinical efficacy of chemotherapy, resistance remains a problem in some patients with GCT. Approximately 10-20% of patients diagnosed with a GCT will not respond to cisplatin based chemotherapy or will relapse despite further treatment, representing a further challenge for the treating physician [2]. The principal cause of therapeutic failures in cases of advanced GCTs involves the phenomenon of resistance to cisplatin based chemotherapy.

Metallothioneins (MT) are small, cysteine-rich, metal binding proteins which are involved in trace metal homeostasis and metabolism, detoxification of toxic metals, development of resistance towards metal containing drugs and scavenging of free radicals [3, 4]. It has been suggested that cellular resistance may be mediated by reduced permeability of tumor cells to drugs, accelerated DNA repair in cisplatin damaged cells and an increase in chemoprotective thiols including MT [5, 6]. The protective roles

doi: 10.5173/ceju.2015.01.486

of MT against oxidative stress and metal toxicity also suggest that MT may have a functional role in cisplatin resistance [7]. Experimental studies have shown that tumor cell lines with acquired resistance to cytotoxic alkylating agents and cisplatin overexpress MT [8]. However, the functional roles of MTs in the clinical setting are less evident.

In the present study, we examined the expression of MT in untreated testicular GCT specimens and compared it with the clinical response to cisplatin based chemotherapy in order to investigate the association between MT expression and cisplatin resistance.

MATERIAL AND METHOD

The medical records and tumor tissue blocks of 39 primary untreated GCT patients who underwent radical orchiectomy were investigated after approval of the study by the local ethics committee of our institution. Median age was 25 years (range, 17-52) and median follow–up was 41 months (range, 3-156). Tumors were staged according to the International Union Against Cancer TNM classification and graded according to WHO histopathological typing [9, 10]. The histological subtype of the study group consisted of 10 seminomas (25.7%), 10 embryonal cell carcinomas (25.7%), 3 teratomas (7.6%) and 16 (41%) mixed GCTs.

Immunohistochemistry

Archival histopathological slides from 39 patients which were stained with hematoxylin and eosin (H-E) were retrieved and reviewed. The most representative blocks were selected, cut into 5 μm thick sections and placed on poly-L-lysine pretreated glass slides. Immunohistochemistry was performed using the monoclonal primary mouse anti-MT antibody E9 (Zymed Laboratories, San Francisco, USA), which was prepared from immunization of Balb/c mice and is able to detect immunreactive MT in formalin fixed paraffin embedded human tissues by the streptavidin-biotin method (DAKO, LSAB Universal kit, Carpenteria, USA). Appropriate negative controls were obtained by omitting the primary antibody from the staining procedure. Human normal testis was used as a positive control.

All slides were evaluated twice by pathologists on separate occasions without any knowledge of patients' clinical outcome. MT staining intensity in the tumor samples was evaluated semiquantitatively according to the percentage of MT positive cells. Tumors with ≥75% staining were classified as showing strong staining and graded as [++], tumors with <75% immunostaining were classified as showing weak immunostaining and graded as [+] and [-] if no immunostaining was observed. The staining intensity evaluation has been validated in other tissues as described earlier [11].

Standard chemotherapeutic regimen for patients with advanced GCT consisted of 3 or 4 cycles of bleomycin, etoposide and cisplatin (BEP). All patients were re-evaluated after completion of chemotherapy. Patients considered as complete responders (CR) were those with normal tumor markers and no residual mass following chemotherapy, as well as patients who had necrosis and fibrosis of mature teratoma following post-chemotherapy residual mass resection. Patients with 50% or more decrease in the diameter of measurable lesions were considered as partial responders. Patients still having elevated levels of tumor markers after chemotherapy and patients with persistent vital carcinoma following post-chemotherapy residual mass resection were considered as non-responders (NR).

The immunostaining characteristics were compared with the clinical response in patients who underwent cisplatin based chemotherapy. Associations between MT expression and the clinicopathological features were assessed by the chi-square test. Results were considered statistically significant at a $p < 0.05$.

