ELSEVIER

European Journal of Cancer 40 (2004) 2064–2070

European Journal of Cancer

www.ejconline.com

The distribution of drug-efflux pumps, *P*-gp, BCRP, MRP1 and MRP2, in the normal blood–testis barrier and in primary testicular tumours

J. Bart [a], H. Hollema [a], H.J.M. Groen [b], E.G.E. de Vries [c], N.H. Hendrikse [d], D.T. Sleijfer [c], T.D. Wegman [d], W. Vaalburg [d], W.T.A. van der Graaf [c,*]

[a] *Department of Pathology, Groningen University Hospital, P.O. Box 30.001, Groningen 9700 RB, The Netherlands*
[b] *Pulmonary Medicine, Groningen University Hospital, P.O. Box 30.001, Groningen 9700 RB, The Netherlands*
[c] *Department of Medical Oncology, Groningen University Hospital, P.O. Box 30.001, Groningen 9700 RB, The Netherlands*
[d] *PET-center, Groningen University Hospital, P.O. Box 30.001, Groningen 9700 RB, The Netherlands*

Received 26 June 2003; received in revised form 10 May 2004; accepted 11 May 2004
Available online 2 July 2004

Abstract

The drug-efflux pumps *P*-glycoprotein (*P*-gp) and multidrug resistance-associated protein 1 (MRP1) are present in the blood–testis barrier (BTB) and may hamper the delivery of cytotoxic drugs to the testis. The precise localisation of *P*-gp and MRP1 in testicular tissue and the presence of the efflux pumps MRP2 and breast cancer resistance protein (BCRP) in the BTB are unknown. We therefore studied the localisation of these pumps in the BTB in normal testis ($n = 12$), in non-seminoma ($n = 10$) seminoma ($n = 10$), and testicular lymphoma ($n = 9$). Slides were scored semi-quantitatively for *P*-gp, MRP1, MRP2 and BCRP and blood vessels with factor VIII antibody. In normal testis, *P*-gp and BCRP were strongly expressed by myoid cells and luminal capillary endothelial wall and *P*-gp also by Leydig cells. MRP1 was observed at the basal side of Sertoli cells and on Leydig cells. MRP2 was only weakly expressed by myoid cells. Seminomas and non-seminomas expressed *P*-gp and/or BCRP and/or MRP1, lymphomas strongly expressed *P*-gp, weakly expressed BCRP and did not or showed weak expression of MRP1. There was very little staining for MRP2 in the tumours. Newly formed vessels in all tumours only expressed *P*-gp and BCRP. *P*-gp, BCRP and MRP1 are present in different cell layers of the normal testis, suggesting the optimal protection of spermatogenesis. In germ cell tumours, this expression pattern may explain the chemoresistance observed to *P*-gp, BCRP and MRP1 substrates. In germ cell tumours and testicular lymphomas, *P*-gp and BCRP expression by tumour cells and by newly formed vessels may also contribute to chemoresistance. These findings underscore the importance of removing the affected testis in cases of primary germ cell tumours and testicular lymphomas, irrespective of whether the patient has already undergone chemotherapy.

Keywords: Blood–testis barrier; Germ cell tumour; *P*-glycoprotein; Breast cancer resistance-related protein; Multidrug resistance-associated protein; Testicular tumour

1. Introduction

The testes are a sanctuary for tumour cells during the administration of chemotherapeutic agents, such as vincristine and doxorubicin, for the treatment of lymphoblastic leukemias [1,2]. Although complete remission to first-line chemotherapy is common in these patients, isolated testicular relapses and testicular combined with central nervous system (CNS) relapses have been reported [3]. Patient outcome following anthracycline-based chemotherapy for testicular lymphomas seems to be worse than for lymphomas at other sites, even after orchidectomy of the affected testis [4]. Metastatic germ cell tumours are extremely sensitive to chemotherapy

* Corresponding author. Tel.: +31-50-361-2821/2824; fax: +31-50-361-4862.
E-mail address: w.t.a.van.der.graaf@int.azg.nl (W.T.A. van der Graaf).

0959-8049/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2004.05.010

regimens consisting of cisplatin, bleomycin and etoposide [5], but are, in general, resistant to cytotoxic agents that are known substrates of the drug-efflux pumps; *P*-glycoprotein (*P*-gp), breast cancer resistance protein (BCRP) or multidrug resistance protein 1 (MRP1). Cisplatin is a substrate of multidrug resistance protein 2 (MRP2) [6,7]. However, after initial chemotherapy, in almost one third of patients, residual viable tumour remains at the primary tumour site [8,9]. It is thought that the blood–testis barrier (BTB), which protects developing germ cells against harmful agents, hampers delivery of certain cytotoxic agents to the testis and tumours [10,11]. The BTB consists of both a passive and active part with regard to drug transport. Three cell layers, endothelial cells, myoid cells and Sertoli cells, form the passive part of the BTB. The cells within these three layers are connected by tight junctions. The endothelial cells of the testicular capillaries lack fenestrations. Sertoli cells form the seminiferous tubule, in which germ cells divide and develop. The myoid cells form the *Tunica propria*, a thin layer around the seminiferous tubule [12,13]. The active part of the BTB consists of efflux pumps, but the composition of this part is less well understood. It is already known that the efflux pumps, *P*-gp and MRP1, are expressed in the testis, but the expression of other pumps, such as MRP2 and BCRP, is unknown [10,14,15]. These members of the ABC transporter family are transmembrane proteins that are able to transport many substrates in an adenosine triphosphate (ATP) dependent manner. They transport a wide range of agents, such as cytotoxic drugs (vinca-alkaloids, anthracyclines, taxoids and epipodophyllotoxins), protease inhibitors, calcium antagonists and corticosteroids [16,17]. Although there are differences in substrate specificity between *P*-gp, BCRP and MRP1 and 2, they have many substrates in common. MRP1 is expressed on the basolateral side of Sertoli cells [15], but the exact arrangement of these four efflux pumps in different cell layers of the BTB is unknown. Insight into their exact arrangement may help us to understand the function of the BTB in normal and malignant testicular tissues. For this reason, we elucidated in this study the localisation of the drug-efflux pumps, *P*-gp, BCRP, MRP1 and MRP2, in the different cell layers in normal testicular tissue and in previously untreated primary seminomas, non-seminomas and testicular lymphomas.

2. Patients & methods

2.1. Chemicals

Biotinylated rabbit-anti-mouse secondary antibody (RAMBIO), rabbit-anti-rat (RARABIO), conjugated strepta-B-complex (ABC) and factor VIII antibody were purchased from DAKO (Glostrup, Denmark). The Avidin/Biotin blocking kit was from Vector Laboratories Inc. (Burlingame, CA, USA), 3.3-diaminobenzidine tetrahydrochloride from Sigma (St. Louis, MO, USA), phosphate-buffered saline (PBS: 6.4 mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 M NaCl; 2.7 mM KCl, pH 7.4) and imidazole from Merck (Darmstadt, Germany) and bovine serum albumin (BSA) from Serva Electrophoresis GmbH (Hamburg, Germany).

The monoclonal antibody, C494, used for the detection of *P*-gp, was purchased from Signet Laboratories (Dedham, MA, USA). The monoclonal antibodies, BXP21, MRPr1 and M_2III-6, were kindly provided by Dr. R. J. Scheper, Free University Hospital, Amsterdam, The Netherlands [18–20].

2.2. Histology/immunohistochemistry

Samples of normal testicular tissue ($n = 12$), and of non-pretreated non-seminoma ($n = 10$), seminoma ($n = 10$) and testicular lymphoma ($n = 9$) from patients treated from 1997 to 2002 were retrieved from our hospital's pathology archive. Normal testicular tissue was obtained from patients who underwent bilateral orchidectomy as hormonal treatment for prostate cancer. The paraffin-embedded samples were sliced into 4 μm sections, placed on positively charged glass slides, and dried. The slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). Subsequently, the slides were washed 3× in 1% BSA/PBS. For histology, the slides were stained with haematoxylin/eosin. For immunohistochemical staining of the efflux pumps, antigen retrieval was performed by heating the slides three times for 5 min in an autoclave at 115 °C in 20 mM blocking reagent (Boehringer Mannheim, Mannheim, Germany) at pH 6.0 (for *P*-gp and MRP1) or by heating in a microwave with citric acid (0.1 mM) for 10 min. Endogenous peroxidase was blocked by incubation with 1% H_2O_2 in PBS over 30 min and pre-incubated with avidin/biotin blocking reagent for 15 min and aspecific antigens were blocked with 1% rat serum in PBS. The slides were incubated with *P*-gp-specific antibody C494 (0.6 μg/ml in PBS with 1% BSA), BCRP-specific antibody BXP21 (5 μg/ml in PBS with 1% BSA), MRP1-specific antibody MRPr1 (2.5 μg/ml in PBS with 1% BSA) and MRP2-specific antibody M_2III-6 (5 μg/ml in PBS with 1% BSA) for 1 h at room temperature in a humidified chamber. The primary antibodies were detected with a biotinylated rabbit anti-mouse secondary antibody (1:300 diluted in 1% BSA/PBS) followed by incubation with peroxidase conjugated streptABComplex (1:100 diluted in 1% BSA/PBS and 1% AB-serum). As a chromagen, 3,3-diaminobenzidine tetrahydrochloride (25 mg) and imidazole (50 mg) in PBS (50 ml) with 50 μl of 30% H_20_2 was used. After counterstaining with Mayer's haematoxylin for