RESULTS

MT staining was heterogenous within each tumor and subcellulary MT was localized both in the cytoplasm and nucleus in most of the tumor cells. In general, cytoplasm stained more frequently and none of the tumors showed nuclear staining alone (Figures 1, 2, 3). Of the 39 tumor samples, 3 (7.7%) evidenced no MT expression, while 26 (66.7 %) and 10 (25.6%)

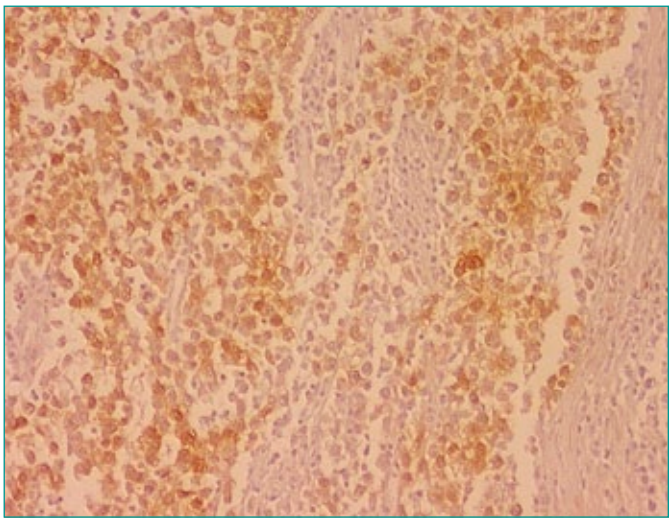

Figure 1. *Seminoma with weak [-] MT immunoreactivity (x 100).*

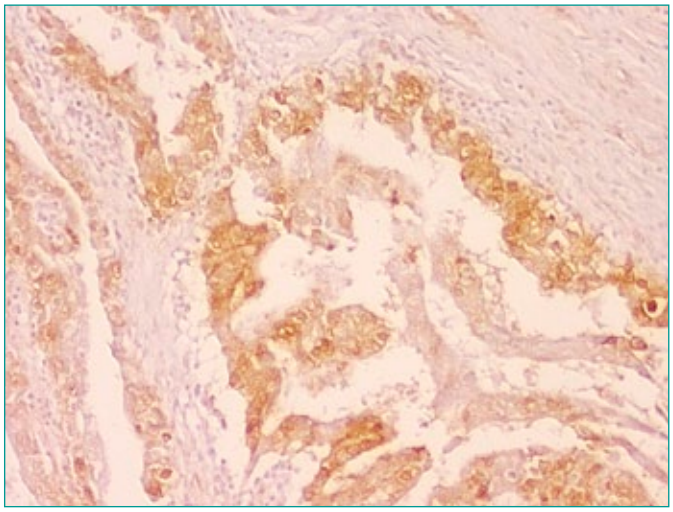

Figure 2. *Embryonal carcinoma showing [=] MT immunoreactivity (x 100).*

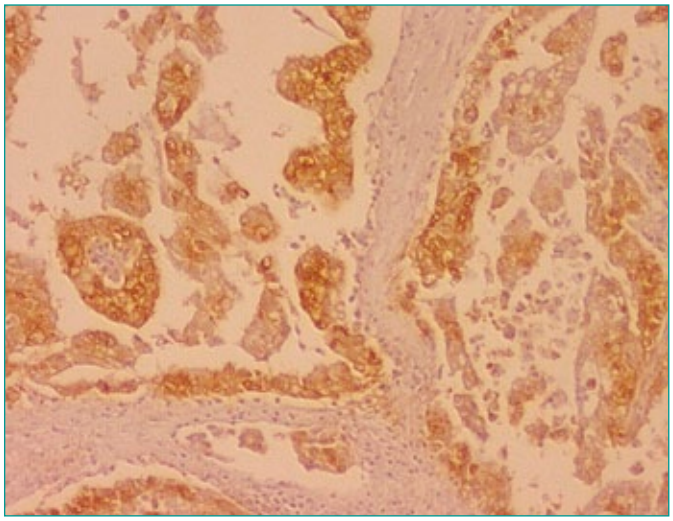

Figure 3. *Representative tissue block of an embryonal cell carcinoma with intense [++] MT staining (x 100).*

specimens showed [+] and [++] staining patterns, respectively.

The distribution of tumors according to histologic subtype and MT expression is listed in Table 1. None of the seminomas showed [++] staining. Although seminomas tend to stain weaker than non-seminomas, the difference of staining intensity between these subtypes was not significant. The distribution of tumors according to clinical stage and MT staining intensity is presented in Table 2. Ninety percent of tumors with clinical stage I or II disease showed MT expression, whereas 100 % of advanced stage tumors showed [+] or [++] staining. No correlation was observed between increasing stage in germ cell tumors and MT staining intensity (p = 0.09).

None of the patients received radiotherapy or chemotherapy before orchiectomy. One patient with advanced seminoma (clinical stage III) received cisplatin based chemotherapy with [+] MT staining. The patient had a complete response following chemotherapy and remained disease free during follow-up. Twenty-two of 29 patients with non-seminomatous germ cell tumors underwent cisplatin based chemotherapy with a median of 3 (range, 2-8) cycles. Two patients died of progressive disease, 3 patients had persistently elevated tumor markers after chemotherapy and 1 had a vital carcinoma following post-chemotherapy residual mass excision. These patients were considered as resistant to chemotherapy. There was no significant relationship between the presence of staining for MT and the response to chemotherapy (Table 3) (p = 0.53).