2 min, slides were dehydrated through graded ethanols (70%, 96% and 100%) and mounted with coverslips. Liver and bronchial tissue slices served as positive controls for *P*-gp and MRP1 staining, respectively. As negative controls, liver and bronchial tissue also served, processed in the same way as described above, but without incubation with the monoclonal antibody.

Blood–vessels were detected with factor VIII monoclonal antibody. Staining was performed with a microprocessor-controlled automated device (Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer's instructions. Counterstaining of the nuclei was performed with haematoxylin.

Two observers independently assessed the expression of *P*-gp, BCRP, MRP1, MRP2 and factor VIII. *P*-gp, MRP1 and factor VIII expression was studied in adjacent slides of normal testicular tissue and the most viable tumour parts. *P*-gp, BCRP, MRP1 and MRP2 expression was assessed semi-quantitatively using a scale of 0–3 (0: no staining, 1: very weak staining, 2: intermediate staining, 3: strong staining). Strong staining was defined as comparable to the strongest-stained slice of all tissue slices. Samples were considered negative if less than 10% of a specific subtype of cells were stained. The final scoring was based on the percentage of positive cells and the semi-quantitative staining result. Factor VIII was used to localise the sites where newly formed vessels in the tumours were formed.

3. Results

3.1. Normal testicular tissue from normal testes

Representative photographs of the immunostaining results are presented in Fig. 1. The different cell types, which belong to the different layers of the BTB expressed in a reciprocal manner either BCRP and *P*-gp or MRP1. Table 1 presents the averaged semi-quantitative evaluation of *P*-gp, BCRP, MRP1 and MRP2 expression in the testicular tissue. *P*-gp and BCRP are expressed on the luminal side of the endothelial cells and on the apical side of the myoid cells. MRP1 is expressed on the basolateral side of the Sertoli cells. The hormone-producing Leydig cells express *P*-gp and MRP1, but not BCRP. Capillary endothelium of normal testes showed strong *P*-gp, but not MRP1 expression. MRP2 is expressed on the myoid cell layer only.

3.2. Normal testicular tissue in the proximity of a testicular tumour

In the testicular tumour cases, the pre-existing normal testicular tissue showed the same pattern of efflux pump expression as in the normal testis (Table 1).