Table 1. *Distribution of tumors according to histopathological subtype and MT expression*

	MT (%)				
Histological subtype	n	[-]	[+]	[++]	p
Seminoma	10	1 (10)	9 (90)	–	0.19
Embryonal carcinoma	10	–	6 (60)	4 (40)	
Teratocarcinoma	3	–	3 (100)	–	
Mixt GCT	16	2 (12.5)	8 (50)	6 (37.5)	

Table 2. *Correlation of clinical staging and MT immunostaining of germ cell tumors*

Clinical stage					
Tumor histology		I	II	III	
Seminoma (n=10)*					
MT	[-]	1 (10)	–	–	–
	[+]	8 (80)	–	1 (10)	–
	[++]	–	–	–	–
Non-seminoma (n=29)					
MT	[-]	2 (6.9)	–	–	–
	[+]	7 (24.2)	5 (17.2)	4 (13.8)	–
	[++]	5 (17.2)	2 (6.9)	–	4 (13.8)

*p=0.09 between seminomas and non-seminomas

Table 3. *MT expression and clinical response to cisplatin based chemotherapy*

	MT (%)			
	[-]	[+]	[++]	p
CR	1 (5.9)	10 (58.8)	6 (35.3)	
NR	-	5 (83.3)	1 (16.7)	

CR: Complete responders; NR: non-responders

DISCUSSION

The immunohistochemical staining patterns of MT have been reported with archival paraffin embedded tumor tissues in various types of human tumors. It was suggested that overexpression of MT in ovarian, prostate and colon tumors could have protective effects against cisplatin [12, 13, 14], whereas other reports did not support this perception [15].

A number of *in vitro* experimental studies have shown that tumor cell lines with acquired resistance to cisplatin overexpress MT [8, 16]. However, other investigators have reported an inverse correlation between resistance and MT or total amount of sulphydryl groups both in cell lines and clinical samples [17, 18]. For instance, Masters et al. reported high MT levels in cisplatin sensitive testicular tumor cell lines [19]. Thus, experimental evidence suggesting a role of MT in cisplatin resistance appears to be inconclusive.

The importance of MT in human GCT has been examined clinically in 4 previous studies. However, the data on the predictive value of MT expression regarding cisplatin resistance has been divergent [18, 20, 21,22]. In a previous study, the intensity and extent of MT staining assessed in tissue sections of 9 embryonal cell carcinomas. The authors observed a considerable heterogeneity in the MT content among individual cells and proposed that MT may be considered as an onco-developmental product [23]. The same investigators later assessed the degree of MT immunostaining in 33 primary testicular GCT specimens [20]. They noted a distinct difference between MT staining in seminomas and non-seminomas. Non-seminomas tended to stain heavily for MT, especially in the more advanced stages [20]. Three patients with advanced seminoma and 15 of 23 patients with non-seminomatous testis tumors received cisplatin based chemotherapy. Tumors from 2 patients presented with a complete response. One of the two patients died due to progressive disease during initial chemotherapy and 2 patients with resistance to first line chemotherapy were reported to show heavy MT staining [20]. In that study, although a direct correlation between cisplatin resistance and MT content had not been established, the authors highlighted the possibility of such a relationship depending on inferential clinical data. In contrast, in 77 patients with germ cell testicular tumors, high MT immunostaining was found to predict a better response rate to chemotherapy, opposing the hypothesis that MT over-expression contributes to cisplatin resistance in this tumor type [21]. The authors attributed these discrepancies to the immunohistochemical staining techniques. Moreover, an intense MT staining in 85% of seminomas was observed and 78% of poor responders to chemotherapy showed no or little staining for MT (all with non-seminomas of advanced stage) [21]. According to these findings, authors suggested that MT expression might be a marker of chemo-sensitivity and its absence, when it occurs, indicated a resistant tumor type [21].

Meijer et al. investigated MT levels and functionality both in the cell line model and in 14 specimens of human GCT, as well as in post-chemotherapy residual vital GCT [18]. Metallothionein levels of cell lines were found to be inversely correlated with sensitivity to cisplatin. In agreement with the *in vitro* data, immunohistochemical detection of MT was reported to be high in 11/14 primary human germ cell tumors. No difference was documented in MT protein expression between primary GCT of responding (n = 6) or non-responding (n = 8) tumors to cisplatin based chemotherapy [18]. Interestingly, all post-chemotherapy residual vital GCTs tested showed a decreased or undetectable level of MT expression compared with their primary tumors. This finding attributed to the tissue specific expression of MT isoforms [18]. The authors concluded that MT expression in primary GCTs did not discriminate between responding and non-responding patients, and therefore could not be used to predict response to chemotherapy [18].