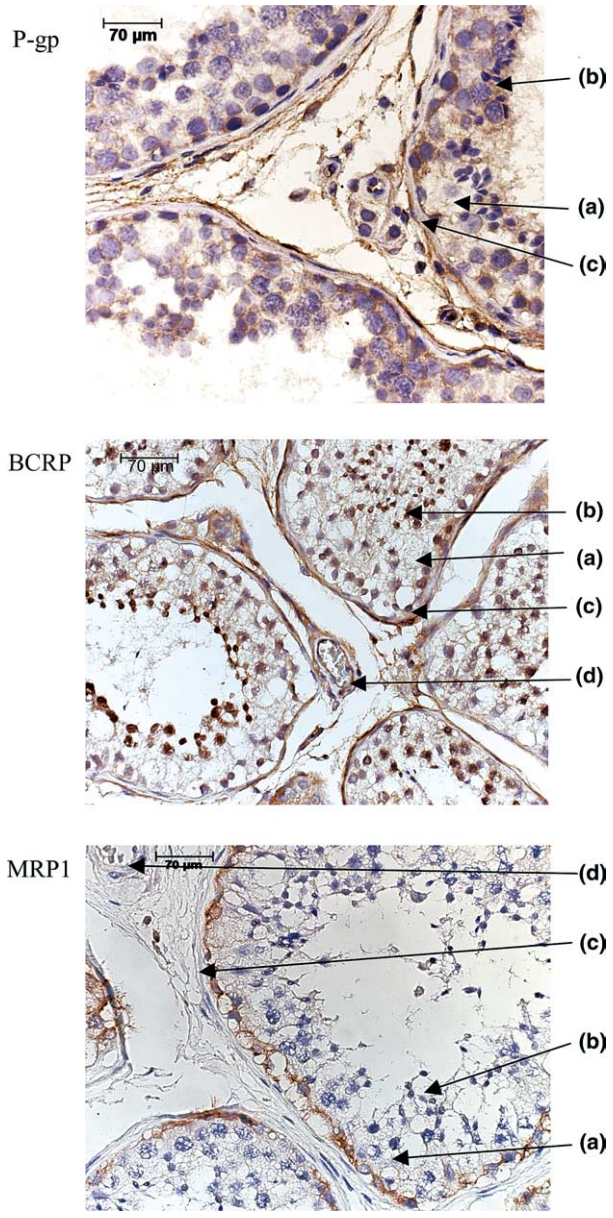

Fig. 1. Immunohistochemical staining for *P*-gp, BCRP and MRP1 in normal testicular tissue. Monoclonal antibodies C494, BXP21 and MRPr1 were used for *P*-gp, BCRP and MRP1, respectively. Magnification 400×. Arrows: (a) Sertoli cells, (b) developing germ cells, (c) myoid cells, (d) endothelial cells. MRP2 data not shown because this was negative.

3.3. Testicular tumours

Semi-quantitative evaluation of staining of different testicular tumours is shown in Table 2. Representative examples are shown in Fig. 2(a)–(f). Seminomas and non-seminomas expressed either *P*-gp, BCRP or MRP1 alone or together. Lymphomas predominantly showed (strong) *P*-gp or BCRP expression, but only weak or no MRP1 expression. MRP2 was only very weakly expressed in some seminomas and on the endothelium of some of the investigated tumours. The factor VIII

Table 1
Average staining result in normal testicular tissue in a normal testis and in the proximity of different tumours

	P-gp	BCRP	MRP1	MRP2
Normal testis (n=12)				
Myoid cell layer	●◐○	●◐○	○○○	●○○
Sertoli cells	◔○○	○○○	●◐○	○○○
Leydig cells	●◐○	○○○	●◐○	○○○
Endothelium	●◐○	●◐○	○○○	○○○
Proximity of a testicular tumour (n=29)				
Myoid cell layer	●○○	●◐○	○○○	◐○○
Sertoli cells	◐○○	○○○	●◐○	○○○
Leydig cells	●○○	○○○	●○○	○○○
Endothelium	●○○	●◐○	○○○	○○○

●, expression of the pump; ○, no expression

Table 2
Average staining result on tumour cells and on endothelium in tumour vessels

	P-gp	BCRP	MRP1	MRP2
Tumour cells				
Seminoma (n=10)	●○◐	●○○	●◐○	◐○○
Non-seminoma (n=10)	●◐○	○○○	●○○	○○○
Lymphoma (n=9)	●○◐	●○○	◐○○	○○○
Endothelium of tumour vessels				
Seminoma (n=10)	●○◐	●○○	○○○	◐○○
Non-seminoma (n=10)	●○○	○◐○	○○○	◔○○
Lymphoma (n=9)	●○◐	●○○	○○○	◔○○

●, expression of the pump; ○, no expression

staining revealed the localisation of the newly formed tumour vessels. These vessels appeared in most tumours as hairy patterns penetrating between tumour cells (Fig. 2(g)). In all samples, *P*-gp and factor VIII staining showed the same pattern at the same localisation (Fig. 2(b) and (g)). In addition, this hairy pattern found for *P*-gp staining, even in slices that had negative factor VIII staining. The hairy pattern of factor VIII expression reflects angiogenesis and thus indicates that early stages of newly formed vessels already express *P*-gp.