In another study, the immunohistochemical staining pattern of proteins involved in the regulation of apoptosis, cell cycle control, and drug export and inactivation were investigated in samples of unselected GCTs (n = 20) in patients achieving a complete remission following chemotherapy (n = 12) and in chemotherapy refractory patients (n = 24) [22]. Mature teratoma components (n = 10) within tumor samples from all groups were analyzed separately. Metallothionein immunostaining was identified in 35%, 58% and 45% of unselected GCTs, chemotherapy responsive tumors and chemotherapy refractory tumors, respectively [22]. In that study, no significant difference was observed in any of the potential regulators of chemotherapy sensitivity, including MTs, between the samples of responding or chemotherapy refractory tumors. Because MT was detectable in tumors regardless of treatment outcome or histology, the authors suggested that the presence of MT immunostaining was not sufficient to confer resistance [22].

In the present study, we examined the immunuhistochemical expression pattern of MT in a cohort of testis tumor patients and also focused explicitly on the implications of MT overexpression in the subgroup of patients who received cisplatin based che-

motherapy. Despite the findings of Eid et al., who reported that MT overexpression in GCT indicates a favorable response to cisplatin therapy, no such relationship was detected in our patient population using monoclonal antibody E9. Consistent with the findings of three previous studies [20, 21, 22], we found no significant association between overexpression of MT and sensitivity to cisplatin based chemotherapy. In the present series, of the 23 patients receiving cisplatin based chemotherapy, 17 achieved a complete response. Of those, 16 (94 %) showed weak or strong MT staining and all of the non-responders showed [+] or [++] MT staining. On the other hand, it is noteworthy that all of the patients who failed to achieve a complete response following cisplatin based chemotherapy had some degree of MT staining. The process of cisplatin resistance is determined by multiple factors on different cellular levels, such as changes in cellular drug uptake and efflux, leading to decreased drug accumulation [24]. Nevertheless, a direct measure of the correlation between cisplatin resistance and MT content in tumors may be obscured by the complexity of cellular defenses against toxicity. Furthermore, genetic variability of the patients themselves might also affect the expression of MT or sensitivity to cisplatin. It appears that in human GCTs, MT may be associated, but is certainly not required, for the induction of resistance to cisplatin. In addition, the controversial results of the previous studies could possibly be attributed to the methods applied using antibodies that were unable to distinguish specific MT isoforms, metal bound and metal free forms of the protein [4]. Subcellular distribution of MT differs between cell types and this may be more important than MT levels in cellular protection against cisplatin damage. The low number of patients may be considered the limiting factor for the power of the present study. In particular, the lack of statistical significance between the groups in the present study might be attributed to the inclusion of limited number of patients. Further studies including larger number of patients are warranted to overcome this ubiquitous problem.

CONCLUSIONS

MT expression in primary germ cell tumors did not differ between responding and non-responding patients and therefore may not be useful in predicting response to chemotherapy. Constitutive expression of MT does not seem to depend on the phenotype of the tumor, and mechanisms of drug resistance in primary testicular germ cell tumors is probably multifactorial. Further research is required to investigate the molecular mechanisms behind the resistance of cisplatin in GCT.