4. Discussion

In the present study, we elucidated the precise localisation of *P*-gp, BCRP, MRP1 and 2 in normal testes and in normal testicular tissues in the proximity of a primary testicular tumour. The expression of MRP2 in the BTB is negligible and will not be extensively discussed. The distribution of the other efflux pumps over different tissue layers suggests a histologically serial order of *P*-gp and BCRP versus MRP1. According to the detoxifying function of these pumps, it is suggested that the pump direction of *P*-gp and BCRP in myoid cells and in endothelial cells, as well as of MRP1 in Sertoli cells is outwards from the seminiferous tubule. This pump direction will result in the maximal protection of developing germ cells. However, so far, it is unknown whether expression of efflux pumps on two cell layers (MRP1 in Sertoli cells and *P*-gp/BCRP in myoid cells) is advantageous in comparison with expression in one cell layer. For example, Leydig cells express both *P*-gp and MRP1 on their cell membrane, but are they better or worse protected than germ cells in the intratubular lumen? It is noteworthy that alkylating agents, such as cyclophosphamide and ifosfamide – agents that are not *P*-gp substrates – frequently result in male infertility, due to irreversible azoospermia [21]. Testicular cancer treatment with cisplatin-containing regimens frequently results in at least temporary azoo- and oligospermia [22], but also in irreversible DNA damage [23]. In contrast, the use of MDR drugs, such as doxorubicin, does not necessarily lead to infertility. Interestingly, hormone production by Leydig cells remains largely intact after cytotoxic treatment [24]. The question of whether the localisation of *P*-gp and MRP1, separately or in combination with other pumps, is advantageous for protection of the testis requires further investigation. For instance, transport studies should be carried out at a cellular level, in viable tissue slices and in knock-out animal models. The resistance of germ cell tumours to *P*-gp or MRP1 substrates may, at least partly, be explained by *P*-gp and MRP1 expression at the BTB and in the tumour cells.

Treatment for metastatic germ cell tumours consists of cisplatin-based regimens. Because the primary treatment consists of orchidectomy, cisplatin penetration in the testis can only be deduced from data of an occasionally removed primary tumour after chemotherapy and from the contralateral testis and bilateral germ cell tumours. The incidence of contralateral germ cell tumours decreases after cisplatin-containing therapy [25]. More than two thirds of synchronous bilateral primary testicular germ cell cancers could be cured with cisplatin-containing chemotherapy [26], but it is also known that in cases of delayed orchidectomy after chemotherapy viable tumour remains [8]. The chemosensitivity of germ cell tumours to cisplatin can be attributed to a combination of factors, including a lack of anti-apoptotic BCL-2 family members and an intact apoptotic cascade downstream of p53. Interestingly, it is suggested that *P*-gp expression on germ cell tumours is associated with a more malignant phenotype and with poor treatment outcome. This is probably due to a correlation of *P*-gp expression with a lack of functional p53 and metallothionein [27,28]. Furthermore, a lack of MRP2 is mentioned as a factor in the sensitivity of germ cell tu-