References

1. Feldman DR, Bosl GJ, Sheinfeld J, Motzer RJ. Medical management of advanced testicular cancer. JAMA. 2008; 299: 672-684.
2. Garcia-Valesco A, Duran I, Garcia E, Taron M, Ballestin C, Castellanos C, et al. Biological markers of cisplatin resistance in advanced testicular germ cell tumors. Clin Transl Oncol. 2012; 14: 452-457.
3. Namdarghanbari M, Wobig W, Krezoski S, Tabatabai NM, Petering DH, Mammalian metallothionein in toxicology, cancer and cancer chemotherapy. J Biol Inorg Chem. 2011; 16: 1087-1101.
4. Thirumoorthy N, Sunder AS, Kumar KTM, Kumar MS, Ganesh GNK, Chatterjee M. A review of metallothionein isoforms and their role in pathophysiology. World J Surg Oncol. 2011; 9: 54-61.
5. Michalke BJ: Platinum speciation used for elucidating activation or inhibition of Pt-containing anti-cancer drugs. J Trace Element Med Biol. 2010; 24: 69-77.
6. Theocharis SE, Margeli AP, Klijanienko JT, Kouraklis GP. Metallothionein expression in human neoplasia. Histopathology 2004; 45: 103-118.
7. Pedersen MO, Larsen A, Stoltenberg M, Penkowa M. The role of metallothionein in oncogenesis and cancer prognosis. Prog Histochem Cytochem. 2009; 44: 29-64.
8. Koropatnick J, Kloth DM, Kadhin S, Chin JL, Cherian MG. Metallothionein expression and resistance to cis-platin in a human germ cell tumor line. J Pharmacol Exper Therap. 1995; 275: 1681-1687.
9. Sobin LH, Gospodarowicz M, Wittekind C. TNM classification of malignant tumors. UICC International Union Against Cancer, ed 7, London, Wiley-Blackwell, 2009, ISBN: 978-1-4443-3241-4.
10. Mostofi FK, Sesterhenn IA. Histological typing of testis tumors. WHO International Histological classification of Tumors, ed 2, Berlin, Springer, 2005, ISBN 978-3-540-42256-3.
11. Tüzel E, Kirkali Z, Yörükoğlu K, Mungan MU, Sade M. Metallothionein expression in renal cell carcinoma: Subcellular localization and prognostic significance. J Urol. 2001; 165: 1710-1713.
12. Dziegiel P, Forgacz J, Suder E, Surowiak P, Kornafel J, Zabel M. Prognostic significance of metallothionein expression in correlation with Ki-67 expression in adenocarcinomas of large intestine. Histol Histopathol. 2003; 18: 401-407.
13. Smith DJ, Jaggi M, Zhang W, Galich A, Du C, Sterrett SP, Smith LM, Balaji KC. Metallothioneins and resistance to cisplatin and radiation in prostate cancer. Urology. 2006; 67: 1341-1347.
14. Surowiak P, Materna V, Maciejczyk A, Pudelko M, Markwitz E, Spaczynski M, et al. Nuclear metallothionein expression correlates with cisplatin resistance of ovarian cancer cells and poor clinical outcome. Virchows Arch. 2007; 450: 279-285.
15. Ioachim EE, Goussia AC, Agnatis NJ, Machera M, Tsianos EV, Kappas AM.

Prognostic evaluation of metallothionein expression in human colorectal neoplasms. J Clin Pathol. 1999; 52: 876-879.

16. Siegsmund MJ, Marx C, Seeman O, Schummer B, Steidler A, Toktomambetova L, et al. Cisplatin resistant bladder carcinoma cells: enhanced expression of metallothioneins. Urol Res. 1999; 27: 157-163.

17. Piulats JM, Jiménez L, García del Muro X, Villanueva A, Viñals F, Germà-Lluch JR. Molecular mechanisms behind the resistance of cisplatin in germ cell tumors. Clin Transl Oncol. 2009; 11: 780-786.

18. Meijer C, Timmer A, de Vries EGE, Groten JP, Knol A, Zwart N, et al. Role of metallothionein in cisplatin sensitivity of germ cell tumors. Int J Cancer. 2000; 85: 777-781.

19. Masters JRW, Thomas R, Hall AG, Hogarth L, Matheson EC, Cattan AR, Lohrer H. Sensitivity of testis tumour cells to chemotherapeutic drugs: Role of detoxifying pathways. Eur J Cancer. 1996; 32A: 1248-1253.

20. Chin JL, Banerjee D, Kadhim SA, Kontozoglou TE, Chauvin PJ, Cherian MG. Metallothionein in testicular germ cell tumors and drug resistance. Clinical correlation. Cancer. 1993; 72: 3029-3035.

21. Eid H, Géczi L, Bodrogi I, Institoris E, Bak M. Do metallothioneins affect the response to treatment in testis cancer? J Cancer Res Clin Oncol. 1998; 124: 31-36.

22. Mayer F, Stoop H, Scheffer GL, Scheper R, Oosterhuis JW, Looijenga LHJ, Bokemeyer C: Molecular determinants of treatment response in human germ cell tumors. Clin Cancer Res. 2003; 9: 767-773.

23. Kontozoglou TE, Banerjee D, Cherian MG. Immunohistochemical localization of metallothionein in human testicular embryonal cell carcinoma cell lines. Virchows Arch A Pathol Anat Histopathol. 1989; 415: 545-549.

24. Nitzsche B, Gloesenkamp C, Schrader M, Hoffmann B, Zengerling F, Balabanov S, et al. Anti-tumor activity of two novel compounds in cisplatin-resistant testicular germ cell cancer. Br J Cancer. 2012; 107: 1853-1863. ■