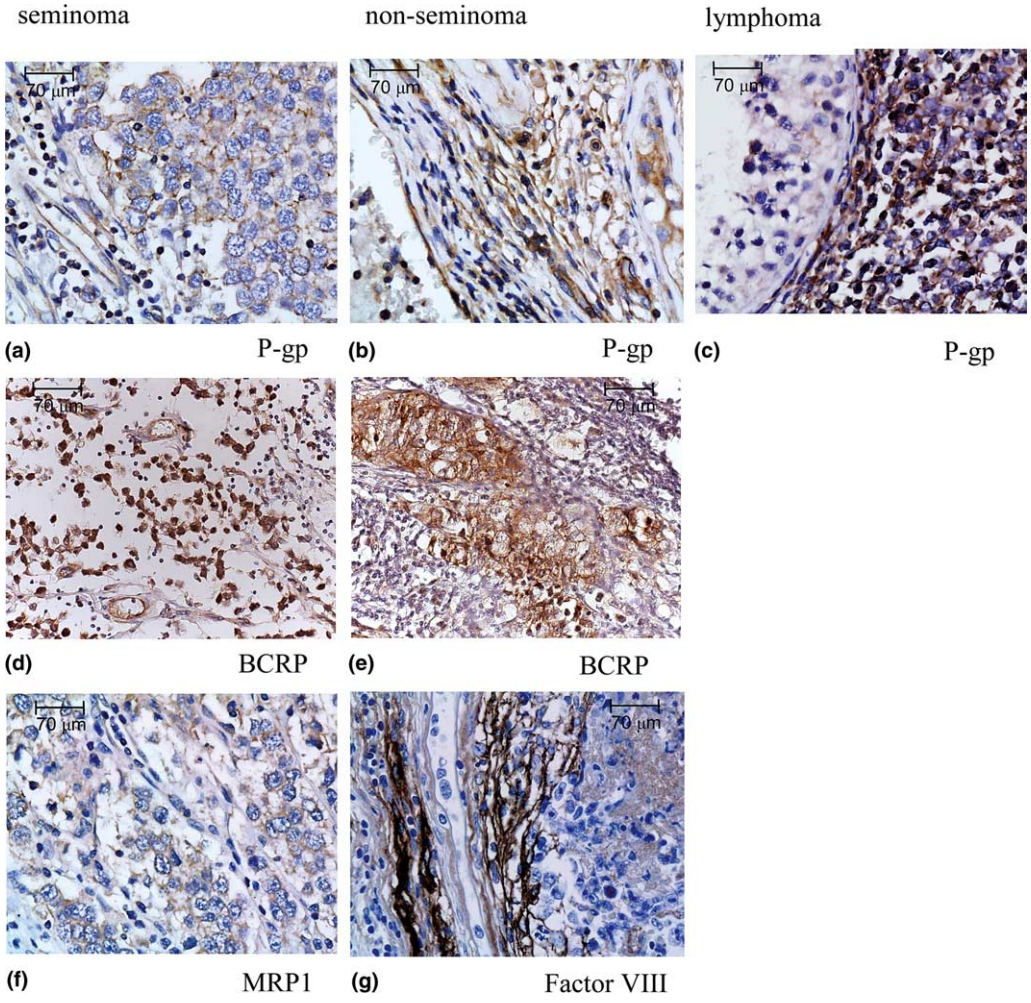

Fig. 2. Representative examples of immunohistochemical staining of *P*-gp, BCRP, MRP1 and factor VIII in different tumour types. (a) *P*-gp staining in seminoma, (b) *P*-gp staining in non-seminoma, (c) *P*-gp staining in lymphoma, (d) BCRP staining in seminoma, (e) BCRP staining in non-seminoma, (f) MRP1 staining in seminoma, (g) factor VIII staining in non-seminoma. Magnification 400×. MRP2 data not shown because this was negative.

mours to cisplatin [29]. Cisplatin is considered a substrate for MRP2, which is hardly expressed in the testis, but it is not a substrate for *P*-gp, BCRP or MRP1. Therefore, it might potentially reach high levels in the testis. However, the higher percentage of viable post-chemotherapy residual tumour cells in the primary testicular tumour compared with residual metastases in retroperitoneal lymph node dissection specimens still suggests a functional BTB for cisplatin, which cannot fully be explained by efflux pump activity [8,30]. Treatment of (testicular) lymphomas usually consists of combination chemotherapy including cyclophosphamide, doxorubicin, vincristine, and prednisolone [31]. Doxorubicin and vincristine are well-known substrates of *P*-gp and BCRP. Although testicular lymphomas show a relatively good response to first-line chemotherapy, the survival of these patients is relatively poor and relapse in the contralateral testicle is not uncommon, especially when no scrotal radiotherapy has been applied [4,32,33]. In our series, primary testicular lymphomas showed a strong *P*-gp and a much weaker BCRP expression, but no or only weak MRP1 expression. Obviously, strong *P*-gp expression alone does not necessarily lead to resistance for all of these cytotoxic compounds. Since primary treatment of testicular lymphomas consists also of orchidectomy, poor treatment results can possibly be attributed to the strong *P*-gp expression by this tumour. To our knowledge, little is known about the role of BCRP in lymphomas. Although it is expressed weakly, it might contribute to chemoresistance. It can also be speculated that the expression of efflux pumps is upregulated by chemotherapy. It is known that Leydig cells excrete factors, which induce *P*-gp expression in the BTB [18]. Possibly these factors are also involved in the strong *P*-gp expression of primary testicular lymphomas.

Almost all tumours in the present study showed *P*-gp and most of the tumours had BCRP expression in the newly formed vessels. Many studies addressed *P*-gp tumour expression, while less attention has been paid to

P-gp expression in the newly formed tumour vessels. As shown, *P*-gp is present in very early stages of neo-angiogenesis. Endothelial cells in neo-angiogenic tumour vessels are often formed by differentiation of a subset of $CD34^+$ pluripotent stem cells, recruited from the bone marrow [34]. These $CD34^+$ cells express *P*-gp and show even stronger expression of BCRP [35]. Most likely, these ABC transporters protect stem cells from harmful agents, and probably from differentiation agents [36,37]. Differentiated stem cells express less efflux pumps than pluripotent stem cells [38]. It can therefore be speculated that endothelial cells that form these early stages of tumour vessel growth are relatively immature, and have not yet lost *P*-gp and BCRP expression on their cell surface. Alternatively, tumours may excrete factors, such as basic fibroblast growth factor or glial fibrillary acidic protein, known to be necessary for the induction of *P*-gp expression in brain capillaries [39]. It is obvious that factors like these are necessary for *P*-gp expression in tumour capillaries, and that tumours excrete them to protect themselves.

This study illustrates that either *P*-gp and BCRP or MRP1 are expressed in different cell layers of the normal testis, which suggests the optimal protection of spermatogenesis. The expression of efflux pumps in germ cell tumours may, at least partly, explain the resistance of these tumours to substrates of *P*-gp, BCRP and MRP1. In addition, in germ cell tumours and in testicular lymphomas, *P*-gp expression in newly formed vessels may contribute to chemoresistance. These findings are in favour of the common practice to remove the affected testis in cases of primary germ cell tumours and testicular lymphomas, irrespective of whether the patient has already undergone chemotherapy.

Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

Acknowledgements

Supported by grant RUG 99-1882 of the Dutch Cancer Society and the Nijbakker Morra Foundation.

References

1. Miniero R, Saracco P, Pastore G, *et al*. Relapse after first cessation of therapy in childhood acute lymphoblastic leukemia: a 10-year follow-up study. Italian Association of Pediatric Hematology–Oncology (AIEOP). *Med Pediatr Oncol* 1995, **24**, 71–76.
2. Vukelja SJ, Swanson SJ, Knight RD, Weiss RB. Testicular relapse in adult acute lymphocytic leukemia: a case report and literature review. *Med Pediatr Oncol* 1989, **17**, 170–173.
3. Pui CH, Campana D, Evans WE. Childhood acute lymphoblastic leukaemia – current status and future perspectives. *Lancet Oncol* 2001, **2**, 597–607.
4. Zucca E, Conconi A, Mughal TI, *et al*. Patterns of outcome and prognostic factors in primary large-cell lymphoma of the testis in a survey by the International Extranodal Lymphoma Study Group. *J Clin Oncol* 2003, **21**, 20–27.
5. Shelley MD, Burgon K, Mason MD. Treatment of testicular germ-cell cancer: a cochrane evidence-based systematic review. *Cancer Treat Rev* 2002, **28**, 237–253.
6. Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* 2000, **92**, 1295–1302.
7. Itoh Y, Tamai M, Yokogawa K, *et al*. Involvement of multidrug resistance-associated protein 2 in *in vivo* cisplatin resistance of rat hepatoma AH66 cells. *Anticancer Res* 2002, **22**, 1649–1653.
8. Leibovitch I, Little Jr JS, Foster RG, Rowland R, Bihrle JP. Delayed orchiectomy after chemotherapy for metastatic non-seminomatous germ cell tumors. *J Urol* 1996, **155**, 952–954.
9. Simmonds PD, Mead GM, Lee AH, Theaker JM, Dewbury K, Smart CJ. Orchiectomy after chemotherapy in patients with metastatic testicular cancer. Is it indicated? *Cancer* 1995, **75**, 1018–1024.
10. Bart J, Groen HJM, van der Graaf WTA, *et al*. An oncological view on the blood–testis barrier. *Lancet Oncol* 2002, **3**, 357–363.
11. Seymour JF, Solomon B, Wolf MM, Janusczewicz EH, Wirth A, Prince HM. Primary large-cell non-Hodgkin's lymphoma of the testis: a retrospective analysis of patterns of failure and prognostic factors. *Clin Lymphoma* 2001, **2**, 109–115.
12. Pelletier RM, Byers SW. The blood–testis barrier and Sertoli cell junctions: structural considerations. *Microsc Res Tech* 1992, **20**, 3–33.
13. Kerr JB. Functional cytology of the human testis. *Baillieres Clin Endocrinol Metab* 1992, **6**, 235–250.
14. Cordon-Cardo C, O'Brien JP, Casals D, *et al*. Multidrug-resistance gene (*P*-glycoprotein) is expressed by endothelial cells at blood–brain barrier sites. *Proc Natl Acad Sci USA* 1989, **86**, 695–698.
15. Wijnholds J, Scheffer GL, van der Valk MA, *et al*. Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage. *J Exp Med* 1998, **188**, 797–808.
16. Bart J, Groen HJM, Hendrikse NH, van der Graaf WTA, Vaalburg W, de Vries EGE. The blood–brain barrier and oncology: new insights into function and modulation. *Cancer Treat Rev* 2000, **26**, 449–462.
17. Muller M. Human ATP Binding Cassette transporters; URL: http://www.med.rug.nl/mdl/humanabc.htm.
18. Flens MJ, Izquierdo MA, Scheffer GL, *et al*. Immunochemical detection of the multidrug resistance-associated protein MRP in human multidrug-resistant tumor cells by monoclonal antibodies. *Cancer Res* 1994, **54**, 4557–4563.
19. Scheffer GL, Kool M, Heijn M, *et al*. Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5 and MDR3 *P*-glycoprotein with a panel of monoclonal antibodies. *Cancer Res* 2000, **60**, 5269–5277.
20. Diestra JE, Scheffer GL, Catala I, *et al*. Frequent expression of the multidrug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J Pathol* 2002, **198**, 213–219.
21. Schwartz CL. Long-term survivors of childhood cancer: the late effects of therapy. *Oncologist* 1999, **4**, 45–54.
22. DeSantis M, Albrecht W, Holtl W, Pont J. Impact of cytotoxic treatment on long-term fertility in patients with germ-cell cancer. *Int J Cancer* 1999, **83**, 864–865.

23. Stahl O, Eberhard J, Jepson K, *et al*. The impact of testicular carcinoma and its treatment on sperm DNA integrity. *Cancer* 2004, **100**, 1137–1144.
24. Gerl A, Muhlbayer D, Hansmann G, Mraz W, Hiddemann W. The impact of chemotherapy on Leydig cell function in long term survivors of germ cell tumors. *Cancer* 2001, **91**, 1297–1303.
25. van Basten JP, Hoekstra HJ, van Driel MF, Sleijfer DT, Droste JH, Schraffordt Koops H. Cisplatin-based chemotherapy changes the incidence of bilateral testicular cancer. *Ann Surg Oncol* 1997, **4**, 342–348.
26. Heidenreich A, Holtl W, Albrecht W, Pont J, Engelmann UH. Testis-preserving surgery in bilateral testicular germ cell tumours. *Brit J Urol* 1997, **79**, 253–257.
27. Eid H, Geczi L, Magori A, Bodrogi I, Institoris E, Bak M. Drug resistance and sensitivity of germ cell testicular tumors: evaluation of clinical relevance of MDR1/*P*-gp, p53, and metallothionein (MT) proteins. *Anticancer Res* 1998, **18**, 3059–3064.
28. Eid H, Bodrogi I, Csokay B, Olah E, Bak M. Multidrug resistance of testis cancers: the study of clinical relevance of *P*-glycoprotein expression. *Anticancer Res* 1996, **16**, 3447–3452.
29. Mayer F, Stoop H, Scheffer GL, *et al*. Molecular determinants of treatment response in human germ cell tumors. *Clin Cancer Res* 2003, **9**, 767–773.
30. Oliver RTD, Ong J, Blandy JP, Altman DG. Testis conservation studies in germ cell cancer justified by improved primary chemotherapy response and reduced delay, 1978–1994. *Brit J Urol* 1996, **78**, 119–124.
31. Kluin-Nelemans HC, Zagonel V, Anastasopoulou A, *et al*. Standard chemotherapy with or without high-dose chemotherapy for aggressive non-Hodgkin's lymphoma: randomized phase III EORTC study. *J Natl Cancer Inst* 2001, **93**, 22–30.
32. Tondini C, Ferreri AJ, Siracusano L, *et al*. Diffuse large-cell lymphoma of the testis. *J Clin Oncol* 1999, **17**, 2854–2858.
33. Zouhair A, Weber D, Belkace'mi Y, *et al*. Outcome and patterns of failure in testicular lymphoma: a multicenter rare cancer network study. *Int J Radiat Oncol Biol Phys* 2002, **52**, 652–656.
34. Peichev M, Naiyer AJ, Pereira D, *et al*. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood* 2000, **95**, 952–958.
35. Zhou S, Schuetz JD, Bunting KD, *et al*. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001, **7**, 1028–1034.
36. Bunting KD, Zhou S, Lu T, Sorrentino BP. Enforced *P*-glycoprotein pump function in murine bone marrow cells results in expansion of side population stem cells *in vitro* and repopulating cells *in vivo*. *Blood* 2000, **96**, 902–909.
37. Schiedlmeier B, Kuhlcke K, Eckert HG, Baum C, Zeller WJ, Fruehauf S. Quantitative assessment of retroviral transfer of the human multidrug resistance 1 gene to human mobilized peripheral blood progenitor cells engrafted in nonobese diabetic/severe combined immunodeficient mice. *Blood* 2000, **95**, 1237–1248.
38. Bunting KD. ABC Transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* 2002, **20**, 11–20.
39. Sobue K, Yamamoto N, Yoneda K, *et al*. Induction of blood–brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. *Neurosci Res* 1999, **35**, 155–164.